

**The effect of *Toxoplasma gondii* on host
behaviour: studies on evolution and
mechanisms of action**

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PhD Thesis

Declarations

All work presented in this thesis is the candidate's own, under the supervision of Prof. Joanne Webster and Dr. Poppy Lamberton. Any work by others has been appropriately referenced.

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Abstract

Toxoplasma gondii affects a range of intermediate and secondary host species, including humans, with cats (*Felidae*) being the definitive host. The mechanism of action of *T. gondii* in its natural rat host can provide profound insights as to evolutionary trajectory of parasite manipulation. I used a range of non-invasive behavioural and physiological assays to assess the impact of infection on different aspects of innate rat behaviour. One key unique focus was an examination of the potential effect(s) of novel genetically modified parasite lines that overexpress tyrosine hydroxylase (TgTH) in comparison to wildtype *T. gondii*. The aim here was to test the hypothesis that TgTH, through its subsequent impact on neuromodulator levels, plays a significant mechanistic role in host behaviour modification.

Consistent with previous studies, there was a clear effect of *T. gondii* on host behaviour, with effects seen in generalised anxiety and feline response tests. However contrary to predictions, there was little or no significant differences between wildtype and TgTH overexpressor infected rats in feline response tests, implying that TgTH does not play a major role in feline or olfactory related predation response. The implications of the role of TgTH in generalised anxiety and activity levels were more mixed. This suggests that other mechanisms of action, probably multiple, are involved.

This work increases our understanding of the potential mechanisms this parasite has evolved to attain specific manipulation of the rodent intermediate host to increase transmission success to the feline definitive host. The results thus have both evolutionary implications for parasite-altered behaviour in intermediate host species, and applied implications in terms of the potential impact on health and behaviour of all infected hosts, including humans.

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Chapter 1

General Introduction

The widespread protozoan *Toxoplasma gondii* is an important example of how a parasite can manipulate mammalian host behaviour¹. The manipulation effects of *T. gondii* in their intermediate hosts have been shown to be specific behavioural alterations caused by the parasite as evolutionary adaptations to increase the chance of transmission to the feline definitive host². For example, infection appears to remove rats' (a natural intermediate host) innate aversion to feline odour, even turning it into a suicidal 'fatal feline attraction' in some cases³. This effect appears to be specific to feline odour rather than other predatory or non-predatory mammal odours^{4, 5}. If these behavioural changes in rats were a mechanism by which the parasite increased its risk of being transmitted to its definitive host, it would follow that the behavioural changes could specifically increase transmission to felids, as only in a feline gut can the sexual stage of the parasite occur.

My experimental system involved *in vivo* non-invasive behavioural tests, looking at behavioural traits related to responses directly to the feline definitive host, as well as general anxiety behaviours and novelty seeking behaviours. My thesis focuses on elucidating the potential mechanism or mechanisms by which *T. gondii* alters behaviour in its intermediate host, and what this entails for infection within the human brain and its subsequent potential impact on certain human affective disorders, in particular that of schizophrenia. There is a hypothesis that *T. gondii* cysts may directly affect neuromodulator levels in the host brain, by producing increased levels of dopamine which influence host behaviour⁶. The role of dopamine in the reward process was classically associated with the ability to experience pleasure, i.e. the emotion of obtaining a reward; recent data suggest, however, a more motivational role, i.e. the emotion of craving a, perhaps unnatural, reward⁷⁻⁹. This may provide an explanation as to the mechanisms behind 'fatal feline attraction' or more risky behaviours displayed by infected rats. The outcomes of this research will help elucidate the mechanisms by which parasites may manipulate host behaviour and in terms of human infection and its potential link with schizophrenia, this gives us further insights into potential prevention and treatment options.

Background to *Toxoplasma gondii* and toxoplasmosis infection

The parasitic protozoan *T. gondii* has a broad range of intermediate and secondary hosts, which includes all warm blooded organisms¹⁰, such as small mammals, cattle, birds,

primates, and humans. However, only members of the family *Felidae*, the cat family, serve as definitive hosts to the parasite. It is as yet unclear as to whether all members of *Felidae* are definitive hosts of the parasite, and different species may display different capacities for oocyst shedding¹¹. Prevalence estimates range from around 5% - 90% in domestic cats (*Felis domesticus*) around the world¹¹. An infected cat may shed unsporulated oocysts in its faeces. Around 2% of infected cats excrete oocysts at a given point in time¹¹. Although the proportion of infectious cats is not high, an infectious cat can shed millions of oocysts, which are capable of surviving in external environments, such as soil, for over one year¹². Infection of cats can depend on the prevalence of infection in birds and small mammals in the area, which in turn become infected by ingesting oocysts. This may lead to hotspots of *T. gondii* infection¹³. Transmission via the oocyst stage is the only mode of transmission to herbivores, and can also occur in omnivores and carnivores, for example humans may also be directly infected by exposure to oocysts in the soil. Intermediate hosts in nature become infected after ingesting soil, water or plant material contaminated with oocysts.

Oocysts are one of the three infectious stages of *T. gondii*, the other two being tachyzoites and bradyzoites. The tachyzoite infectious stage forms in the intermediate host directly after ingestion of the oocysts. Tachyzoites may be found in blood, semen, saliva, urine, sputum, tears and milk¹⁰. There is no or low risk of transmission through most of these routes, however transmission may occur via milk from a mother to offspring, and recently there has been some preliminary evidence for sexual transmission¹⁴. Parasitemia during pregnancy may result in transplacental transmission. Repeated congenital infections may occur in small mammals such as mice, rats and hamsters, which may produce several infected litters without requiring re-infection from an outside source^{13, 15}.

Tachyzoites localize in neural and muscle tissue and develop into tissue cyst bradyzoites, another form of transmission. Tissue cysts can persist in the muscle tissues of live animals for years, potentially whole lifespans¹³. Cats become infected after consuming intermediate (rodents and birds, predated upon by felids) or secondary (other endothermic, dead-end) hosts harbouring tissue cysts (Fig. 1). Animals bred for human consumption may also become infected and develop tissue cysts; therefore this is also a potential route of transmission to humans.

In humans, prevalence of infection is high, with estimates of infection in the UK ranging from 23- 33%¹⁶ and 4% - 92%¹¹ worldwide. It is often considerably higher in other countries particularly where there is a culture of eating rare or undercooked meat, such as France and Ireland¹¹. Half a billion of the human population alive today has been exposed to the

T. gondii parasite, as indicated by seropositivity. However there are large variations in prevalence in humans and animals from different geographical areas, both within and between countries¹¹. Causes of these variations could be due to numerous factors, for

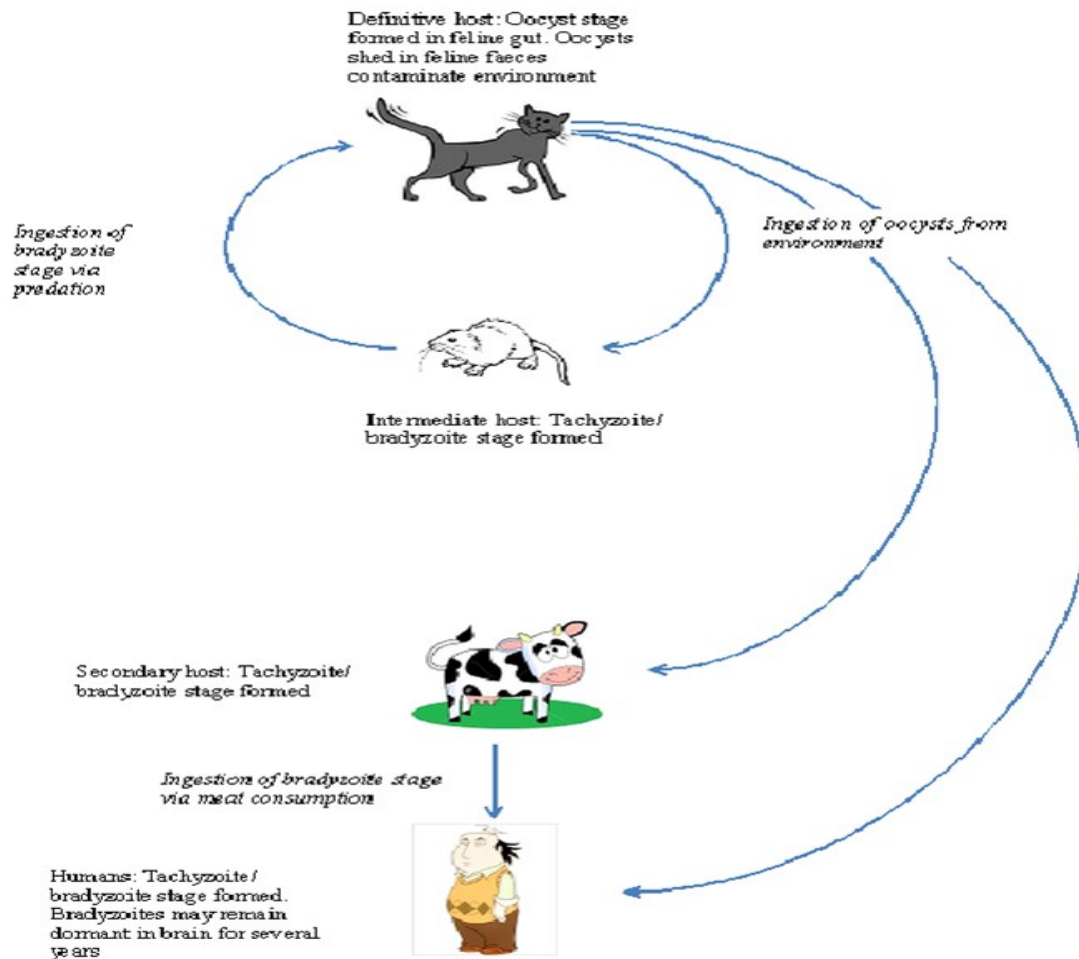


Figure 1: Life cycle of *Toxoplasma gondii*

example, environmental factors may affect the sporulation and survival time of oocysts in the environment^{11, 17}, prevalence appears higher in warm climates and low lying areas than in cold or mountainous regions¹³ and cultural habits such as cat ownership, cooking and eating habits¹³ may affect transmission via the bradyzoite stage¹⁸. Hygiene may also play a role with infection occurring through ingesting oocysts from cat faeces, from unwashed hands or vegetables.

Whilst latent toxoplasmosis in humans was originally considered asymptomatic in immunocompetent individuals, there is now a growing body of evidence which suggests that infection may be linked, in the long term, to an increased risk of schizophrenia and other human affective disorders¹⁹⁻²². The high prevalence of *T. gondii* infection in several human populations highlights the importance of research into the subtle effects the parasite may

have during chronic infection of the human brain. This chronic infection of latent *T. gondii* in the brain is established due to a balance between host immunity and the parasite's evasion of the immune response. The proliferation of tachyzoites during the acute stage of infection is suppressed by interferon- γ (IFN- γ)-dependant, cell-mediated immune responses, and to some degree by humoral immunity. This leads to the development of chronic infection by *T. gondii* cysts, which preferentially locate in the brain.

Diagnosis of *T. gondii* can be made by biological, serological or histological methods. Tests often used for humans include the dye test, the modified agglutination test (MAT), and the enzyme-linked immunosorbent assay (ELISA)¹¹. Clinical signs of toxoplasmosis are non-specific and therefore cannot be relied on for accurate diagnosis. After diagnosis *T. gondii* in humans is normally treated using sulfonamides with pyrimethamine¹¹. These drugs act on tachyzoites, and therefore control active infection but do not eliminate chronic infection. Currently there is a lack of drugs active against the bradyzoite stage, although some active compounds such as azithromycin and the hydroxynaphthoquinone 566C80 display anti-cyst effects^{23, 24}. The doses of drugs used vary depending on whether the patient is a pregnant woman, a child, or an immunosuppressed individual, and on the symptoms of the toxoplasmosis^{11, 25}.

Manipulation of anxiety and predation-related fear by parasites and pathogens

Behavioural and neurophysiological traits and responses associated with anxiety and predation-related fear have been well documented in rodent models¹. Certain parasites and pathogens which rely on predation for transmission appear able to manipulate these, often innate, traits to increase the likelihood of their life-cycle being completed. This could occur through a range of mechanisms, such as alteration of hormonal and neurotransmitter communication and/or direct interference with the neurons and brain regions that mediate behavioural expression¹. Whilst some post-infection behavioural changes may reflect 'general sickness' or a pathological by-product of infection, others may have a specific adaptive advantage to the parasite and be indicative of active manipulation of host behaviour²⁶. There are a few key mechanisms by which anxiety and predation-related fears are controlled in mammals, and some evidence exists for how some infectious agents may manipulate these mechanisms, including for example *T. gondii*^{6, 27}. Selective pressures have potentially resulted in this parasite evolving strategies to alter the behaviour in its natural intermediate rodent host. Latent infection has also been associated with a range of altered behavioural profiles, from subtle behavioural alterations to severe pathologies, in other secondary host species including humans^{19, 20, 28, 29}. In addition to enhancing our knowledge

of the evolution of parasite manipulation, to further our understanding of how and when these potential changes to human host behaviour occur, and how we may prevent or manage them, it is imperative to elucidate the associated mechanisms involved.

Parasite-induced behavioural alterations

Whilst behavioural alterations caused by *T. gondii* are believed to have specifically evolved to increase the parasite's chance of successful transmission, this is not the only way in which a parasite can alter the behaviour of its host²⁶.

- Sickness behaviour:

Morbidity in the host, often displayed as general malaise or 'sickness', that may facilitate transmission of the parasite. This is often observed with vector-borne diseases, where a decrease in host energy and activity levels can increase the likelihood of being bitten by an insect vector, via an increase in host landing rate and a reduction in fly swatting behaviours^{30, 31}. Examples of this can be seen in rodents infected with *Trypanosoma brucei brucei*³² and various *Plasmodium* spp.^{33, 34}, as well as sneezing and/or coughing, which facilitates the transmission of aerosolised pathogens, via increasing their volume and distance travelled³⁵.

- General pathology:

Morbidity (or mortality) in the host that is of no obvious selective benefit to host or parasite. This may be the case with neurosyphilis caused by *Treponema pallidum*, which is associated with cognitive deterioration and neuronal loss long after the period of infectivity³⁶.

Schistosoma mansoni infection has also been associated with reduced exploratory behaviours, cognitive abilities and nociception in the human host³⁷⁻⁴⁰ that is of no apparent selective benefit to the parasite which relies on contact with freshwater snails for its transmission.

- Parasite Manipulation:

Behavioural alterations of the host with a specific selective benefit to the parasite. This is the case with *T. gondii*, where a reduction in fear of feline odour and increased activity are thought to be specific selective advantages to the parasite, increasing its chance of

transmission by predation to the feline definitive host^{2, 41}. Other heteroxenous parasites which appear to cause their intermediate hosts to be predated upon more than expected by their definitive hosts include *Plagiorynchus cylindraceu* in isopods⁴², *Sarcocystis cernae* in voles⁴³, and *Euhaplorchis californiensis* in fish^{44, 45}. Another example of parasite manipulation is perhaps *Lyssavirus* spp., causative agents of rabies. In wild hosts such as dogs and bats, the virus' mechanisms within the central nervous system may facilitate transmission, for example dogs with furious rabies show higher levels of aggression and biting⁴⁶ that increase chance of transmission via infected saliva to susceptible hosts blood and body tissues⁴⁷. Seoul virus, similar to the rabies virus in dogs, is thought to increase aggressive behaviours in rats where the resulting wounding may facilitate transmission via contact with virus present in saliva or excrement^{48, 49}.

- By-product:

Behavioural alterations displayed in accidental/dead-end hosts as a side-effect of selective adaptations in the parasite's natural host species. One example of this could be the rabies virus in humans. Humans are a dead-end host where the virus causes hyperactivity, phobic spasms and severe agitation⁵⁰ that is of no selective benefit to the virus. This can also be seen in the case of *T. gondii*-infected sea otters showing abnormal behaviour and increased predation by sharks⁵¹, despite sharks being a dead-end host to the parasite. Behavioural alteration as a by-product also appears to be the case in *T. gondii* infection of humans, where adaptations designed to facilitate transmission in the rodent intermediate hosts cause related effects in humans: such as lowered reaction times⁵², subtle personality alterations and reduced novelty-seeking²⁸. This may also explain the potential epidemiological link between *T. gondii* and schizophrenia in chronic human infections^{19, 20}, discussed in detail below.

Behavioural affects in rodents

T. gondii infection in rats appears to be more common than in mice, and can range from negligible prevalence to 43%^{11, 53}. The means by which cats become infected in the wild is epidemiologically important; however this varies greatly by location, depending on the most common prey of cats, and their prevalence of infection. It is thought that cats may be less likely to prey upon larger animals, such as adult rats or rabbits; however a positive correlation has been found between feline infection and the body mass of intermediate hosts⁵⁴. The role of rodents in the transmission of *T. gondii* in the wild is therefore not yet completely understood¹¹. Numerous trophically transmitted parasites have been shown to

alter the behaviour of their intermediate hosts in a way that increases their vulnerability to predatory definitive hosts⁵⁵. Examples of parasites which do this include the cestode *Schistocephalus solidus* and *Acanthocephalus dirus*⁵⁶.

Behavioural effects in rats

The predilection of *T. gondii* for the brain of its intermediate host places the parasite in a prime position to manipulate host behaviour. It has been shown that *T. gondii* increases activity levels in wild and wild: laboratory (Lister hooded) hybrid brown rats, *Rattus norvegicus*⁵⁷ whilst parasites with direct life cycles both from experimental (*Syphacia muris*) and natural (*Leptospira* spp., *Cryptosporidium parvum*, *Coxiella burnetti*, *Hymenolepis nana*) infections did not⁵⁷. *T. gondii* infected wild brown rats also display low neophobia in comparison to their uninfected counterparts⁵⁸. Both these behavioural changes may have evolved to facilitate transmission to the definitive host species, through increased predation by felids⁵⁸.

The most convincing piece of evidence for the manipulation hypothesis comes from a study with wild: laboratory (Lister hooded) rats which showed that *T. gondii* infection appeared to alter a rat's innate aversion to feline odour, and in fact in more active rats, turned it into an attraction³. When infected and uninfected rats were exposed to odours such as their own smell, a neutral water smell and rabbit (a non-predatory mammal) smell, both infected and control groups behaved similarly. The only difference was found in the rat's reaction when exposed to feline odour. This would suggest that the behavioural change in the rats is a subtle alteration in cognitive perception in the face of predation risk, rather than a general impairment of the olfactory system. Other studies comparing infected and uninfected Lister hooded laboratory rats' reactions to feline and non-feline predatory mammal odours (dog⁵, mink⁴) demonstrated that *T. gondii* associated behavioural changes were specific to the feline definitive host, strongly supporting the theory that the behavioural changes induced by *T. gondii* are an evolutionary adaptation that specifically increase the parasite's chance of reaching its definitive feline host species.

Wistar rats infected with *T. gondii* have also been shown to display lower anxiety levels relative to their uninfected counterparts when tested with a standard anxiety test, the elevated plus maze⁵⁹. The anxiolytic effect detected by the elevated plus maze and the social interaction tests also investigated were not dose-graduated, although when the dose was increased locomotion began to decrease significantly. The decline in general motor activity of the group of rats infected with the higher inoculation prevents the possible

detection of an anxiolytic effect in the rat. This evidence suggested that *T. gondii* induces an anxiolytic effect on rats, but there may be a cut-off point at which high levels of infection may cause a pathology that overcomes the subtle behavioural change found at lower levels of infection. Serological and histological investigation suggests that the anxiolytic effects are associated with anti-*T. gondii* IgG levels in serum and with the pattern of brain cyst distribution⁵⁹. The predominant invasion of limbic areas and the damage of glial and neural cells may also contribute to the anxiolytic mechanism^{59, 60}.

Behavioural effects in mice

Whilst subtle behavioural effects have been shown in *R. norvegicus*, the same effect is not always observed in mice, where conflicting evidence exists. One study in 7 week old C57BL/6J mice did indicate that animals with chronic *T. gondii* infection displayed increased exploratory locomotion, reduced unconditioned fear and more risky behaviours⁶¹, consistent with the behaviour expected by the adaptive manipulation hypothesis. However another study in 8 week old C57BL/6 mice chronically infected with the avirulent ME49 *T. gondii* strain showed that acquired infection with *T. gondii* can result in widespread brain pathology, motor co-ordination and sensory deficits⁶². Brain pathology in this study showed that all levels of the brain and brainstem had some minimal to moderate meningoencephalitis with gliosis, although social interaction and cognitive behaviour remained unchanged⁶². Another *T. gondii* study with 10 week old BALB/c: C57BL/6 crossed mice indicated that pathology and behavioural changes were transient, and disappeared before the 12th week post inoculation⁶³. In 5-7 week old Swiss Webster outbred mice, chronic, adult acquired *T. gondii* infection has been shown to cause neurological and behavioural abnormalities secondary to inflammation and loss of brain parenchyma and perivascular inflammation is common⁶⁴. This suggests that genetically susceptible mice with persistent *T. gondii* infection may develop inflammatory and neurodegenerative responses⁶⁴.

In these studies with mice, the general pathology that affects the mice's sensorimotor abilities may also be associated with increased predation risk as an outcome of infection. However these behavioural changes are likely to be nonspecific by-products of pathological symptoms of toxoplasmosis, rather than specific manipulation activity by the parasite. This is because these behavioural changes are not solely linked to increased predation risk, by cats or other predators, but would also reduce the mouse's feeding, mating and reproductive success, which would not increase the parasite's transmission.

Infected mice have been shown to display increased general movement, but decreased rearing and digging movements⁶⁵. Adult acquired infection was associated with increased immobility, whilst congenital infection was associated with decreased immobility⁶⁶. Unlike rats, it was shown that infected mice displayed less preference for central, exposed areas than controls⁶⁷. It was also shown that infected mice displayed higher preference for the familiar arm of a Y-maze, whilst uninfected mice displayed higher preference for a novel arm⁶⁸. These findings suggest that infected mice may actually display reduced novelty-seeking/ anxiety-related behaviours, which opposes the findings in rats⁵⁸. One theory as to why this may be the case is that infection may in fact be altering novelty discrimination mechanisms, leading to the naturally neophobic rats and the naturally neophilic mice being influenced in opposite directions by infection⁶⁹. These results are important to take into account in the current study, as they indicate that behavioural changes may be specific to the species of animal, the time of exposure to the parasite, and that different behavioural profiles may be altered in a range of ways⁶⁶. It is unclear as to why *T. gondii* infection may affect different intermediate hosts in different ways. Some explanations of this could be, for example, the prevalence of *T. gondii* in these species of intermediate host, or perhaps even the importance of felines for transmission in populations where *T. gondii* may be sustained largely by vertical transmission and/or repeated carnivory⁷⁰.

The effect of *T. gondii* on its intermediate host species is highly variable. Differences are likely to be caused by the host species or strain, the parasite strain, the time of exposure, the level of infection, and the age and sex of the host. Genetic variation in host susceptibility to the parasite is also likely to play a major role. The differences in the effects of *T. gondii* simply between rodent models illustrate the caution that should be taken when extrapolating the effects to humans.

Behavioural effects in humans

There is a growing body of evidence to suggest that *T. gondii* infection may also affect the general human population in subtle ways^{28, 71-74}. Studies investigating the personality characteristics of *T. gondii* seropositive and seronegative individuals using personality tests⁷⁵ show that differences between infected and non-infected individuals are strongly affected by gender, with infected men having lower superego strength and higher vigilance, and infected women showing higher superego strength and higher warmth⁷⁶. There has also been evidence to show that people in motor vehicle accidents have a higher prevalence/ infection intensity of *T. gondii* infection than the general population^{71, 77-79}. This could be explained by higher risk-taking behaviours of infected individuals, or due to infected individuals displaying

reduced psychomotor performance. It is yet to be elucidated as to how this links into the apparent predation risk associated behavioural changes observed in rats², and this is further investigated in this study. Evidence for reduced psychomotor performance in infected individuals is supported by a study into reaction times of seropositive and seronegative adults, where the computerised reaction time test indicated that adults with latent infections performed worse than the control group²⁸. Infection status however explained less than 10% of the overall variability in performance²⁸.

T. gondii and schizophrenia

Whilst the subtle behavioural changes described above apply to the general human population, there is further evidence that indicates that *T. gondii* may increase the risk of developing schizophrenia in those individuals already susceptible to the disease^{19-22, 80, 81}, plausible due to the chronic nature of *T. gondii* infection. There is increasing recent interest in this, for instance a PubMed search with the words '*Toxoplasma*' and 'schizophrenia' in the title indicated 69 publications in the last five years, as compared to 35 in the previous five years. Although great advances have been made in the understanding and treatment of mental illnesses in recent years, the causes often remain to be elucidated. There have been many theories over the years linking infectious agents with behavioural changes and diseases associated with the brain. Several infectious agents are known to chronically infect human brain tissue, such as the protozoa – *Plasmodium*, *Trypanosoma*, and *T. gondii*, which have all been shown to have the ability to affect host behaviour due to their positioning within the brain or central nervous system²⁰. The outcomes of infection are dependent on the balance between the parasites and the host's response.

A meta-analysis of several studies, in 17 countries over five decades, demonstrated that the prevalence of *T. gondii* antibodies is significantly higher in individuals with schizophrenia than in control populations (odds ratio: 2.73). This odds ratio exceeds that for most genetic studies, strongly supporting a potential etiological role of *T. gondii* in schizophrenia¹⁹. Further work has investigated the association of *T. gondii* infected schizophrenia patients with clinical and demographic factors, and mortality⁸⁰. Schizophrenia patients with serological evidence of *T. gondii* infection were more likely to be female, and were shown to have an increased risk of dying from natural causes⁸⁰. All other demographic and clinical characteristics did not appear to be associated with *T. gondii* infection. The gender difference raises important epidemiological questions, particularly as in the general population there is no consistent difference between male and female prevalence of *T. gondii* infection. However a gender-related personality difference in *T. gondii* seropositive

individuals has been observed supporting the theory that gender may play a role in the development of schizophrenia in *T. gondii* seropositive individuals.

T. gondii seropositive patients with schizophrenia showed a five-fold increase in rate of mortality due to natural causes⁸⁰. Although this finding does not imply causality, it highlights important future areas of research. Evidence has been collected that shows early infection by *T. gondii*, as a foetus or shortly after birth, may increase the risk of developing schizophrenia later on in life⁸² and/or may interact with other risk factors. Antibodies to *T. gondii* have also been found to be more prevalent in serum samples of patients with first-episode schizophrenia when compared with controls. This is another indication that infection status is marked at the beginning of the illness itself⁸³. Even within schizophrenia patients, effects of *T. gondii* infection can be observed. A magnetic resonance imaging study showed that reduction of grey matter in schizophrenia patients was only observed in *T. gondii* seropositive patients and not in *T. gondii* seronegative patients or healthy controls⁸⁴. It is interesting to note that several of the medications currently used to treat schizophrenia also interfere with the replication of *T. gondii*. Infection with *T. gondii* may have wide-reaching implications for mental health, ranging from subtle behavioural changes within the general population to an increased risk of schizophrenia. A further understanding of the etiological link between *T. gondii* infection and the onset of schizophrenia may provide further insights into the treatment of psychiatric disease.

Potential mechanisms of action and genetic analysis

Although the effects of *T. gondii* on host behaviour have been well studied, there is still very limited knowledge as to the mechanism by which these behavioural changes might occur. It has been suggested that the parasite's effects in the brain may be indirect, via the host's immune response⁸⁵ and/or that the parasite may have a direct effect due to its localisation within a particular brain region, or by indirectly and/or directly affecting neuromodulator levels⁶.

The immune response

T. gondii elicits a strong cell-mediated immune response in healthy individuals and it is not a virulent pathogen in immunocompetent hosts. After the development of cell-mediated immunity, the tachyzoite stage is cleared from the host tissues, but the slowly multiplying bradyzoite stage persists. The bradyzoite stage is isolated from the host immune system by a cyst wall, these dormant cysts able to persist chronically within the tissues of the

intermediate host. The cyst stage of *T. gondii* appears to affect a variety of brain cells, including astrocytes, neurons and microglia. These cells also produce cytokines as a response to infection, which control inflammatory responses⁸⁶. Cyst reactivation is prevented during chronic infection by parasite-specific T cells which release high levels of IFN- γ . Immunocompromised hosts are often unable to prevent cyst reactivation, often leading to toxoplasmic encephalitis in immunocompromised AIDS patients⁸⁷.

These local immune responses in the brain required to keep *T. gondii* dormant alter cytokine levels which could then subsequently influence neuromodulator levels and host behaviour. The production of IFN- γ in response to *T. gondii* infection may be involved in a mechanistic pathway as elevated levels of IFN- γ have been shown to be associated with altered behaviour, such as increased biting behaviour⁸⁸ and reduced emotionality⁸⁹ in mice. *T. gondii* is also thought to increase kynurenic acid in the brain⁸⁵, which is associated with decreased levels of extra-cellular dopamine in the striatum^{90, 91}, but increased activity of dopaminergic neurons⁹². In this way the immune response to the parasite may allow it to indirectly affect neuromodulator levels in the brain, and thereby host behaviour.

Localisation in the brain

A mechanism by which *T. gondii* may alter host behaviour may involve the parasite having a direct effect on the brain, particularly given the parasite's localisation in the brain. *T. gondii* cysts have been shown to be ubiquitously distributed throughout all brain regions, with a high frequency in neuronal cells⁶⁰. In *T. gondii* infected rodents which show neurodegeneration and behavioural changes related to non-specific pathologies, this could be due to blockage or interference with the functions of infected neurons. It has however been shown that infected neuronal cells can continue to form synapses⁶⁰. These pathologies alone are unable to explain specific behavioural changes observed in *T. gondii* infected rodents, particularly as many important behavioural characteristics such as social status and mating success can be left unaltered by infection⁹³. It also would not explain the apparent specificity of the parasite on altering behaviour to increase predation by its feline definitive host.

One explanation for the specific behavioural alterations caused by *T. gondii* could be the potential preferential localisation of *T. gondii* cysts to specific brain areas, although this has not at present been shown. Three brain regions of particular interest include the olfactory bulbs, the amygdala, and the nucleus accumbens. The specificity of the behavioural alteration to feline odour, as well as the apparent attraction of infected rats to feline odour

rather than simply a diminished behavioural trait, suggests that a general reduction in olfaction is unlikely. There is the possibility that *T. gondii* could produce inactivation of a few olfactory receptors that specifically detect feline odour, supported by the fact that different predator species activate specific olfactory brain regions in rats⁹⁴. The amygdala is thought to mediate specific fears, whilst other brain regions may evoke generalised anxiety response after prolonged exposure to a stressful environment⁹⁵. A predilection of *T. gondii* for the amygdala could therefore be involved in altering species specific, stimuli specific fear responses. The final brain region of interest, the nucleus accumbens, is a collection of neurons within the striatum thought to play a role in reward, pleasure and fear⁹⁶. Rodent studies have shown that an increase in extracellular dopamine in the nucleus accumbens can lead to a hyperdopaminergic state, i.e. a long-lasting increase in dopamine metabolism⁹⁷. This is particularly interesting in relevance to my research as if *T. gondii* infection does increase levels of dopamine in this brain region, the effects may be long lasting beyond the point of initial infection, perhaps allowing the increase in reward-seeking and motivational behaviours associated with dopamine⁷ to continue over the host's lifespan. The direct effect of the parasite by its localisation in the brain is plausible; however differential localisation is inconsistent between studies at present⁶⁰, and there is no total saturation of all brain regions with the parasite. This may depend on parasite strain, as different strains have different abilities to persist and form cysts, therefore comparisons between Type I, II and III strains would be useful in future studies, as well as newer variants that have been recently discovered such as Type X⁹⁸. In this study I chose a Type II strain thought to be an avirulent cyst-forming strain, so as to avoid pathology but to allow cyst-dependent effects to be observed. The effect of cysts may also depend largely on host species. It has also been recently suggested that behavioural changes do not require persistence of brain cysts, but occur during early acute infection in a permanent manner⁹⁹; and it has been shown that rats that display immunohistochemically positive neurons rather than actual cysts can also show behavioural alterations¹⁰⁰. Therefore it is possible that the parasite also works via additional mechanisms, which are not mutually exclusive. My study aims to provide further information on these additional mechanisms, which will then further enable us to elucidate which aspects of *T. gondii* altered behaviour are influenced by cyst localisation.

Neurotransmitter modulation

The hypothesis that *T. gondii* cysts directly affect neuromodulator levels in the host brain, by producing increased levels of dopamine which influence host behaviour is of particular interest in my study due to the recent sequencing of the *T. gondii* genome¹⁰¹. This provided an insight into how the parasite's genetic structure may allow it to do this¹⁰¹, enabling me to

use genetically modified parasites to investigate the mechanisms of action. *T. gondii* was found to contain two genes that encode for tyrosine hydroxylase, an enzyme that produces L-DOPA, a precursor of dopamine. Tyrosine hydroxylase metabolises both phenylalanine and tyrosine, with a substrate preference for tyrosine. Phenylalanine is catabolised to tyrosine, and the tyrosine is catabolised to produce L-DOPA. The catalytic domain descriptive of this class of enzymes is conserved within the parasite enzyme, and interestingly it displays similar kinetic properties to metazoan tyrosine hydroxylases, although it contains a unique N-terminal extension with a signal sequence motif. Therefore the enzyme produced by the parasite has similar properties to the enzyme produced in the brain of the mammalian host, suggesting that it may act within the host brain to augment or alter innate behavioural profiles of the host. My study uses the novel approach of using genetically modified (GM) parasites that overexpress this enzyme, to further understand its effects on host behaviour and physiology.

The two genes that encode for the enzyme are TgAaaH1 and TgAaaH2. TgAaaH1 is constitutively expressed, whilst TgAaaH2 is induced during the formation of the bradyzoite stage of the parasite, i.e. the cyst stage¹⁰¹. This was ascertained by quantitative RT-PCR of mRNA levels extracted from purified *T. gondii* pellets¹⁰¹, the same technique used in this study by my collaborators at the University of Leeds to quantify gene expression levels in the GM parasite lines. The fact that higher levels of the enzyme are produced during bradyzoite differentiation is intriguing, as this is the stage of the life cycle with the lowest metabolic activity, indicating a strong potential role for the encoded product. Expression of TgAaaH2 mRNA increases up to ten-fold in parasites during conversion from the fast growing tachyzoite stage to the cyst-forming bradyzoite stage, whilst TgAaaH1 mRNA levels remain constant. It is uncertain as to whether expression of TgAaaH2 is strictly stage specific, but current evidence suggests this to be the case¹⁰¹. The reasons for *T. gondii* to develop stage specific expression of tyrosine hydroxylase are currently unknown, but the dual function of the enzyme may indicate different roles and metabolic requirements in each life cycle stage. As my research is conducted after the formation of the bradyzoite stage, the insights provided may be more relevant to the function of the constitutively expressed TgAaaH1 gene.

This is the first time that an aromatic amino acid hydroxylase has been found in an apicomplexan parasite¹⁰¹. When searched for in other apicomplexan genomes, the genes were not found in *Eimeria*, *Cryptosporidium*, *Theileria* or *Plasmodium*, however an ortholog was found in *Neospora caninum*, lending strong support to this theory as *N. caninum* is the only one of these other apicomplexan which also form a tissue cyst stages, and which

requires predation of the intermediate host by the definitive host (canids) to complete the life cycle. In addition the signal peptide motif conserved in the tyrosine hydroxylase gene in *T. gondii* is also found in its *N.caninum* ortholog. An interesting further study, not within the scope of this thesis, would be to see if *Sarcocystis neurona*, which relies on predation by its opossum definitive host for the completion of its life cycle, also has an ortholog of this gene. Such a study could then be tied in with behavioural studies looking at *S. neurona* infected rats and their response to predation risk by the definitive host.

There are a number of theories for the role of L-DOPA synthesis in *T. gondii* and the increased dopamine levels which have been found in the brains of infected rodents¹⁰². One hypothesis is that L-DOPA is involved in tissue cyst wall formation, supported by knowledge that *Eimeria maxima* contains L-DOPA in its oocyst wall glycoproteins¹⁰³, although these oocysts differs substantially from the tissue cyst of *T. gondii*. L-DOPA may also be associated with increasing chemically reactive molecules containing oxygen, in host cells, as it can cause oxidative damage¹⁰⁴. However, the current most popular theory is that the increased dopamine during infection is linked to the observed behavioural changes seen in rodents, humans and other mammals. *T. gondii* infects neuronal and glial cells, and the cysts form a latent infection, placing it in a prime position to affect host behaviour via the chronic production of dopamine, which is involved in reward-seeking and motivational behaviours⁷. My study will add to this theory by providing further insights into the role of *T. gondii* produced tyrosine hydroxylase (TgTH) in influencing different aspects of host behaviour, physiological factors, the interactions between these, how this may involve the dopaminergic system, and what we may extrapolate from this to human infection.

Aims and Objectives

The mechanisms of parasite manipulation are not fully understood, and my study aims to provide valuable insights into determining the role of *T. gondii* and parasite-produced neuromodulators in altering behaviour in the rodent intermediate host. Effects of *T. gondii* are affected by the strain/ genotype of the parasite⁵; host genes^{27, 105}; host cytokine response^{27, 63, 106}; and the route of infection by the parasite²⁷ (i.e. via ingestion, congenitally or perhaps sexually, and via which parasite stage). In this study the latter three will be kept constant, whilst examining the effect of dopamine in behavioural alterations indirectly by varying the parasite's genotype, using the novel approach of using GM parasites to elucidate the importance of the particular genes associated with the production of *T. gondii* produced tyrosine hydroxylase (TgTH). I examined the link between *T. gondii* infection in rodents and their behaviour in comparison to uninfected rodents, initially with standard wildtype parasite

lines. I then expanded my research to include inoculation with GM parasite strains that overexpress the genes *TgAaaH1* and *TgAaaH2*, which are thought to lead to elevated dopamine levels in the brain. Studies were performed both on behavioural phenotype and on molecular and immunohistological examination of unexposed control rats, wildtype *T. gondii* infected rats, and *TgTH* overexpressor *T. gondii* infected rats. Rat sex was controlled for and investigated in the analyses to help further understand gender differences in this animal host system.

The central aim of my PhD was to use the *T. gondii*- rat model to further investigate the phenomenon of parasite-altered behaviour, and to elucidate the mechanisms by which parasites alter behaviour in mammals including humans. This provides us with a better understanding of potential ways that neural pathways are affected in *T. gondii* infected humans, which will further our knowledge of the epidemiological link observed between *T. gondii* infection and the potential development of schizophrenia. I used a battery of novel and classical non-invasive behavioural assays on the epidemiologically and clinically significant rat-*T. gondii* model to test the type of behavioural alterations being caused by the parasite. These are thought to be of specific selective benefit to the parasite, and hence fall under the category of parasite manipulation. The results of these tests had both theoretical and applied implications²⁶.

The specific objectives of the study were:

- 1) To analyse behaviour of rats – uninfected and *T. gondii* infected – in a number of behavioural tests, designed to measure general anxiety related behaviours and more specific responses to feline (definitive host) and other mammalian odours. I analysed behaviour using EthoVision XT (Noldus, Wageningen, Netherlands), a software programme designed to track and analyse animal movements. This will expand on previous manually recorded studies by being able to include activity and speed of movements as well as enabling more detailed analysis within a shorter time frame.
- 2) To analyse the effects of using GM *T. gondii* strains that overexpress the genes *TgAaaH1* and *TgAaaH2*, in order to better determine the link between *T. gondii* infection, the dopaminergic pathway, and behavioural changes.
- 3) To combine behavioural data with data on levels of dopamine and other neuromodulators in the brains of uninfected rodents and infected rodents from both wildtype and GM *T. gondii* strains, to further elucidate the mechanistic pathway by which behaviour is influenced.
- 4) To use molecular techniques to identify *T. gondii* antibody titre levels and steroid hormone levels within both unexposed rats and those exposed to both wildtype and GM *T. gondii*

strains and use these to help elucidate further the potential link between *T. gondii* infection and schizophrenia.

This thesis thus consists of four data chapters focussing on the behavioural and physiological effects of wildtype and TgTH overexpressor *T. gondii* infection within the laboratory rat host. Within Chapter 2 I aimed to investigate the effects of both wildtype and novel TgTH overexpressor *T. gondii* infection on generalised anxiety. I hypothesised to find that *T. gondii* infection reduces generalised anxiety behaviour, as this would lead to increased risk taking behaviours and chance of predation by the definitive host. The aim of Chapter 3 was to assess the effects of wildtype and TgTH overexpressor *T. gondii* infection on fear response to feline (definitive host) odour. I predicted that infection would reduce fear response and might in fact cause an attraction to feline odour. Comparisons between odours of different feline species were hypothesised to provide further evidence on the specificity of parasite manipulation, and perhaps capacity of these species as definitive hosts of the parasite. Chapter 4 aimed to investigate the effects of wildtype and TgTH overexpressor *T. gondii* infection on novelty discrimination and recognition memory traits. Infection was predicted to reduce novelty discrimination. Whilst it is known that *T. gondii* produces the enzyme tyrosine hydroxylase, it is as yet unknown as to how this influences behavioural alterations observed in infected rodents, and which behavioural alterations it impacts. The specificity of behavioural alteration caused by *T. gondii* infection suggests that multiple pathways in the complex mammalian brain are likely to be involved to maximise transmission success, and my behavioural assays therefore enable us to distinguish which behavioural traits are influenced by TgTH, and which may be influenced by alternative pathways. From the behavioural assays conducted in Chapters 2, 3 and 4, I predicted to find enhanced behavioural alterations in TgTH overexpressor infected rats compared to wildtype infected rats in some but not all of the behavioural assays, depending on the role TgTH plays in that particular behavioural alteration. The aim of Chapter 5 was to assess the physiological effects of wildtype and TgTH overexpressor *T. gondii* infection on neuromodulator levels, antibody titres and steroid hormone levels. Neuromodulator levels were predicted to show L-DOPA and dopamine levels to be higher in wildtype *T. gondii* infected rats than unexposed controls, and higher still in TgTH overexpressor *T. gondii* infected rats. I predicted to see interactions between infection status and hormone levels and/ or antibody titres, which may provide further explanation as to the gender-specific effects seen with *T. gondii* infection. The general discussion of Chapter 6 places the combined results into a broader context and considers the implications of *T. gondii* infection on adaptive parasite manipulation hypotheses, evolutionary significance in the wild, and relevance to human health.

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Chapter 2

Toxoplasma gondii infection affects generalised anxiety and exploration in the rat, which may in part be associated with *T. gondii*- produced tyrosine hydroxylase (TgTH) increasing activity levels

Summary

The protozoan parasite *Toxoplasma gondii* has previously shown conflicting results in terms of its potential impact on host anxiety levels. A hypothesis exists that the parasite reduces generalised anxiety in the rat intermediate host, increasing the risk of predation by the feline definitive host in order to complete the parasite's life cycle. Anxiety-related behaviours are believed to involve the dopaminergic pathway, and this may therefore be part of the mechanism by which the parasite influences anxiety-related traits. A range of non-invasive, classic behavioural assays based on approach-avoidance behaviour were conducted to assess the impact of the parasite on generalised anxiety. The novel approach of using genetically modified parasites that overexpress *T. gondii* produced tyrosine hydroxylase (TgTH) gives us further insights into the mechanisms behind this, and whether there is involvement of the dopaminergic system. My findings indicate that in some tests there may be evidence that *T. gondii* is involved in reduction of anxiety levels, and that this could be associated with increased activity, with more active animals more likely to enter and explore a risky environment. These alterations may lead the intermediate host of the parasite to be more likely to be predated upon by the definitive host, thus completing the parasite's life cycle. The findings of one test indicate that the TgTH gene may be involved in increased activity levels in females, but caution must be taken as this was not consistent between tests or experiment rounds.

Introduction

Generalised anxiety is broadly defined as anxiety which does not vary with time, and is an enduring feature of the animal demonstrating it¹. Generalised anxiety may reduce an animal's risk of predation over all, but is distinct from the phobic anxiety response elicited by predator odour¹. The protozoan *Toxoplasma gondii* has been shown to alter host behaviour in several ways, many of which are linked to anxiety. Indeed, humans with latent toxoplasmosis have been shown to take more risks in driving, which may be a function of reduced anxiety levels, alongside other explanations such as impaired concentration and psychomotor performance². Tests performed have included a reaction to specific traps, which indicated that, under certain conditions, *T. gondii*-infected rats were trapped earlier than control rats³. The open field test has indicated that infected rats display increased activity⁴, increased exploration of novel objects³, and increased exploration of novel environments⁵, all of which might lead to an increased predation rate. Treatment of infected rats with both anti-*T. gondii* and anxiolytic drugs has been shown to reduce several predation-risk associated behavioural traits⁶. This may have implications for treatment of humans with anxiety related disorders, particularly as the potential link with *T. gondii* infection strengthens.

Recent sequencing of the *T. gondii* genome revealed two parasite genes (TgAaaH1 and TgAaaH2) that encode for the enzyme tyrosine hydroxylase⁷. This enzyme is involved in the synthesis of L-DOPA, a precursor to dopamine and is known to occur in humans. Dopamine in humans plays a role in anxiety and stress responses^{8,9}, for instance dopamine depletion in the prefrontal cortex has been shown to induce anxiogenic behaviours in rats¹⁰, and anxiolytic drugs have been shown to display dopamine receptor antagonism¹¹. *T. gondii* has been shown to increase dopaminergic production *in vitro*¹², leading to the hypothesis that *T. gondii*-produced tyrosine hydroxylase (TgTH) is, at least in part, involved in the mechanism behind behavioural alterations caused by the parasite. To test this hypothesis I used the novel approach of assessing the behavioural alterations in rats induced by infection with wildtype *T. gondii* compared with unexposed controls, and genetically modified (GM) strains of *T. gondii* which overexpress the gene TgAaaH2. Previous studies in humans and mice have indicated that gender may play an important role in behavioural outcomes post-infection¹³⁻¹⁵. Many laboratory rodent studies to date only use one gender, often male, in order to limit variation and avoid complications arising from female oestrus cycle impacting behaviour^{16,17}. Due to the differences exhibited between the sexes however, knowledge on the specific behavioural alterations of both male and female rats is therefore an important

component of *T. gondii* research, and should not be excluded. This study includes analyses both controlling for sex, but also investigating the variation between them.

Anxiety tests are a standard procedure often performed on rodents. Rats are a suitable animal model for testing for anxiety-related behaviours, due to their natural approach-avoidance behaviour. This behaviour has evolved due to an evolutionary conflict between the need to explore novel situations in the hope of discovering new food sources, and the need for caution and avoidance to minimise the risk of predation. Anxiety tests allow the rat to freely explore two environments, one of which should induce more anxiety than the other. The time or the ratio of entries into the more anxiety-inducing environment provides us with a measure of the rat's level of anxiety. Such tests can be used to further investigate the behavioural alterations caused by *T. gondii* infection on generalised anxiety behaviours in its intermediate host, and potential mechanisms behind this. I have used a battery of complementary and/or contrasting non-invasive behavioural assay tests, both novel and that have been used in previous *T. gondii* studies, to elucidate whether anxiety is reduced through *T. gondii* infection (elevated plus maze test¹⁸ and the hole board test), and to further understand how generalised anxiety is reduced, and whether this is associated with balancing defects or light sensitivity (the light-dark Suok test¹⁹), hippocampal function, the serotonergic system (the marble burying test and the burrowing test), or the olfactory system (the object and odour exploration tests).

In this study I predicted that *T. gondii* infected rats would display reduced levels of anxiety, spending more time in open arms in the elevated plus maze, more time in the light zone in the light-dark Suok test, and more time in proximity to the novel odour or object in the exploration tests. I predicted there may be a higher number of head-dips in the holeboard test, a lower number of marbles buried in the marble burying test, and a lower level of burrowing in the burrowing test. These tests in conjunction with the novel approach of using GM modified *T. gondii*-TgTH overexpressors were used to ascertain the effect of this gene on anxiety-related behaviour. Based on previous findings of decreased anxiety in infected compared to uninfected rats^{20, 21}, I hypothesised that rats infected with parasites that overexpress the TgTH gene may display decreased anxiety compared to wildtype infected rats whilst rats infected with both wildtype and TgTH overexpressor genotypes will have lower anxiety levels than the uninfected controls. This study helps to elucidate the potential links between latent toxoplasmosis, anxiety related behaviours, and the involvement of the dopaminergic pathway in rodents²², thus giving us further insights as to the evolutionary and medical implications of these.

Materials and Methods

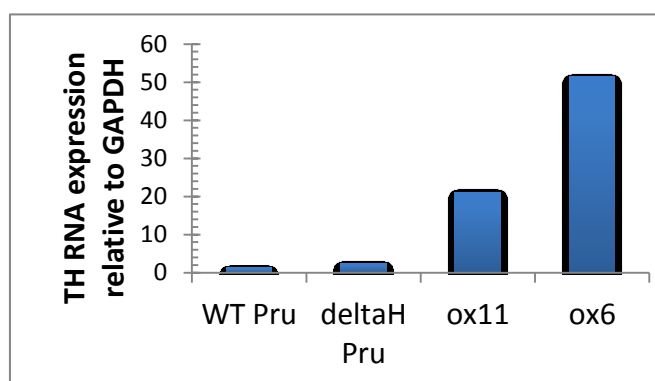
Host maintenance

Equal numbers of male and female Lister-hooded rats were obtained at 3 weeks of age from Harlan UK Ltd., and in order to minimise any generalised non-specific stress/ anxiety level, were accustomed to handling until adulthood. Rats were housed in groups of four, until male rats reached 500g weight, after which such males were housed in pairs. Female rats did not reach this weight margin. Rats were housed in aerosol controlled plastic cages with solid bottoms, with wood shavings and woodchips used as bedding. Bedding was changed two to three times weekly, and the cage sterilised weekly. Rats were fed and watered *ad libitum* on a pelleted rodent diet and sipper tubes. Environmental enrichment was provided through addition of items such as cardboard tunnels and shredded paper.

Sample sizes were calculated using Mead's resource equation, $E = N - T^{23}$. (See appendix 2 for further details on calculations). All tests conducted on rats were fully PPL and PIL licensed and classed as 'mild'. All rats were euthanized at the end of the study via a schedule 1 method, and there was no parasite-induced morbidity or mortality throughout the study.

Parasite strains

All parasites were cultured in HFF (Human foreskin fibroblast) cells by Dr. Greg Bristow at the Institute of Integrative and Comparative Biology at the University of Leeds and the level of TgTH RNA expression was determined by qPCR. TgTH



overexpressor strain infected rats were infected with strains

overexpressing the genes encoding tyrosine hydroxylase TgAaaH2, which overexpressed in both the tachyzoite and bradyzoite stages, determined by quantitative RT-PCR of mRNA. Recombinant lines were developed that overexpresses TgTH several-fold over the wildtype, parental Prugniard strain (Fig. 1). These contained the TgAaaH2 gene under the control of a *T. gondii* tubulin promoter. In the second experiment round the Ox11 line was used. This was one of a collection of overexpressor lines that overexpress TgTH and was mid-range in

Figure 1: Generation of overexpression strains at University of Leeds. Log scale of TH RNA expression in the tachyzoite stage, normalised to GAPDH RNA expression

the level expressed. In the third experiment round the Ox6 line was used. This was the highest in the level of TgTH expressed (Fig. 1). As well as the two overexpressor lines Ox11 and Ox6, two 'wildtype' strains (WT Pru and deltaH pru) were used in my study (Fig.1). These were Pru (type II) strains of *T. gondii*, which was chosen as it is an avirulent cyst-forming strain²⁴. Rats were infected at approximately 3 months of age. Parasite strains were all maintained in the laboratory at the University of Leeds for several generations.

The three experiment rounds had treatment groups as follows:

First experiment round: Unexposed controls (n=12) and wildtype 1 strain (WT Pru) (n=12) infected animals

Second experiment round: Unexposed controls (n=6), wildtype 2 strain (deltaH Pru) (n=12) and TgTH overexpressor 1 strain (Ox11) (n=12) infected animals

Third experiment round: Unexposed controls (n=6), wildtype 1 strain (n=11) and TgTH overexpressor 2 strain (Ox6) (n=16) infected animals.

In the first and third experiment rounds, the wildtype parasite was an original stock Pru strain of *T. gondii*. In the second experiment round the 'wildtype' parasite was actually a modified Pru stain. This modification was independent of the TgTH genes, and therefore had wildtype-level TgTH expression, but was a strain altered for the purpose of mutagenesis within the laboratory. This parasite had the ku80 and the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) genes knocked out. The ku80 gene is involved in non-homologous end joining, and knocking out this gene deletes the dominant pathway mediating random integration of targeting episomes²⁵. The HXGPRT gene is a metabolic gene that provides the parasite with resistance to mycophenolic acid²⁶. This allowed us to control for subtle strain differences that did not involve overexpression of TgTH. For the purposes of analysis I have referred to this strain as 'wildtype' as the expression of the TgTH gene was to the same level, although this strain was in fact a genetically modified mutant. Two replicate lines that overexpressed the TgAaH2 gene were used in order to ensure any effects observed were related to TgTH overexpression from this gene, rather than from any other unrelated strain effects.

T. gondii parasites were released from HFF cells in Leeds, counted, and then transported from Leeds to London in Dulbecco's Modified Eagle's Medium (DMEM) solution. *T. gondii* parasites were syringe-lysed using a 27 gauge needle to release tachyzoites, then centrifuged. The DMEM was removed, and the parasite material resuspended in sterile phosphate buffer solution (PBS) and densities estimated. Infection was conducted via interperitoneal (i.p.) injection with 0.2 ml per rat containing approximately 1×10^6 tachyzoites

in sterile PBS. This amount was chosen based on a previous study which also used i.p. inoculation of Pru strain tachyzoites in rats²⁷, and did not result in the development of any sickness pathologies or complications. Uninfected sham control rats were IP injected with 0.2 ml of sterile PBS.

Behavioural assays

Behavioural testing of rodents generally falls into two categories – conditioned and unconditioned behaviours²⁸. The behavioural repertoire of a rodent can be affected by many factors such as their sex, food supply, and dominance hierarchies²⁸. The behavioural tests I performed were all unconditioned behavioural tests. *Unconditioned tests* include ethologically based paradigms that consider the animals' spontaneous or natural reactions and all those chosen here did not involve any pain or discomfort¹. Conditioned behavioural tests were not chosen as I wanted all tests to be non-aversive and applicable by examining natural behavioural traits whilst mimicking environmental situations encountered by wild rats. These experiments were designed to test the hypothesis of anxiety reduction in Lister-hooded rats infected with wildtype and TgTH overexpressor *T. gondii* strains, in order to assess whether consistent results can be found in this rat strain, and to gain information on the mechanisms of action behind these behavioural alterations, such as the dopaminergic pathways potential involvement.

Rat behaviours were recorded and analysed using the behavioural tracking software Ethovision XT (Noldus, Wageningen, Netherlands). This was used to record data such as rat ID, date, number of faecal boli produced during the test, and coded condition (unexposed, wildtype strain, TgTH overexpressor strain). The ability of automated tracking to record velocity of movement, and thereby activity levels of rats, is of particular importance in *T. gondii* research and was currently under researched²⁹. There is a convincing body of evidence that *T. gondii* increases activity levels in both rats⁴ and mice^{30, 31}, which may be predicted to increase the chance of predation by the feline definitive host³². In terms of the association between *T. gondii* and schizophrenia, symptoms of schizophrenia, such as psychomotor agitation and hyper-responsivity to psychotomimetic drugs, are modelled in rodents by testing locomotor responses and hyperactivity³³. Thus one could perhaps propose that the increased activity observed in *T. gondii*-infected rats and the mechanisms behind this may be helpful in further understanding schizophrenia symptoms. This is of interest as it may suggest that the evolutionary drivers we hope to understand behind *T. gondii* manipulation may have further applied implications in medical research and understanding the proposed link between *T. gondii* infection and schizophrenia in humans³⁴.

³⁵. All apparatus used were cleaned in between testing each rat using Trigene, to remove any olfactory cues, and dried before use. Testing occurred at 6 weeks post-inoculation, as at this stage it was expected the parasite would have reached the chronic bradyzoite stage of infection²⁰ when we expect subtle behavioural alterations during a chronic toxoplasmosis infection^{36, 37}.

The elevated plus maze

The elevated plus maze (Fig. 2) measures generalised anxiety behaviour, and is based on the classic approach-avoidance theory²⁸. It has been used as a model of state, unconditioned anxiety for over two decades, and is well documented and reviewed^{18, 38, 39}, and is therefore comparable with other related



Figure 2: Rat in closed arm of elevated plus maze

tests. It is an easy to use, non-aversive, automated assay of unconditioned responses based on natural behaviours seen in wild rats⁴⁰, and can detect subtle changes which I predicted to arise from *T. gondii* infection³². The behavioural assay relies upon the rat's proclivity toward dark, enclosed spaces (approach) and an fear of heights and open spaces (avoidance)¹⁸. It has been shown previously that rats infected with up to 1000 of the virulent RH *T. gondii* strain tachyzoites show increased open arm exploration, although at higher levels of infection pathology overrides this effect²⁰.

The elevated plus maze comprises of two open and two enclosed (30 cm high walls) arms, each 50 cm long and 10 cm wide and elevated 50 cm off the ground arranged in a plus shape. The assessment of anxiety behaviour is measured as the duration of time spent on the open arms.

The rat was taken out of its cage and placed at the junction of the open and closed arms, facing the open arm opposite to the experimenter. Ethovision XT then recorded the rat for a period of 5 minutes¹⁸ when rats demonstrate the most robust avoidance responses⁴¹. Ethovision XT is able to record the number of entries made by the rat into the open and closed arms, and the time spent on the open and closed arms. The elevated plus maze was cleaned between uses as described above.

The light-dark Suok test

The light-dark Suok test (Fig. 3) is built around the same classical approach-avoidance theory as the elevated plus maze. The Suok test evokes two potential threats – the fear of height, reinforced by the difficulty of retaining balance, and the fear of novelty. As there is a close association between balance control and anxiety⁴², this test provides us with further information on this anxiety-vestibular link¹⁹ as well as assessing them simultaneously. Certain neural circuits are shared by pathways that mediate autonomic control, vestibular control and anxiety⁴². These are modulated by the monoaminergic system, that directly links balance disorders with emotional dysregulation. As *T. gondii* infection may influence anxiety behaviours in rats²² and has been shown to cause sensorimotor deficits in mice⁴³, this test allows for both aspects of behavioural alteration to be studied. Dopamine is involved in the modulation of vestibular pathways⁴⁴, and in anxiety-related behaviour⁴⁵, therefore alterations in this behavioural test in wildtype and TgTH overexpressor *T. gondii* infected rats may indicate involvement of the dopaminergic system, and analysis of neuromodulator levels within the brains of infected and uninfected rats providing further insight into these alterations and the mechanisms behind them.

The apparatus consisted of a wooden alley (240 x 5 x 1 cm³) separated into 16 sectors (15



Figure 3: Rat in the light zone of the light-dark Suok test

cm each). This was elevated to a height of 20 cm by two vertical stands, with a 30 cm two-sector central zone. The room was darkened by covering the ceiling lights with black and red polythene film. Red film was used as it has been shown that rats perceive dim red light as darkness, due to their

dichromatic vision, enabling the

investigator to see, but the rat to be in presumed darkness⁴⁶. The light zone was created by placing five 60W lamps 50 cm above one half of the apparatus to illuminate it.

The rats were transported from the holding room to the experimental room for acclimation 1 hour before testing. The animal was placed in the centre of the apparatus with the snout facing the dark end. The movements of the rat were then detected using Ethovision XT for 10 minutes. Unfortunately in the first set of experiments Ethovision XT was sometimes

unable to correctly detect the animal, due to the large light gradient of the background. This meant that velocity was unable to be used as a response measure. This was modified in the second set of experiments as only the dark zone and centre zone were recorded, allowing velocity in the dark zone to be measured. Duration of time in the light zone was calculated as: *Total duration – (duration in dark zone + duration in centre zone)*.

The number of faecal boli were recorded, as this is an additional anxiety indicator for rats⁴⁷. A limitation of the experiment was that behaviour may have been affected by the presence of the experimenter in the room, necessary in case the rat fell off the beam. Disturbance was kept to a minimum, and remained constant for each trial.

The marble-burying test⁴⁸

The marble burying test (Fig. 4) is dependent on hippocampal function, and is inhibited by many compounds that are active on the serotonergic system, many of which attenuate anxiety, depression or obsessive-compulsive disorder (OCD)⁴⁸. This is of relevance to toxoplasmosis research as there may be some evidence that *T. gondii* cysts preferentially locate in the hippocampus⁴⁹, and that *T. gondii* may have links with OCD in humans⁵⁰. This may also link in with *T. gondii* altered host anxiety levels²², as anxiety levels may be



Figure 4: The marble burying test

disrupted in OCD⁵⁰ as well as other human mood disorders associated with infection⁵¹⁻⁵⁴. The aim of my study was to look for evidence that may provide support for these links, and indicate any potential involvement of the TgTH genes. Marble burying behaviour may, in part, display elements of defensive burying⁵⁵⁻⁵⁷, which is of particular interest for *T. gondii* as this can be a predation-

related defence mechanism. The test mimics an environmental situation encountered by wild rats, therefore making it more applicable to the natural intermediate host of the parasite in the wild. *T. gondii* infection may affect hippocampal function, as the parasite has been shown to invade hippocampal neurons and glial cells⁵⁸, and the septohippocampal pathway is involved in the innate fear of feline odour²⁷. In terms of *T. gondii* and its association with schizophrenia, there is evidence for the hippocampus playing an important role in the neuropathology of schizophrenia⁵⁹.

Twenty five marbles of 15mm in diameter (John Lewis, London, UK) were placed within a rat cage in a 5 x 5 regular arrangement, each being 2 inches apart on top of approximately 1 cm of woodchip bedding. Rats were placed individually in the cage and after 30 minutes the number of marbles buried completely or to within 2/3 of their depth counted (Fig. 4). The number of faecal boli produced during the test was counted. Marble burying data was collected from the second experiment round.

Burrowing test

The burrowing test can be used to sensitively measure a wide range of behavioural abnormalities⁶⁰. Burrowing behaviour may, in part, display elements of defensive burying^{55-57, 60}, which could be of particular interest for *T. gondii* research if associated to predation-related defence mechanisms. Defensive burying is a paradigm for measuring anxiety, and the burrowing test is a sensitive test that can detect subtle behavioural changes and be linked to anxiety as well as other behavioural dysfunction^{48, 60}. An advantage of this test is that it allows digging behaviour to be quantitatively and manually measured, and can give a more detailed profile than marble burying. Burrowing is decreased by selective serotonin reuptake inhibitors and by changes in interleukin levels in rats⁶⁰, and serotonin levels within rats were measured (Chapter 5) to see if there are any correlations between infection status, serotonin levels and burrowing behaviour.

Burrows were created using soil pipe material (Nu-Line, London, UK) 4 inches in diameter and 33.3 cm long. Red autoclavable bagging was used to seal the end of the burrow. The



burrows were then filled with 1500g of sterile playsand (Argos, London, UK). Burrows were placed in an empty rat cage with normal woodchip bedding covering the surface of the floor, and a lid was placed on top of the cage to contain the rat. The test time was 2 hours and then the volume of playsand remaining in the burrow was

Figure 5: The burrowing test measured, to provide a measure of the amount of sand removed by burrowing (Fig. 5). Percentage of sand removed by burrowing was calculated as $(1500\text{g} - \text{number of grams of sand remaining in burrow after 2 hours}) / 1500 \times 100$. 95% confidence intervals were

calculated by STATA, based on S.E.M. The number of faecal boli produced during the test was also counted.

The Hole board test⁶¹

The Hole board test (Fig. 6) is widely used to measure anxiety levels⁶² and exploration of a novel environment^{61, 63} in rodents, and is therefore replicable and comparable with other related tests. It tests an unconditioned response based on natural behaviours seen in wild rats⁴⁰, is non-aversive and can detect subtle

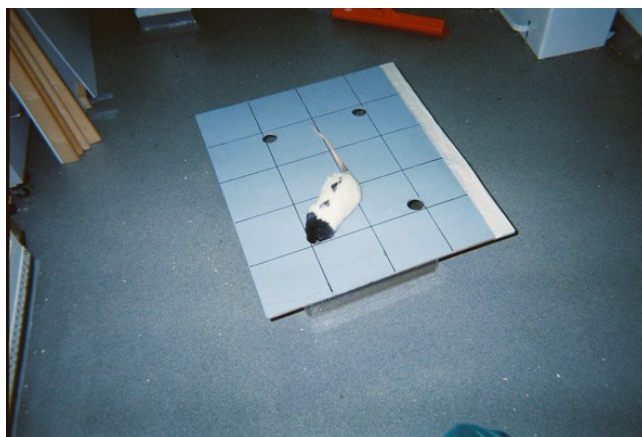


Figure 6: The hole board test

changes such as those which are expected to arise from *T. gondii* infection³². *T. gondii* infected mice have been shown to display increased exploratory behaviours in the holeboard test in previous studies⁶⁴, which was suppressed by GBR 12909, a selective dopamine uptake inhibitor, suggesting that this test may provide further insights into the association between *T. gondii* infection and changes in the dopaminergic neuromodulatory system⁶⁴, and thereby the potential link with schizophrenia in humans^{34, 35}.

A four-hole holeboard was used, consisting of a wooden board measuring 65 x 55 cm, divided into 16 sections. Four central holes were cut of diameter 3 cm. The board was elevated to a height of 20 cm. The number of head dips and faecal boli produced were recorded over a period of 10 minutes. A 'head dip' was defined as the animal inserting its head into the hole up to its ears. If the nose was inserted but the ears were not touching the surface of the hole, this was not counted.

The object and odour exploration tests

The object and odour exploration tests were designed to measure and compare the rat's level of exploration of a previously unencountered visual stimulus, or a previously unencountered olfactory stimulus. There is evidence that *T. gondii* infected rats may be attracted to feline odour⁶⁵, but other research has shown that alterations in anxiety-related behaviours are unlikely to be related to a simple disruption of the olfactory system³⁶. By observing unexposed, wildtype and TgTH overexpressor strain infected rats in the object



Figure 7: The object exploration test

and odour exploration tests, I was able to differentiate between responses to a novel object and response to a novel odour. This allows us to further test the hypothesis that *T. gondii*-altered behaviours are not related to alterations in the olfactory system, and enables us to elucidate how the parasite may

manipulate the host's behaviour.

The object exploration⁶⁶ test was conducted in an arena measuring 100 x 100 cm. An object was placed in a corner of the arena. The amount of time the animal spent in the quadrant of the arena containing the object was recorded, as well as its velocity of movement, frequency of entry and latency to enter the object quadrant. The object used was a smooth red Perspex cylinder (Fig. 7). The positioning of the object was



Figure 8: The odour exploration test

randomised to avoid positional bias. At the start of the test the animal was placed facing away from the object in the opposite quadrant. The test time was 10 minutes. All apparatus was wiped down with Trigene in between tests to remove odours. The odour exploration test was conducted in the same way but with an odour placed in a corner of the arena (Fig. 8). To provide the odour 0.5g of dried ground mint was mixed with woodchips in a bowl.

Statistical analysis

Analyses for the behavioural tests were performed using STATA 11 (StataCorp, Texas, USA). Parametric multivariate regression models were used. All variables and/or their residuals were tested for normality and transformed or categorised if necessary before any parametric testing was conducted. Continuous data were transformed to categorical data where necessary using quantiles. Experiment round and parasite strains were controlled for. For each regression model, multivariate analyses were conducted for each outcome variable

looking at the independent variables infection status, rat sex, velocity of movement, and experiment round where applicable. Interactions were tested for between infection status and each other independent variable, and where this was significant the interaction term remained in the model, otherwise it was excluded. Analyses were conducted separated by rat sex, as sex is shown to strongly affect behavioural traits in humans. Therefore a novel component of my study was to look at the effect of sex both in uninfected and *T. gondii* infected rats, and analyses were also conducted without separation of genders where no interaction effect was noted between infection status and rat sex. For the object and odour exploration tests, one-sample t-tests were used to assess whether animals spent more time in the object or odour zone than would be expected if there was no exploration.

Results

Data from anxiety tests indicates some involvement of *T. gondii* and TgTH in anxiety and activity traits. All but two exposed rats sustained infection, as indicated by serological analyses, and data from these two rats were excluded from the analyses (Chapter 5). No effects of infection were observed on number of faecal boli (an indicator of anxiety level) in any test. I have chosen to represent figures as individual data points rather than means, as it is increasingly being suggested that individual variance allows more effective comparisons of animal behavioural traits than mean trait values^{67, 68}.

Elevated plus maze

The results of the elevated plus maze test indicated that overall, wildtype ($F_{6, 78} = 13.46$, $p=0.03$) and TgTH overexpressor infected animals ($F_{6, 78} = 13.46$, $p=0.001$) displayed a higher duration in open arms than unexposed controls (Fig. 9). Wildtype infected animals were not shown to be significantly different from the other TgTH overexpressor infected animals ($p>0.10$). When looking within experiment round, there was some weak evidence in the second experiment round that TgTH overexpressor infected animals had a higher duration in the open arms than wildtype infected animals ($F_{3, 19} = 21.79$, $p= 0.061$). Females spent longer in the open arms than males ($F_{3, 83} = 18.15$, $p=0.001$), with females spending a mean duration of 60.5 seconds (95%CI, 50.2-70.8) in the open arms and males 40.37 seconds (95%CI, 31.2-49.5)

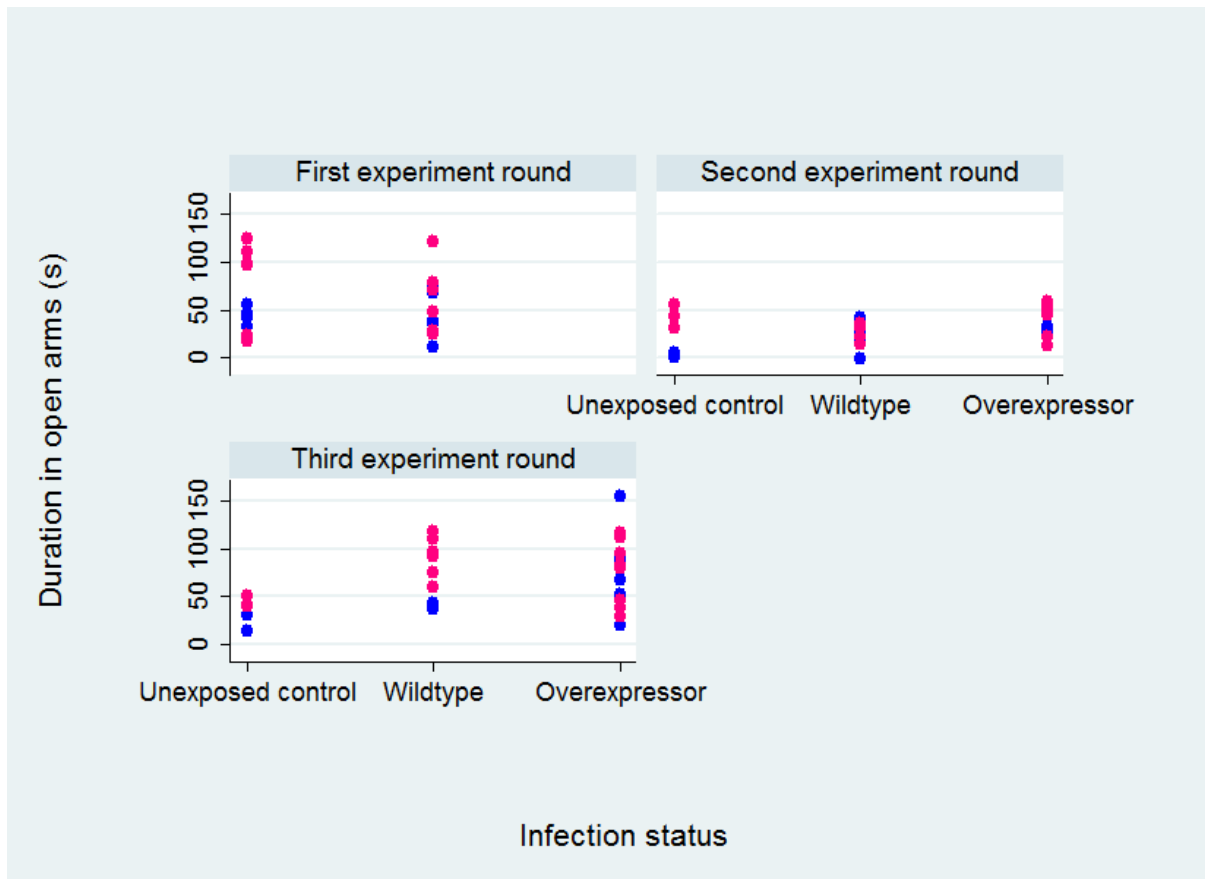


Figure 9: Duration of time spent (in seconds) in the open arms in the elevated plus maze test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from all three experiment rounds. Total duration of the test was 5 minutes. Males are shown in blue and females in pink.

Velocity of movement was not shown to be affected by infection status in this test ($p > 0.10$, Fig. 10). Females were shown to display a higher velocity of movement in the elevated plus maze test ($F_{3,83} = 18.15$, $p < 0.001$). Duration in open arms and velocity of movement are strongly positively correlated in this test ($F_{1,85} = 29.03$, $p < 0.001$). Both duration in open arms ($F_{3,75} = 5.16$, $p = 0.003$) and velocity of movement ($F_{3,76} = 4.74$, $p = 0.004$), were affected by an interaction between experiment round and infection status.



Figure 10: Velocity of movement in the elevated plus maze test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from all three experiment rounds. Males are shown in blue and females in pink.

Light-dark Suok test

When looking at all experiment rounds combined, accounting for the interaction term between infection status and experiment round, no effect of infection status on duration in light zone was observed ($p > 0.10$) (Fig. 11). There were weak interaction effects between infection status and rat sex in this test ($F_{2,77} = 2.61$, $p = 0.08$).

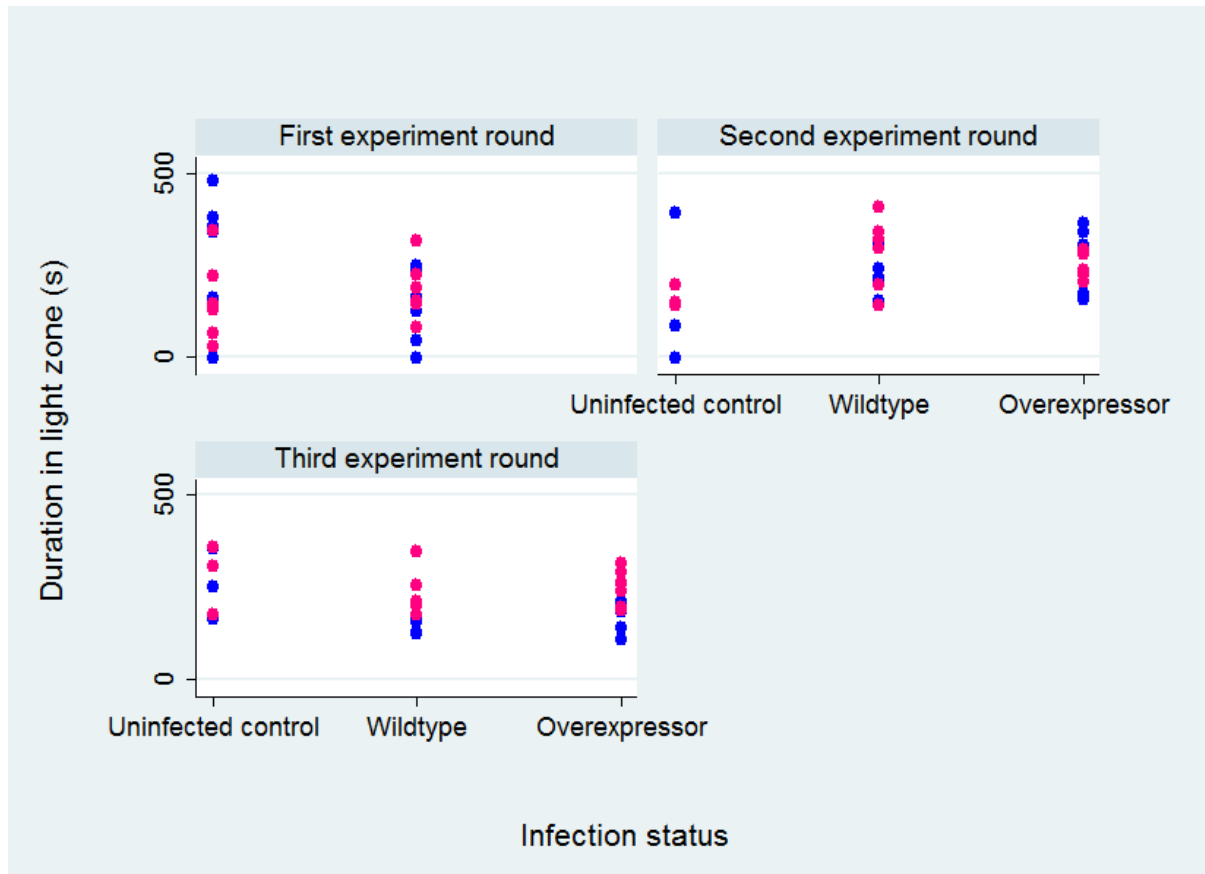


Figure 11: Duration in light zone in seconds, in the light-dark suok test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the three experiment rounds. Total test time was 10 minutes. Males are shown in blue and females in pink.

Within males, there was evidence that velocity of movement was higher in wildtype ($F_{6,54} = 8.61$, $p = 0.032$) and TgTH overexpressor ($F_{6,54} = 8.61$, $p = 0.003$) infected animals than the unexposed controls. Within females, velocity of movement was higher in TgTH overexpressor infected animals than wildtype ($F_{2,23} = 3.60$, $p < 0.032$) and unexposed control animals ($F_{5,26} = 4.93$, $p = 0.008$). These effects were found after accounting for the strong interaction effects between infection status and experiment round ($F_{2,54} = 14.50$, $p < 0.001$), indicating that opposing effects were found in the second and third experiment rounds, and this result appears to be found predominantly in the second experiment round. Duration in light zone and velocity of movement were positively correlated in this test ($F_{1,61} = 18.59$, $p < 0.001$).

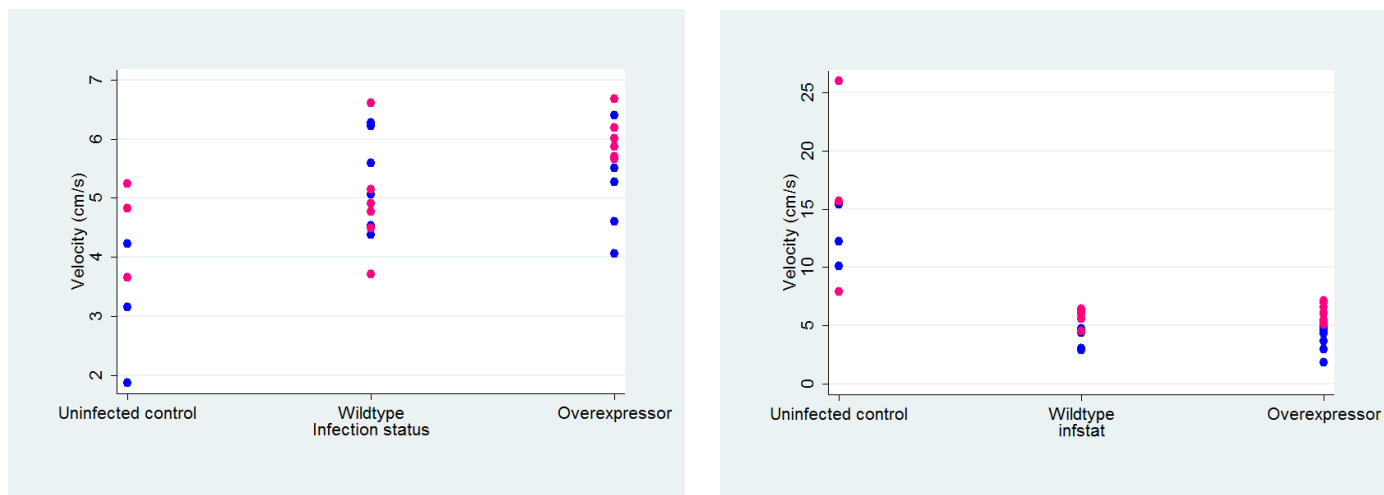


Figure 12: Velocity of movement in the dark zone of the light-dark suok test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Males are shown in blue and females in pink.

Marble-burying test

No effect of infection status was observed for the number of marbles buried in this test. There were no differences between unexposed controls, wildtype and TgTH overexpressor infected animals ($p > 0.10$). Females buried on average 14.1 (95%CI, 12.0 – 16.1) of the 25 marbles in 30 minutes (Fig. 13), significantly more than the 10.8 (95%CI, 7.7 – 13.8) which males buried ($F_{2, 33} = 3.53$, $p = 0.060$). No interaction effects were found ($P > 0.10$).

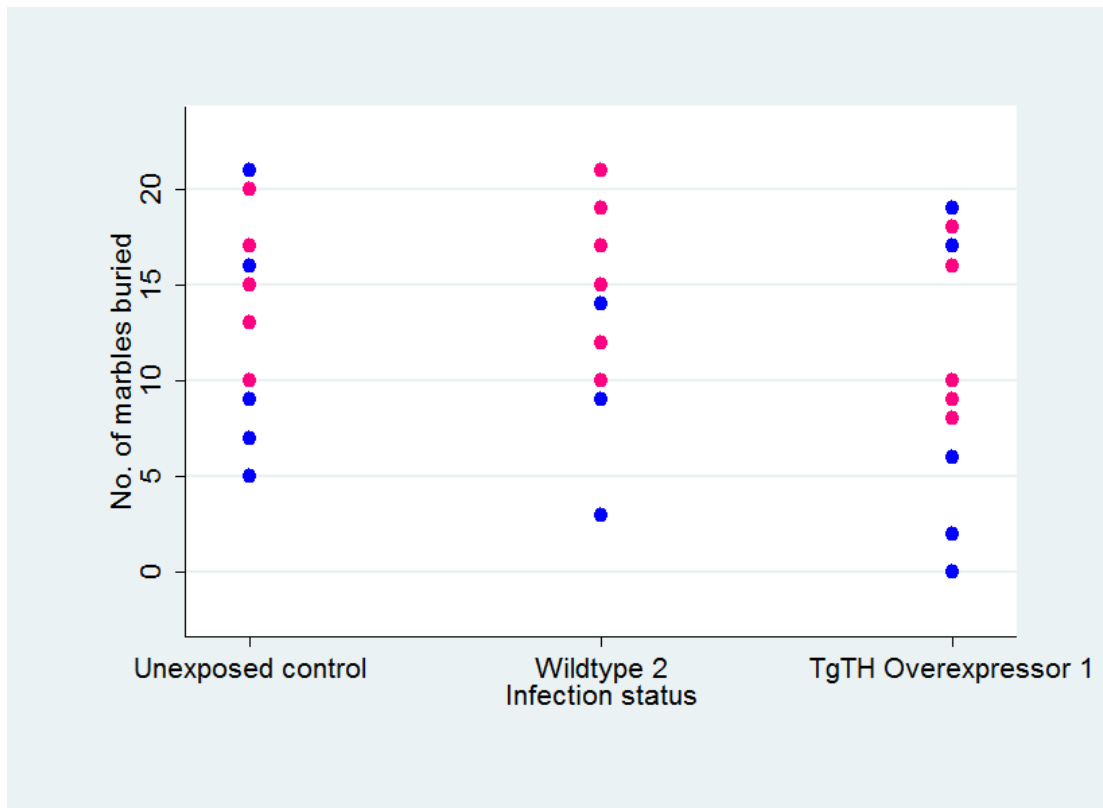


Figure 13: Number of marbles buried in the marble burying test of unexposed control (uninfected rats), *T. gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data are from the second experiment round. Males are shown in blue and females in pink.

The Burrowing test

There was weak evidence that wildtype ($F_{4, 56} = 9.29$, $p=0.097$) and overexpressor ($F_{4, 56} = 9.29$, $p=0.066$) infected animals removed less sand by burrowing than unexposed control animals (Fig. 14). Unexposed control rats removed 69.4% (95% CI, 47.4-91.3) of sand, wildtype infected rats removed 45.6% (95%CI, 26.9 – 64.3) and TgTH overexpressor infected rats removed 43.0% (95% CI, 27.0 – 59.0) over the 2 hour period. Female rats burrowed more than male rats ($F_{2, 60} = 17.33$, $p = 0.020$).

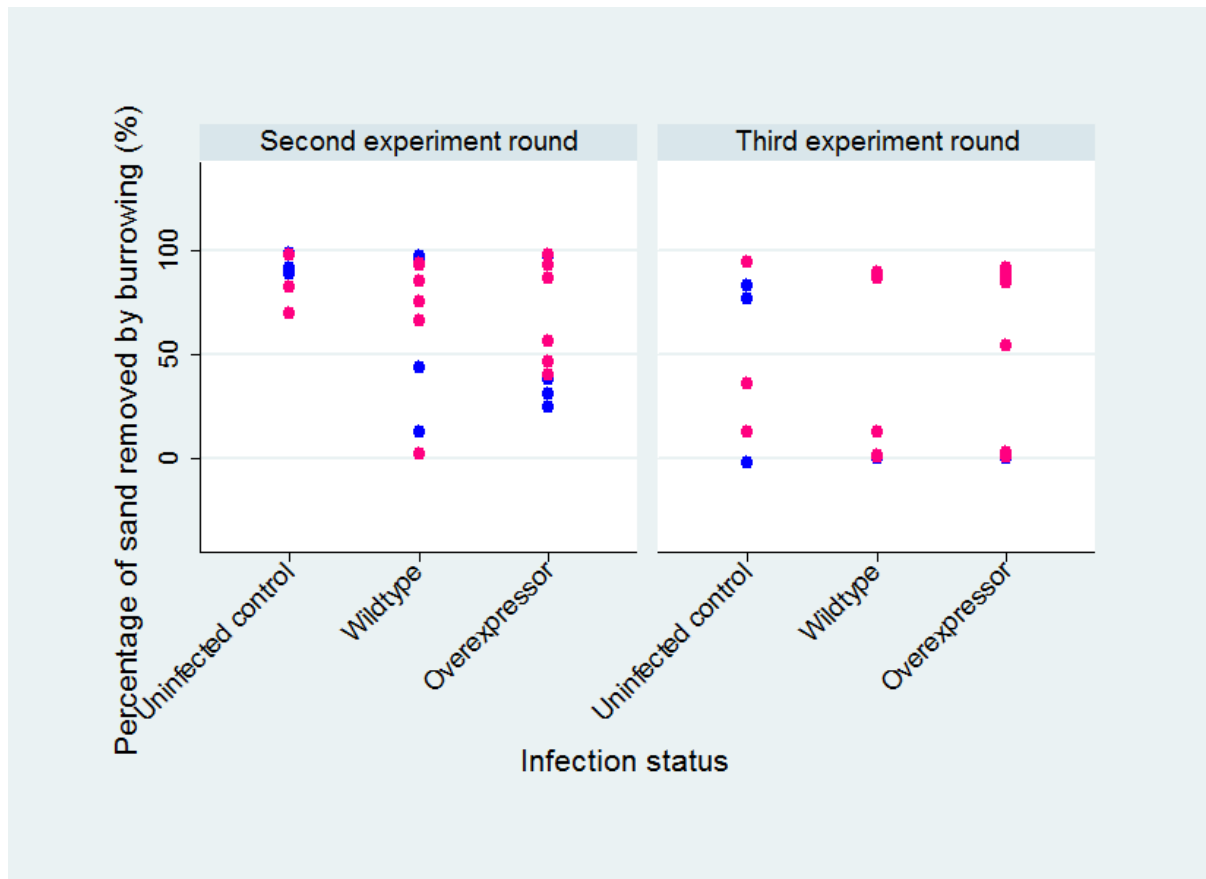


Figure 14: Percentage of sand removed by burrowing in the burrowing test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Males are shown in blue and females in pink.

The Hole board test

The results of the hole board test indicated a significant interaction between infection status and rat sex ($F_{2, 48} = 4.12$, $p < 0.022$), with male wildtype ($F_{3, 22} = 3.89$, $p = 0.005$) and overexpressor ($F_{3, 22} = 3.89$, $p = 0.004$) infected animals displaying a lower number of head dips than unexposed controls, with the mean number of head dips being 12.3 (95%CI, 10.9-13.8) for unexposed control males, 7.4 (95% CI, 5.6-9.1) for wildtype infected males and 7.2 (95% CI, 5.1-9.3) for TgTH overexpressor infected males. Within females, the opposite effect was observed, with wildtype infected animals displaying a higher number of head dips than unexposed controls ($F_{4, 24} = 5.16$, $p = 0.058$), and mean number of head dips being 7.7 (95%CI, -0.3 – 15.7) for unexposed control females, 10.7 (95%CI, 7.2-14.2) for wildtype infected females and 11.2 (95%CI, 9.3-13.1) for TgTH overexpressor infected females (Fig. 15). There were also interaction effects between infection status and experiment round ($F_{1, 49} = 3.13$, $p = 0.08$).

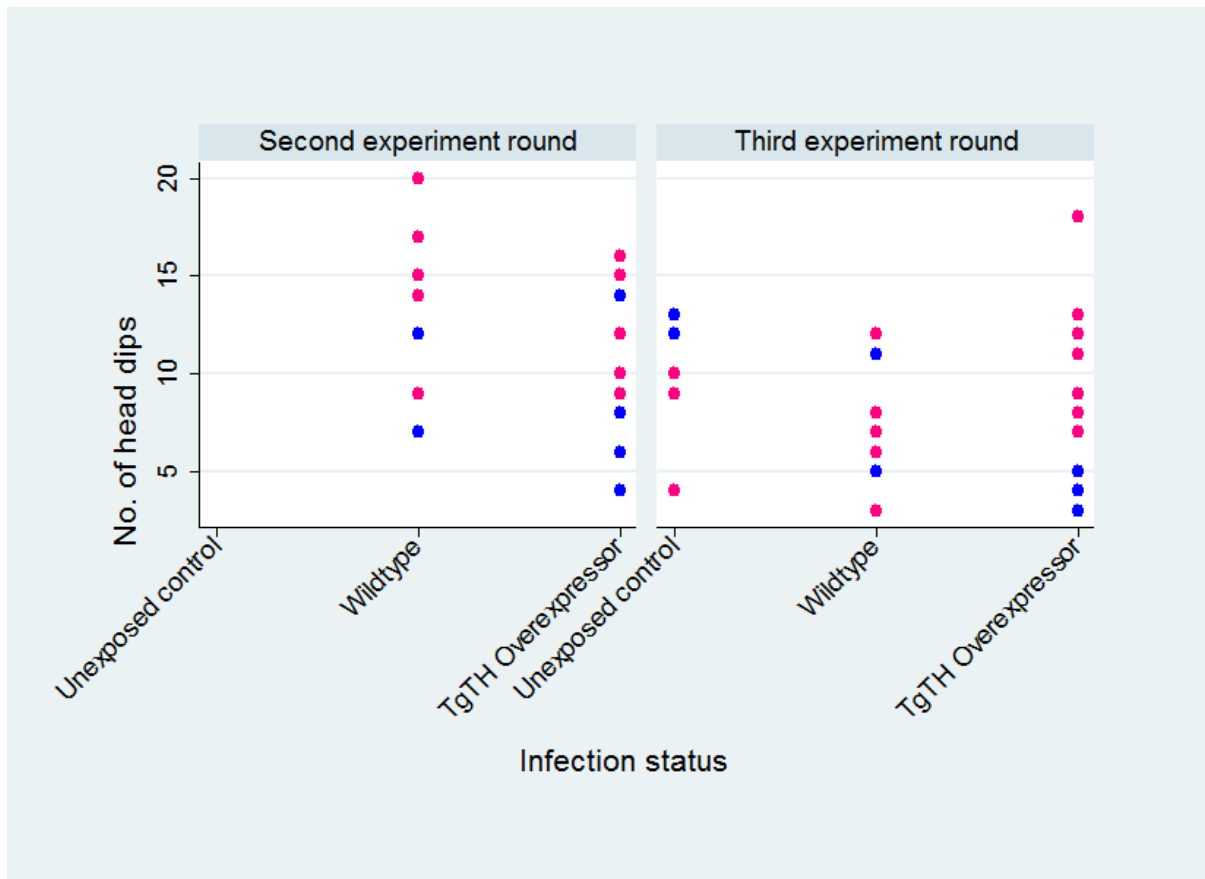


Figure 15: No. of head-dips in the holeboard test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Data was not collected from unexposed rats in the second experiment round. Males are shown in blue and females in pink.

Object exploration

Both wildtype ($t_{11} = 3.01$, $p=0.01$) and TgTH overexpressor infected rats ($t_{10} = 4.44$, $p=0.001$) spent a higher about of time in the object zone than would be expected if there was no exploration of the object (150 seconds, a quarter of the test time, shown by the red reference line). This was not seen in the unexposed control rats (Fig. 16). The results indicate weak evidence that wildtype ($F_{3,25} = 1.39$, $p=0.095$) and TgTH overexpressor ($F_{3,25} = 1.39$, $p=0.092$) infected rats displayed an increased duration in the object zone as compared to unexposed control rats, although this effect was confounded by velocity. Mean duration in object zone was 162 seconds (95%CI, 104-220) for unexposed control rats, 222 seconds (95%CI, 169 – 274) for wildtype infected rats and 224 seconds (95%CI, 187-261) for TgTH overexpressor infected rats. No differences were found between infection status' for mean distance from the object ($p>0.10$). No interaction effects were found ($p>0.10$).

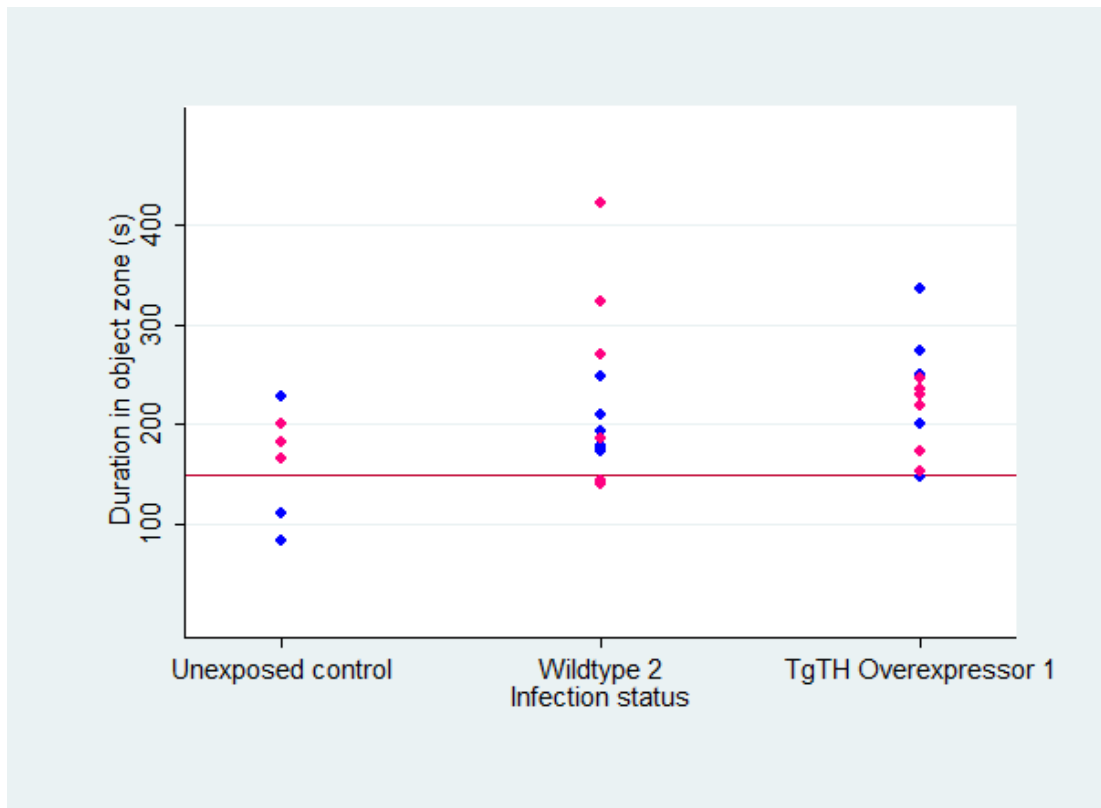


Figure 16: Duration in object zone in the object exploration test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data are from second experiment round. The red reference line indicates 150 seconds, time expected if no exploration. Males are shown in blue and females in pink.

Odour exploration

Wildtype infected rats ($t_{11} = 4.18$, $p=0.002$) spent a higher about of time in the odour zone that would be expected if there was no exploration of the odour (i.e. time was spent randomly distributed around the test zone), shown by the red reference line at 150 seconds (Fig. 17). Within TgTH overexpressor rats, only females showed weak evidence of exploration of the novel odour ($t_5 = 2.46$, $p = 0.057$), whilst TgTH overexpressor males did not ($t_4 = 0.22$, $p = 0.83$). The evidence for exploration of odour in unexposed controls was not significant ($p>0.10$). There were no significant differences of the duration spent in the odour zone between unexposed controls, wildtype and overexpressor infected rats ($p > 0.10$).

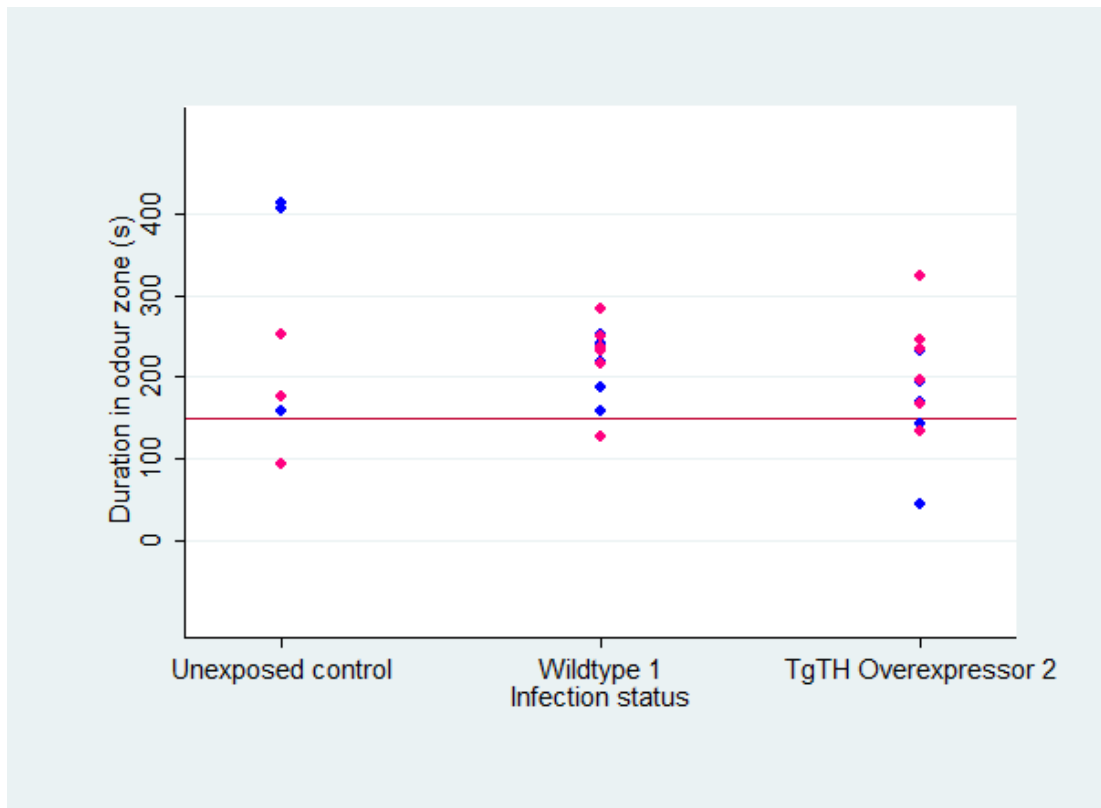


Figure 17: Duration in novel odour zone in the odour exploration test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data are from second experiment round. The red reference line indicates 150 seconds, time expected if no exploration. Males are shown in blue and females in pink.

TgTH overexpressor infected males displayed a higher mean distance to odour than wildtype infected males ($F_{(3, 10)} = 5.35, p=0.034$) and unexposed control males ($F_{(3, 10)} = 5.35, p=0.003$). This effect was not seen in females ($p>0.10$; Fig. 18). Mean distance to odour was 76.4 cm (95%CI, 63.6-89.2) for TgTH overexpressor infected males, 63.7 (95%CI, 58.9-68.6) for wildtype infected males and 49.7 (95%CI, -3.1-102.5) for unexposed control males.

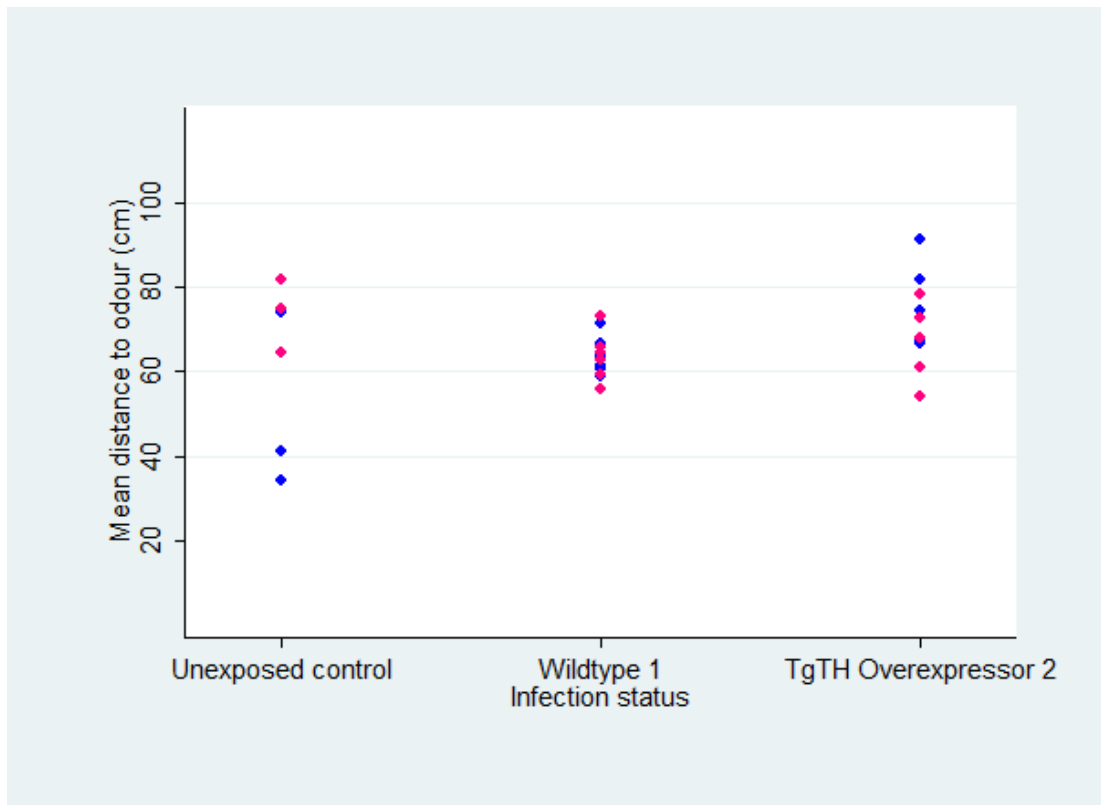


Figure 18: Mean distance to novel odour in the odour exploration test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data are from second experiment round. Males are shown in blue and females in pink.

Discussion

Though there were high levels of individual and experimental round variability observed, the results from some behavioural tests suggest that *T. gondii* infection may be associated with anxiolytic effects in rats. There was potentially some evidence for the role of the TgTH genes demonstrated by the results of the elevated plus maze, as in the second experiment round TgTH overexpressor infected animals spent slightly longer in the open than wildtype infected animals. TgTH overexpressor infected animals did not however overall display significantly different behaviour than wildtype infected animals in this particular test, therefore this evidence may not be conclusive. The elevated plus maze may be measuring more than one facet of behaviour, such as anxiety, activity, and balance, meaning that it can be difficult to fully elucidate the responses from this test alone, and results should be interpreted in conjunction with other behavioural assays. It appears that, in this case anxiety is affected by *T. gondii* infection. The effect of duration in open arms was found independently of velocity of movement. It has been suggested that the behaviour of female rats in the elevated plus maze is more characterised by motor activity than by anxiety⁶⁹, whilst male rats may be driven more by anxiety behaviours. The positive correlation between duration in open arms

and velocity of movement does indeed suggest that anxiolytic behaviour and activity levels are linked, and that more active animals may display less cautious behaviours. This could be a balancing phenomenon, but is still interesting in terms of mechanisms as to whether the parasite is influencing anxiety behaviour through altering activity, or via another mechanism. It has in fact recently been suggested that parasite manipulative effects may act on such correlations of host traits, rather than independently on individual traits⁶⁸. The effect of rat sex on the velocity of movement may be potentially due to the test measuring different variables in males and females, with male responses being driven primarily by anxiety, but female responses in the elevated plus-maze being characterized more by activity than anxiety⁶⁹.

The results of my study are consistent with previous research where infection with *T. gondii* has been shown to have an anxiolytic effect on both male^{4, 20} and female^{4, 70} rats in an elevated plus maze. Similar directions of effects are expected in both sexes, as *T. gondii* is thought to both reduce anxiety levels and increase activity levels⁴. It has also been shown that in some cases, the subtle behavioural changes induced by *T. gondii* can only be observed in the most active rats⁶⁵. My results did not indicate an effect of rat sex independent of velocity, as the main effect of sex appeared to be on activity levels with females displaying higher velocity of movement than males. This contradicts previous findings which showed male and female rats to display different behavioural profiles in the plus maze, with female rats showing reduced aversion to open arms compared to males⁷¹.

When looking at the light dark test overall, no effect was observed of infection status on duration in light zone. This test showed that in the second experiment round, wildtype and TgTH overexpressor infected male animals displayed higher velocity of movement than unexposed controls, and in females, TgTH overexpressor infected animals displayed higher velocity of movement than wildtype and unexposed control female animals in the second experiment round. It is possible that in this test, balancing defects were counteracting the effect of anxiety reduction, as *T. gondii* infection may be involved in anxiety reduction but not in an associated improvement in balance control, with infection not affecting neurological pathways that mediate links between balance and anxiety⁴². As with the elevated plus maze, the anxiolytic outcome and activity levels were strongly positively correlated, therefore the effect of infection on increasing activity levels may be linked to less cautious behaviours. This may lend support to the hypothesis that wildtype parasite, and tentatively TgTH overexpressor parasite, induces anxiolytic behaviours through increasing activity levels. There is no conclusive evidence for the role of TgTH in anxiety reduction, as no difference was observed between wildtype and overexpressor infected animals. The increased velocity

of movement of TgTH overexpressor infected females as compared to wildtype infected females in the second experiment round does, nevertheless, indicate a potential role of TgTH in increasing activity levels, at least within females. It is possible that TgTH may be interacting with sex specific hormones, and therefore its effect is more pronounced in females than in males, or that the effect of TgTH is more pronounced in the generally faster moving female animals. This test therefore provides some weak evidence to support the theory that *T. gondii* may be involved in increase in activity levels, although we cannot say this conclusively as this was not consistent between experiment rounds. The interaction effects seen between infection status and experiment round may be due to the high sensitivity of this test to vestibular function, where some infected rats may have responded differently to the anxiety-vestibular link. This may also explain the reduced variation seen in infected rats as compared with unexposed controls in this test. Mice infected with an avirulent strain of *T. gondii* have been shown to display sensorimotor deficits⁷², and although this has not been observed in rats *per se*, results from mice can be extrapolated to rats and any subtle alterations in balancing behaviour may therefore have an impact in this test.

The burrowing test was another assay that indicated an effect of infection on burrowing behaviour, although there was no evidence for involvement of TgTH in this, as TgTH overexpressor infected animals were not shown to behave differently to wildtype infected animals. As the effects of TgTH in the light dark Suok test were shown to be related to activity levels and velocity of movement, it is possible that in this test where movement of the animal across the arena was not a feature, the effects of TgTH were not observed. It is possible that the results of the burrowing test may be indicating an anxiolytic effect of infection, if burrowing is indeed a measure of anxiety, as defensive burying is, or at the very least indicates some form of behavioural dysfunction of this natural behaviour observed in uninfected rodents in the wild. However although anxiety levels were shown to be affected by infection status in the tests above, this was not supported by the marble burying test. As both marble burying and burrowing behaviour are thought to involve hippocampal function and the serotonergic system, it is unclear as to why an effect was found in the one and not the other, and this may simply indicate that the burrowing test is more sensitive. Therefore the burrowing test may be providing evidence that *T. gondii* infection has some impact on hippocampal function and/ or the serotonergic system, but there is no evidence from either test that TgTH is involved in this neurological pathway.

The hole board test results indicated opposing effects in male and female rats, with infection reducing the number of head dips in males (an anxiogenic effect) but increasing the number of head dips in females (an anxiolytic effect). It is possible that this is due to interaction with

sex specific hormones, and indeed studies in humans have suggested that *T. gondii* infection may have opposing effects on hormones depending on sex¹⁴. However it is important to note that it has been suggested rather than being an indicator of exploration and/or anxiety levels⁷³, head-dipping may instead measure an escape response, which decreases with decreasing fearfulness⁷³. It has been shown previously that the effects of *T. gondii* infection were seen in a 16-hole hole board test⁶⁴, which indicated that *T. gondii* infected female mice displayed increased head dipping behaviour, supporting our findings⁶⁴. This effect was not seen in males, and indeed in my study there may in fact be an opposite effect in males, with head dipping actually reduced in infected individuals in comparison to male uninfected controls. These results support other studies which indicate that *T. gondii* infection may have opposing or differential effect in males and females¹³⁻¹⁵, and reiterate that a wide range of behavioural assays must be conducted to further understand this, particularly as rat anxiety behaviour tests themselves are strongly influenced by sex^{69, 71}.

Exploration of the novel object by all animals was observed in the object exploration task, but only wildtype infected animals displayed evidence of exploration in the odour exploration task. The increased duration in the object zone of wildtype and TgTH overexpressor infected animals as compared to unexposed controls indicates that there was some effect of infection on increasing exploration levels in the animals, but that this might not be due to TgTH expression as the TgTH overexpressor infected animals did not show a greater response. It could be argued that such a response was associated to less cautious behaviour and thereby increased risk of predation, including by the feline definitive host. This result differs from the findings of the directly comparable odour exploration test, where TgTH overexpressor infected males did not show evidence of increased exploration, and displayed a higher mean distance to the novel odour. This in fact opposes the hypothesis that TgTH is involved in reducing neophobia or anxiety levels. However these findings may lend support to the hypothesis that *T. gondii* infection and TgTH may impair novelty discrimination mechanisms⁷⁴. It could be suggested that the overexpression of TgTH has led to the lack of recognition of the novel odour as a novel or anxiogenic stimulus. These results are particularly intriguing as they oppose the effects found in previous studies using feline odour⁶⁵, lending support to the theory that attraction to feline odour is specific manipulation⁵ to increase chance of transmission to the definitive host, which I will further investigate in Chapter 3. It could also be suggested that intensity of infection and TgTH production are involved in attention deficit pathologies^{75, 76}. Another potential explanation of why this was found in the odour exploration test and not the object exploration test may be that TgTH is involved in impairment of olfactory pathways. Previous evidence suggests that *T. gondii* does not cause a blunt impairment of olfaction⁷⁷, but it may be that, for example, glomeruli

within the olfactory bulb dedicated to innate responses are impaired^{36, 78}. This is supported by some studies that suggest a higher cyst density within the olfactory bulb^{49, 79, 80}.

Many of the behavioural tests including the elevated plus maze test, the light-dark Suok test and the hole board test showed in some instances interaction effects between infection status and experiment round, which were controlled for but may explain some of the inconsistencies seen in results in this and previous studies⁸¹. There was also variation within the wildtype and TgTH overexpressor lines, although this was confounded by experiment round. This highlights that the effects of *T. gondii* are subtle, and are subject to high variation depending on individual host, parasite, environmental and laboratory factors^{37, 81}. The results from the generalised anxiety tests tell us that *T. gondii* infection does indeed appear to be involved in reduction in generalised anxiety, and this is likely to increase risk of predation, both by the feline definitive host and by other predators. This may suggest that the selective pressures on *T. gondii* to enter the feline definitive host are strong enough to override the possibility of the intermediate host being predated upon by a non-definitive host predator. In terms of mechanisms, our results from the burrowing test suggest possible involvement of the hippocampus and serotonergic system, as these have been shown previously to influence behaviour in this test⁶⁰. The odour exploration test results imply possible involvement of the olfactory bulb. The evidence for the role of TgTH from the second experiment round of the light-dark test appears to be that it is involved in increasing activity levels, and may have an anxiolytic effect in association with this, as more active animals are more likely to enter and explore a risky environment. The implications of these findings for humans are as yet unknown, and care should be taken when extrapolating from the rat model to humans, but these findings may provide explanation as to the growing body of evidence that *T. gondii* infection in humans is associated with outcomes such as involvement in road traffic accidents^{2, 82-84} and suicide attempts⁸⁵⁻⁸⁷, which can be indicative of greater risk taking and impulsivity^{88, 89}. The subtle alterations in the rat brain suggest that 'by-product' effects in the physiologically similar human brain are likely to arise, particularly over the course of a chronic toxoplasmosis infection which may span several years²⁹.

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Chapter 3

***Toxoplasma gondii* infection enhances predation risk by altering rat activity depending on proximity to feline odour as well as influencing preference to different feline definitive host species odours**

Summary

Toxoplasma gondii has been shown in several previous studies to override a rat's innate aversion of feline odour, in some cases perhaps even turning this into an attraction. This behavioural alteration is believed to increase the risk of predation of the rat intermediate host by a feline definitive host, and has been shown to be specific to feline odour as opposed to other mammalian or non-definitive host predator odours. In this study I examine the effect of the parasite genotype on feline avoidance/ attraction traits, and whether the behavioural alteration is differentially affected depending on the feline definitive host species. The novel approach of using genetically modified parasites that overexpress *T. gondii* produced tyrosine hydroxylase (TgTH) gives us further insights into the mechanisms behind this, and whether there is involvement of the dopaminergic system. My results potentially indicate that the parasite selectively manipulates the response of the rat host by differentially altering activity profiles depending on the rat's proximity to feline odour. It increases the rat's general activity profiles enhancing the chance of encountering a predation risk, but then opposingly decreasing activity levels once in close proximity to feline odour and thus decreasing the rat's chance of escaping predation by the feline definitive host and increasing the total amount of time spent near the feline odour. TgTH does not, however, appear involved in the specific response to feline definitive host odour suggesting that other mechanisms are more closely involved in altering the rats' specific response to feline odour. In addition *T. gondii* also differentially alters host response to feline odour depending on the feline species, with infection appearing to beget preference for wildcat odour over domestic cat odour, a finding which illustrates the importance of feline urinary components in *T. gondii* studies.

Introduction

Toxoplasma gondii is a protozoan parasite with an indirect lifecycle, of which the only known definitive hosts are members of the cat family (*Felidae*)¹. The sexual cycle of the parasite occurs within the feline gut, after which oocysts are shed with the cat's faeces. Many feline species are thought to be definitive hosts of *T. gondii*, and although prevalence of infections are variable, oocyst shedding has been demonstrated both experimentally and naturally in several wild feline species, including for example the wildcat *Felis silvestris*, the Amur leopard cat *F. euptylurus* and the African wildcat *F. lybica*^{1,2}.

T. gondii has a wide range of intermediate hosts, including small birds and mammals, such as the rat (*Rattus norvegicus*)³. In the wild *R. norvegicus* are subject to predation by a number of species, including cats, foxes, mink, and birds of prey. Rats have an innate aversion to the odour of these predators, which should decrease their risk of predation^{4,5}. Laboratory rats with no previous exposure to cats will avoid contact with a substance where it detects cat odour, and will shelter from it⁶. Exposure to cat odour has been shown to increase anxiogenic responses detected in social interaction and generalised anxiety tests, which can be detected for up to an hour after odour exposure⁶. The physiological changes in rats when exposed to cat odour have been demonstrated to include raised corticosterone levels⁷ and greater c-Fos expression in many brain regions, particularly in the medial amygdala, medial hypothalamus and periaqueductal grey⁷. Different predator odours may have varied effects on the rat – for example cat odour elicits specific defence behaviours which are not observed after exposure to fox odour⁸.

The innate aversion to cat odour amongst rodents provides a profound obstacle for the parasite against successful predation by the feline definitive host. Investigations into the effect of *T. gondii* infection on a rat's response to feline odour proved to have surprising results⁹. *T. gondii* appears to alter a rat's perception of cat predation risk, with uninfected rats displaying normal aversion to cat odour, whilst infected rats showed no avoidance of cat odour treated areas, and in some cases even showed attraction ('Fatal Feline Attraction')⁹. This behaviour has been consistently demonstrated over several studies⁹⁻¹², utilising a range of methods *T. gondii* and various odours, including both a four-choice odour test and a two-choice odour test^{10,13}. Infected and uninfected rats showed a significant diversion in their responses to cat odour, when compared with their reaction to rabbit (a non-predatory mammal) odour. Two-choice odour tests indicated that this altered predation risk is specific to felines, and not to other non-definitive host predators, e.g. mink¹³ and dog¹⁴. This adds

weight to the parasite manipulation hypothesis, which suggests that the parasite may be specifically altering the rat's behaviour to increase its chances of entering the feline definitive host, and thereby completing its life cycle¹⁵. This subtle ability to distinguish between a *T. gondii* definitive and a non-definitive host predator odour indicate that this is a specific alteration in the cognitive perception of the predation risk by the rat, rather than a more general destruction of a behavioural trait or of olfaction¹⁶.

Knowledge on the behavioural changes of *T. gondii* infected rats in response to additional feline species other than the domestic cat (*Felis domesticus*)^{9, 10, 13, 14} and the bobcat (*Lynx rufus*)¹¹ already studied, will help elucidate this feline fatal attraction further. In this study we investigate the effects of other feline species in which oocyst shedding has been demonstrated both in experimental and natural conditions^{2, 17}, such as the cheetah (*Acinonyx jubatus*), and the puma (*Felis concolor*). If the theory of parasite manipulation is correct, it is expected that infected rats would also show reduced aversion to these other feline species. It could also be argued that a lack of co-evolution with these other feline species may reduce any parasite manipulation effect. Four studies on seroprevalence of *T. gondii* in cheetahs have indicated prevalences ranging from around 27.3% – 77.7%, whilst fourteen studies on seroprevalence in pumas have indicated ranges of 7% - 100%¹. Many studies on prevalence in domestic cats have indicated ranges from 5.4% - 84.9%¹. Therefore there is no obvious difference in seroprevalences between the three feline species, and as with all species infected with the parasite, prevalence of *T. gondii* is likely to vary widely based on geographical location and sampling distributions¹. However seroprevalence within a population does not correlate to actual numbers of that population or predation risk of the rodent and so selection pressures may have varied between different potential definitive hosts and the intermediate hosts.

Response to urine odour may also depend on differences in urine components of these feline species. Cheetah and puma urine contain no lipids in their urine, whilst lipids are present in the urine of the domestic cat¹⁸. Cauxin levels have are lower in big cats than male domestic cats¹⁹, and cauxin is not present in puma urine at all²⁰. Presence and levels of feline have also been shown to differ between cat species¹⁹.

The precise mechanisms behind the behavioural alterations of *T. gondii* infected rats to feline odour are as yet unknown. Several neuromodulatory and endocrine mechanisms have been suggested to be involved in feline aversion in rodents²¹. The level of involvement of the dopaminergic system in this is as yet unclear, although it has been suggested that feline odour exposure increases turnover rates of dopamine in the hypothalamus and striatum²².

Both the two-choice and four-choice feline response tests enabled me to further investigate response to feline odour in *T. gondii* infected rats, using the novel approach of genetically modified (GM) lines which overexpress the *T. gondii* tyrosine hydroxylase (TgTH) gene in order to ascertain the effect of this enzyme on feline response behaviour. Tyrosine hydroxylase is involved in the production of L-DOPA, a precursor of dopamine. This will therefore give us further insight into the relationship between *T. gondii* infection, the dopaminergic system, and the 'Fatal Feline Attraction' response²¹. Tests were conducted with unexposed control rats, rats infected with wildtype *T. gondii* strains, and rats infected with GM TgTH overexpressor *T. gondii* strains. These behavioural tests allow us to examine the species specificity of *T. gondii* altered response to predator odour, including between different feline species. The differences in capacities of different feline species as definitive host of *T. gondii* are as yet unknown, and if there are differences in behavioural alterations of the *T. gondii* infected rat intermediate host depending on feline species, this will help elucidate the role of different definitive host species in the evolution of parasite manipulation in *T. gondii*. The use of TgTH overexpressor parasite infected rats will reveal to what extent, if any, TgTH gene expression is involved in these species-specific feline responses.

Rat activity levels were also investigated as previous studies suggest that *T. gondii* infection increases activity in rats and mice²³⁻²⁵ and we wish to also understand the potential co-evolution of these additional behavioural alterations. The novel use of behavioural tracking software has enabled me to quantitatively measure the rat's velocity of movement, both overall and when in proximity to different odours. This enabled me to analyse in further detail the patterns of altered activity in *T. gondii* infected rats, and whether this is influenced by exposure to feline definitive host odour or other odours, and the role of the TgTH gene expression in this behavioural alteration.

I hypothesised that rats infected with parasites that overexpress the TgTH gene may display increased feline attraction behaviours compared to wildtype infected rats in the four-choice feline response test. Previous studies have indicated behavioural alterations in feline response may be seen within the most active rats⁹, and my findings in Chapter 2 indicated positive correlations between anxiolytic outcome and velocity of movement; therefore I predicted there may be similar correlations between activity levels and feline attraction behaviours in these feline response tests, as the more active rats may display less cautious behaviours. Even if TgTh expression is involved in feline fatal attraction I did not necessarily expect TgTH overexpressor parasites to alter preference for different feline definitive host species in the two-choice feline response test, but this was deemed plausible in accordance with the role of the feline species as definitive host in the wild. This study will help elucidate

the potential links between latent toxoplasmosis, feline response behaviour, activity levels and the involvement of the dopaminergic pathway in *T. gondii* infected rodents²¹, thus giving us further insights as to the evolutionary implications of these.

Materials and Methods

Host maintenance and parasite strains

Rats were infected with wildtype *T. gondii* and *T. gondii* TgTH overexpressor strain parasites or sham inoculate with phosphate buffer solution as described previously (Chapter 2; Appendix 2). Three experiment rounds were conducted as before (Chapter 2; Appendix 2), with each experiment round(s) stated below for each behavioural test.

Behavioural assays

Feline response assays were conducted at 6-8 months post-exposure. Behavioural tracking was conducted with Ethovision XT (Noldus, Wageningen, Netherlands) as described previously (Chapter 2; Appendix 2).

Four-choice feline response test

The four-choice feline response assay enables the parasites ability to manipulate innate traits such as fear of feline odour to be measured. Through providing the test rodent with a number of odours, this assay may be comparable to a situation in the wild where a rat would



encounter several mammalian odours, including its own. This allows for any specificity of the difference in response to feline odour as compared with other odours to be recorded. Each test was conducted over a long 2½ hour period of time, assessing a rat's response over time rather than just its initial response

Figure 1: Four-choice feline response test (thereby further minimizing risk of Type I or II errors). Although previous studies were conducted over 12 hours, rats generally remained in a given area after 2 hours and therefore my studies were limited to 2½ hours¹⁰. This test was conducted using a previously described experimental model^{9, 10} and measures innate

physiological and behavioural reactions by rats to cat (urine) odour, own (rat) odour, neutral (water) odour and non-predatory mammal (rabbit) odour^{26, 27}. Rats' responses were assessed through the number of entrances into and/or duration spent in zones with different odours.

In short the experimental arena was a box 100 cm x 100 cm, divided into four 50 cm x 50 cm analysis zones (although there was no physical barrier between the zones). The surface of the box was covered with a layer of woodchips to provide a homogenous surface that could be cleaned between trials. Each corner had a white plastic nest box placed equidistant from the edge facing the centre (Fig. 1). Each corner had two small 3 cm diameter plastic bowls, one containing five food pellets, and one containing 10ml water. Food and water are provided *ad libitum* during the test as food deprivation has been shown to alter anxiety-related responses and activity levels in both male and female rats²⁸. The experimenter was not present in the room during the test, and the positioning of the odours was randomised between trials to minimise positional bias. The importance of this was paramount, as a preliminary trial without odours and with uninfected rats highlighted the impact of extraneous noise, odour and light stimuli on the positioning of the animals within this test, and hence the importance of positional randomisation and experimenter absence (Appendix 3).

The cat, rabbit and neutral odour corners were treated with 700µl of undiluted cat urine (Archway Veterinary Surgery, Wiltshire and the Royal Veterinary College (RVC)), rabbit urine (Harlan UK Ltd. and the RVC) and water respectively. The rat odour corner contained 0.5g of woodchips from the floor of the rat's own cage. In between trials, woodchips were emptied from the box and disposed of and the box, nest boxes, food and water bowls all wiped down with Trigene to remove odours from the previous trials, and allowed to dry.

Two-choice feline odour test – Comparison between different feline species



Figure 2: Two choice feline odour test

The two-choice feline odour test enables the measurement of the number of entrances into and/or the time spent in zones with different feline odours, in this instance domestic cat vs. puma and domestic cat vs. cheetah²⁹ representing the preference for, and/or discrimination against the two particular odours. This test aims to elucidate how species specific the alteration of fear to attraction is, and thereby how likely behavioural adaptations are to enhance transmission of

the parasite. As stated earlier, the two-choice test has previously been used to distinguish between infected and uninfected rats responses to feline definitive host predator and non-definitive host predator odours¹³. In the current study, we hoped to distinguish if *T. gondii* infected rats' responses differ between the odours of different feline definitive host predator species.

A two-choice apparatus was used with arms separated from a start arena by a sliding door (Fig. 2). Within each arm a small layer of woodchips was placed. In one arm 0.7ml of domestic cat urine (Archway Veterinary Surgery, Wiltshire and the Royal Veterinary College (RVC), Hertfordshire) was placed. In the other arm, 0.1 – 0.2g of tissue paper soaked in wildcat urine of either cheetah or puma (RVC) was placed.

Rats were placed in the start arm facing away from the choice arms. The sliding door was lifted, and the rats were allowed to freely explore the maze for 5 minutes. As both odours are aversive, a short test time was used to minimise stress for the animal. Position of odours within the two arms was randomised to avoid positional bias and, woodchips were changed and all apparatus wiped down with Trigen to remove odours before and after each test.

Statistical analysis

Dependent variables for the four-choice feline response test were the animal's velocity, duration spent in each zone, frequency of entry and latency to enter each zone. These variables were continually recorded over the 2½hour period using Ethovision XT. STATA 11 was used to analyse each variable using multivariate regression analyses, described in detail in Chapter 2. Rat sex, infection status, velocity, parasite strain and experiment round were independent variables in analyses. Correlations between duration in zones and velocity of movement were tested for. Interaction effects between infection status and velocity of movement in the four-choice test were found using bootstrap with 1000 repeats of multivariate regression analysis. This was considered appropriate to get a better precision estimate for this dataset as it is a small sample size that deviated strongly from the normal distribution.

In the two-choice feline odour test, the amount of time spent in the arm containing domestic cat urine as compared to the arm containing wildcat urine was assessed. Number of faecal boli produced during the test was also counted. Analysis of response variables for the two-choice test was conducted by creating a discrimination ratio, defined as:

Duration in domestic cat zone/ (Duration in domestic cat zone + Duration in wildcat zone).

If there was no difference between the rats' response to domestic cat odour and wildcat odour, a discrimination ratio that does not differ significantly from 0.5 would be expected. A one-sample t-test was used to test this hypothesis, controlling for experiment round as described in Chapter 2. All variables and/or their residuals were tested for normality and transformed or categorised using quantiles where necessary.

Results

Data from the feline response tests indicate differential responses of wildtype *T. gondii* and TgTH overexpressor infected rats to the urine of different species, as well as interactions with activity levels. All but two exposed rats sustained infection (Chapter 5).

Four-choice feline response test

The results of the four-choice feline response test (Fig. 3) indicate weak evidence that wildtype infected animals spent a higher proportion of time in the cat zone than unexposed controls ($F_{8, 76} = 2.56$, $p=0.08$), after accounting for the interaction between infection status and experiment round. This effect was shown to be strongest in females ($F_{1, 10} = 9.25$, $p=0.012$) in the first experiment round. Wildtype infected females in the first experiment round displayed a mean proportion of time in cat zone of 0.44 (95% CI, 0.11-0.78), as compared to 0.12 (95%CI, 0.05-0.19) in unexposed controls females. No effects were seen with TgTH overexpressor infected rats compared to unexposed controls ($p>0.10$) or wildtype infected rats ($p>0.10$). Overall mean values for proportion of time spent in cat zone were 0.22 (95%CI, 0.11-0.32) for unexposed controls, 0.23 (95%CI, 0.13-0.32) for wildtype infected animals and 0.20 (95%CI, 0.15-0.24) for TgTH overexpressor infected animals.

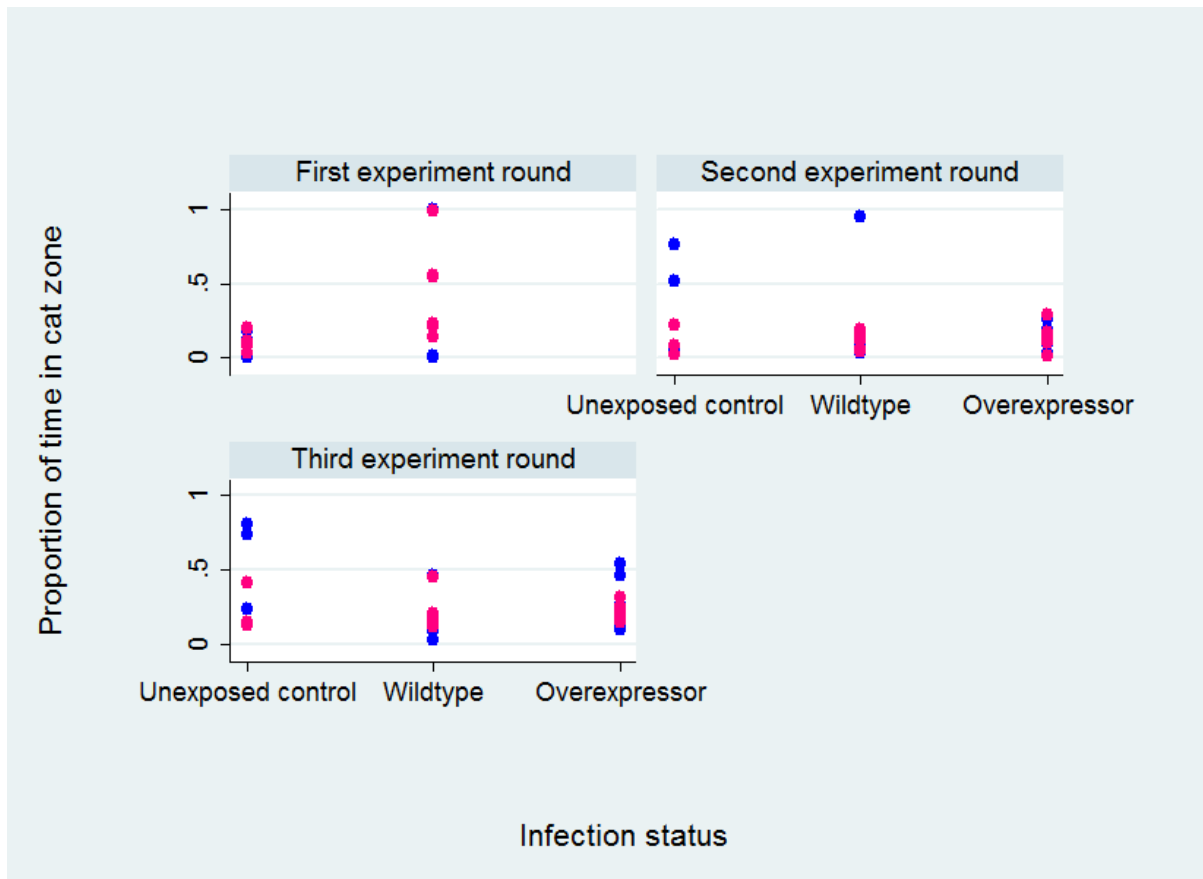


Figure 3: The proportion of time spent in the cat zone in the four-choice chamber of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the three experiment rounds. There were no TgTH overexpressor infected rats in the first experiment round. Males are shown in blue and females in pink.

No effect was seen of infection on velocity of movement ($p > 0.10$). Mean overall velocity of movement was 3.2 cm/s (95%CI, 2.8-3.6) for TgTH overexpressor infected animals, 2.8 cm/s (95%CI, 2.3-3.2) for wildtype infected animals, and 2.9 cm/s (95%CI, 2.3-3.5) for unexposed controls (Fig. 4). Female rats displayed higher overall velocity than male rats ($F_{3,83} = 17.06$, $p < 0.001$). Within all rats, a strong negative correlation was found between proportion of time spent in the cat zone and velocity in the cat zone ($F_{1,85} = 123.51$, $p < 0.001$), and a strong positive correlation was found between proportion of time spent in the cat zone and velocity in non-cat zones ($F_{1,85} = 34.07$, $p < 0.001$).



Figure 4: The velocity of movement in the four-choice chamber of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the three experiment rounds. There were no TgTH overexpressor infected rats in the first experiment round. Males are shown in blue and females in pink. An interaction effect was found between infection status and velocity in non-cat zones (chi-sq = 9.35, $p = 0.009$), with wildtype infected rats (coef. = 0.11, 95% CI 0.08-0.14) displaying

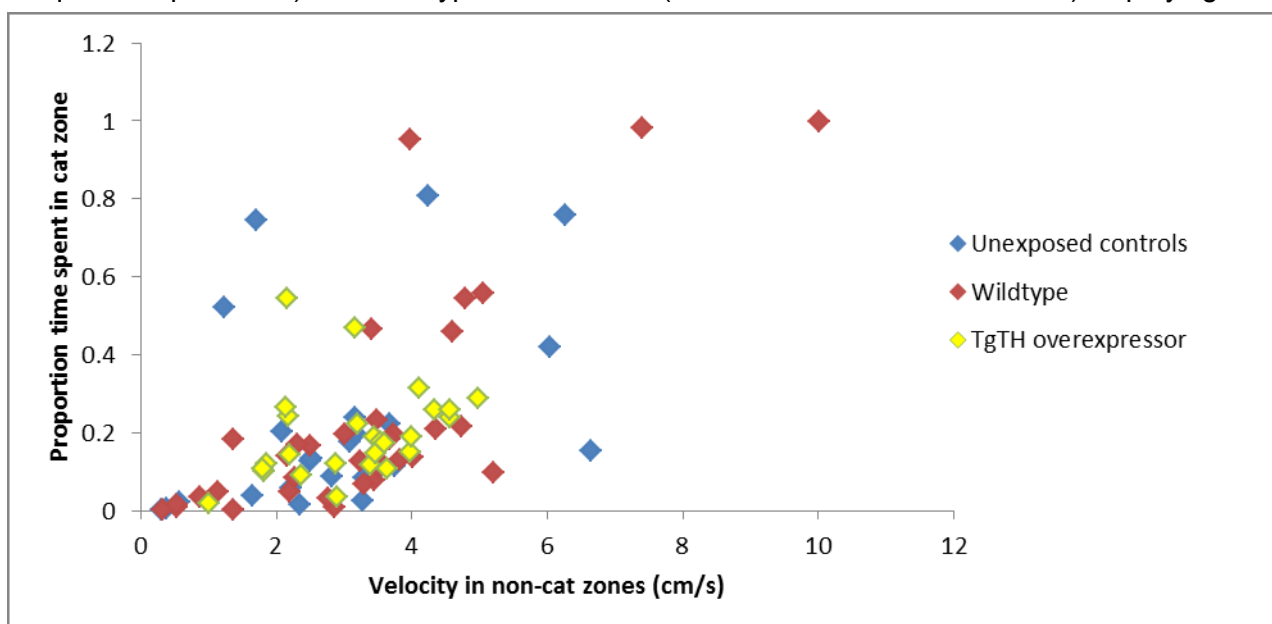


Figure 5: Proportion of time spent in cat zone compared against the mean velocity in non-cat zones in four-choice feline response test for unexposed controls, wildtype and TgTH overexpressor infected rats. a steeper gradient of positive correlation between proportion time spent in the cat zone and velocity in non-cat zones than TgTH overexpressor infected rats (coef. = 0.03, 95% CI -0.01-0.07) (Fig. 5). In other words, wildtype infected rats that moved faster outside of the cat zone also spent more time within the cat zone in relation to the faster moving TgTH overexpressor infected animals. This correlation was also stronger in wildtype infected animals (Adj $R^2 =$

0.57) as compared to TgTH overexpressor infected animals (Adj R^2 = 0.03). No correlation was observed in unexposed control animals ($p > 0.10$).

There was a weak interaction effect between infection status and experiment round when looking at proportion of time spent in the cat zone in this test ($F_{3, 76} = 2.23$, $p = 0.091$).

Two-choice feline response tests

Domestic cat (*F. domesticus*) and cheetah (*A. jubatus*)

The results of the two choice test comparing domestic cat and cheetah odour suggest that wildtype ($F_{4, 56} = 3.26$, $p = 0.003$) and TgTH overexpressor ($F_{4, 56} = 3.26$, $p = 0.006$) infected animals displayed a lower discrimination ratio than unexposed controls. Mean discrimination ratios were 0.61 (95%CI, 0.45 – 0.77) for unexposed controls, 0.40 (95%CI, 0.32-0.47) for wildtype infected animals, and 0.40 (95%CI, 0.35-0.46) for TgTH overexpressor infected animals (Fig. 6). Both Wildtype ($t_{22} = 3.69$, $p = 0.001$) and TgTH overexpressor ($t_{25} = 6.24$, $p < 0.001$) infected rats showed a preference for cheetah odour as both had discrimination ratios significantly below 0.5, whilst unexposed controls displayed a preference for domestic cat odour ($t_{11} = 5.62$, $p < 0.001$).

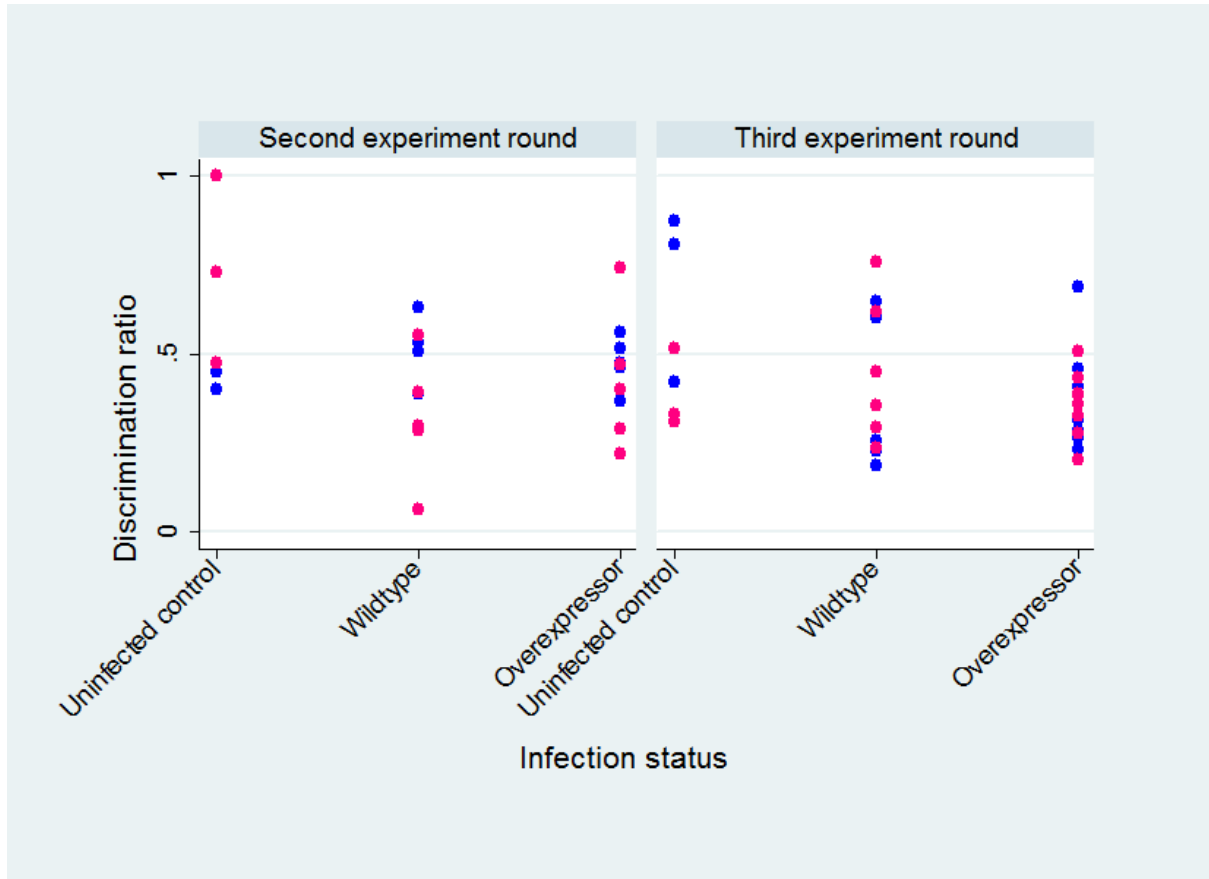


Figure 6: Discrimination ratio in the two-choice domestic cat and cheetah test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Males are shown in blue and females in pink.

An interaction effect was observed between infection status and velocity of movement on discrimination ratio ($F_{2, 53} = 6.62$, $p=0.003$). This suggests that in this test, faster moving rats were responding differently to the odours depending on their infection status (Fig. 7). No overall correlation was observed between velocity of movement and discrimination ratio ($p>0.10$), but within wildtype infected rats, a positive correlation was observed between discrimination ratio and velocity of movement ($F_{1, 21} = 9.38$, $\text{Adj } R^2 = 0.28$, $p=0.006$).

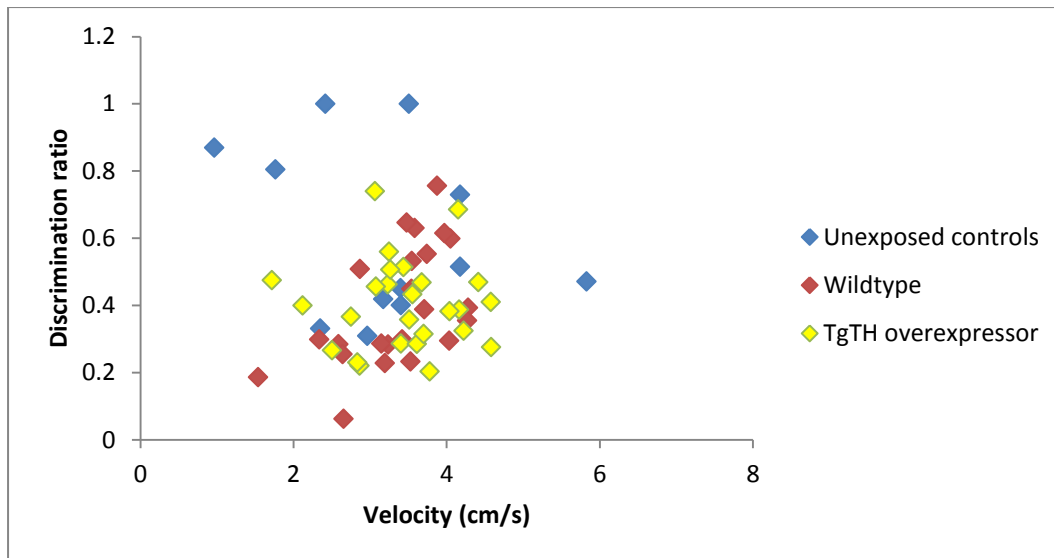


Figure 7: Discrimination ratio compared against the mean velocity in the two-choice domestic cat and cheetah test for unexposed controls, wildtype and TgTH overexpressor infected rats.

Domestic cat (*F. domesticus*) and puma (*F. concolor*)

The results of the two choice test comparing domestic cat and puma odour (Fig. 8) indicate that TgTH overexpressor infected animals displayed higher discrimination ratios than wildtype infected animals ($F_{3,45} = 2.04$, $p = 0.031$). Mean discrimination ratios were 0.48 (95%CI, 0.33-0.64) for unexposed controls, 0.43 (95%CI, 0.36-0.49) for wildtype infected animals, and 0.55 (95%CI, 0.46-0.63) for TgTH overexpressor infected animals. No interaction effects between infection status and any of the other variables including experiment round were found in this test ($p > 0.10$). Wildtype infected rats showed a preference for puma odour as they displayed a discrimination ratio significantly below 0.5 ($t_{22} = -2.24$, $p = 0.035$).

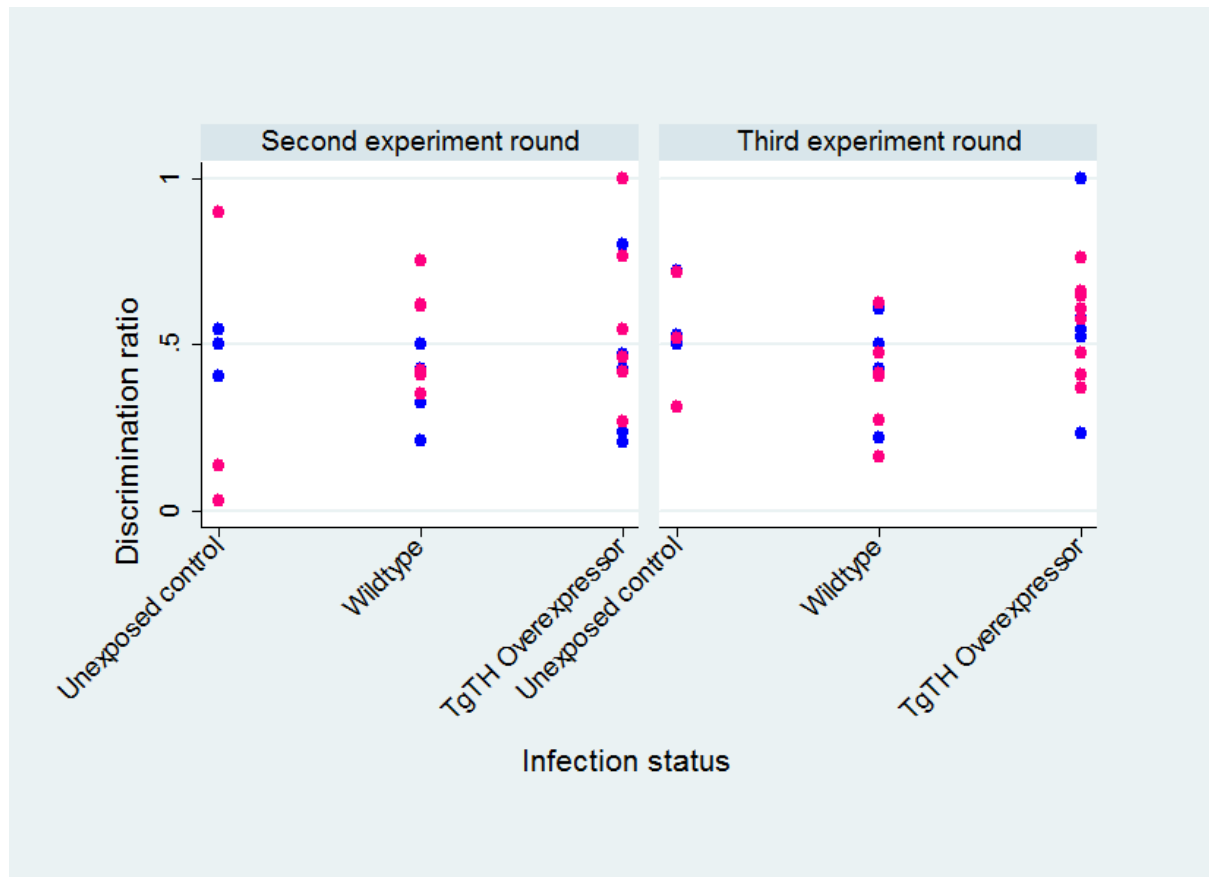


Figure 8: Discrimination ratio in the two-choice domestic cat and puma test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Males are shown in blue and females in pink.

Wildtype ($F_{4, 56} = 7.57, p=0.001$) and TgTH overexpressor ($F_{4, 56} = 7.57, p=0.007$)

infected animals both displayed increased velocities as compared to unexposed control animals (Fig. 9) in this test with wildtype infected rats showing a mean velocity of 3.8 cm/s (95%CI, 3.3-4.4), TgTH overexpressor infected rats 3.6 cm/s (95%CI, 3.2-4.0) and unexposed control rats 2.5 cm/s (95%CI, 1.8-3.3)

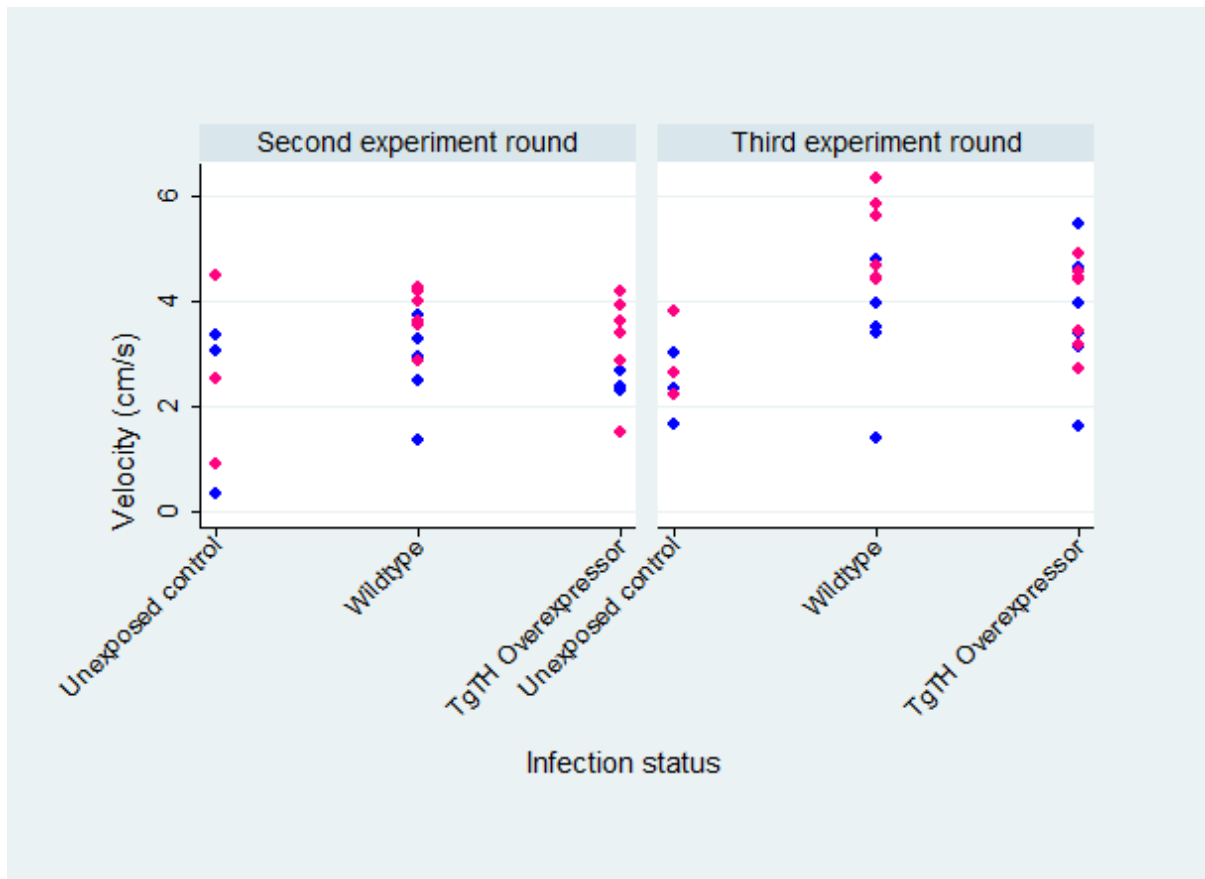


Figure 9: Velocity of movement in the two-choice domestic cat and puma test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. Males are shown in blue and females in pink.

Discussion

I examined the effect of TgTH overexpressor *T. gondii* infection on the 'Fatal Feline Attraction' behaviours previously described⁹ and whether this effect was feline species specific. Consistent with previous studies, I demonstrated some apparent loss of aversion, a 'Fatal Feline Attraction' in wildtype infected rats as compared to their uninfected counterparts^{9, 11, 12, 14}, which was particularly observed in females. However, contrary to our predictions here, no effect was found in TgTH overexpressor infected rats, which showed no difference in proportion of time in the cat zone relative to their wild-type infected or even uninfected counterparts. This result appears to oppose the hypothesis that TgTH overexpressor parasite lines would increase the feline attraction behaviour observed in wildtype *T. gondii* infected rats.

My investigations into activity levels when in proximity to different odours lead to a surprising and exciting result. I observed opposing correlations between 'Fatal Feline Attraction'

behaviours with velocity of movement in the cat zone (negative correlation) and velocity of movement in the non-cat zones (positive correlation). This implies that those rats that spent a higher proportion of time within the cat zone moved faster outside the cat zone, but slower within the cat zone, which would greatly increase the chance of predation. The interaction effect between infection status and velocity in non-cat zones in this test indicated that this effect was greatest in wildtype infected rats. This interaction was only found when looking at velocity in non-cat zones, which supports the theory that these infected rats are behaving differently in terms of their activity levels when in proximity to feline odour. It has previously been suggested that increased activity exhibited by *T. gondii* infected rats increases predation risk as cats are attracted to their prey by movement²³. My findings however suggest that further to this alteration, infection increases speed of movement, or activity levels in general, thereby increasing the rat's chance of encountering a feline, but the rat slows down when in proximity to a cat odour, thereby, increasing the amount of time spent near the feline (through decreasing activity when in proximity to feline odour), whilst also additionally increasing chance of predation by decreasing the rat's ability to escape. It is also important to note that these zone-specific differences in velocity of movement were only found in feline response tests in this study and not in generalised anxiety tests (Chapter 2), providing further evidence to support the hypothesis that *T. gondii* causes specific behavioural alterations to increase the host's chance of predation by a feline. As these effects were seen in all infected animal groups and were not the greatest in TgTH overexpressor infected animals, this suggests that alternative mechanisms to TgTH play a role in these responses.

Varying correlations between two traits may be an important aspect of adaptive parasite manipulation, and one that is much overlooked in studies that focus on only one phenotypic trait, and/or comparisons of mean values. In order to maximise transmission success, it may be that multiple traits are altered, which act additively or synergistically with each other³¹. This is particularly potent for manipulation that has evolved to increase predation by a definitive host, but decrease predation by a non-definitive host^{32, 33}. It has been proposed that correlations between host traits may in fact be the main target of host manipulations, rather than the traits themselves³⁴. A parasite may best increase its probability of transmission from its intermediate host to its definitive host by strengthening or reversing an existing association between host traits. In these cases, a comparison of mean trait values between parasitised and nonparasitised hosts would completely miss the effect of manipulation³¹. In the case of *T. gondii*, it appears that infection may be strengthening and increasing the correlation between proportions of time spent in cat zone and general activity

levels. It then appears to be reversing the direction of this correlation when in proximity to the feline odour.

Whilst the negative correlation between proportion of time in the cat zone and velocity in the cat zone was observed in all rats, the interaction indicated that this effect was strongest and highest in wildtype infected rats. This suggests that TgTH is not necessarily involved in any specific response to feline odour. This is unsurprising as the variety of behavioural alterations observed in *T. gondii* infected rodents suggests that there may be multiple pathways affected by infection. Whilst increases in activity levels caused by the TgTH pathway, as seen in Chapter 2, may increase the rat's chance of encountering a predation risk, it may be that specific responses to the feline definitive host are influenced by alternative pathways that do not involve TgTH. In addition, the overexpressor lines are GM strains that do not represent an optimally co-evolved parasite strain and therefore, although it helps us to elucidate relative influences of dopamine, it could indicate that even if TgTH expression is involved, the altered behaviours may not be further maximised for predation from an additional increase in the production of this dopamine precursor enzyme.

Whilst I did find a stronger loss of aversion in females to feline odour in this test, it is important to note that as females are faster moving than males, in laboratory environments at least, the effects of infection on feline odour may be more pronounced in females. Uninfected female rats have also been shown to display more defensive behavioural patterns to feline odour than male rats^{35, 36}. Factors such as an enriched environment can also affect female rats response to feline odour more than male rats³⁵. Female rats may have a higher baseline emotionality response to predator stress, and their behavioural response may be more sensitive to environmental or physiological factors that alter this than male rats³⁵. This illustrates the importance of using both rat genders in feline response studies involving *T. gondii* infection, as many laboratory studies only use male rats¹¹, which may bias the results.

Response to domestic cat versus cheetah odour indicates that both wildtype and TgTH overexpressor infected rats displayed a preference for cheetah odour, whilst unexposed control rats displayed a preference for domestic cat odour. Again, an interaction was observed between odour preferences and velocity of movement, with faster moving wildtype infected rats displaying higher discrimination ratios. When, however, rats' responses to domestic cat and puma odour were compared, whilst both wildtype and TgTH overexpressor infected animals displayed increased velocities of movement compared to unexposed controls, only wildtype infected rats displayed a higher preference for puma odour. This

supports my earlier results and the theory that TgTH is not involved in specific responses to feline odour.

The findings of the tests may have implications of the wildcats having better capacities as definitive hosts of the parasite than the domestic cat, and/or of having a stronger co-evolution with the parasite. Oocyst shedding has been demonstrated in all three feline species^{2, 17}, but further research is needed to make direct comparisons between these species. There is also the possibility that the infection may be linked to a less specific effect, such as the reduced aversion to a more pungent or intense feline odour for example. This has been shown in previous studies to confound species-specific predator responses in rats³⁷. Concentration and odour strength of urine may well affect behavioural responses, as it has been previously shown that the response of *T. gondii* infected rats to cat urine is dose dependant³⁸. This may link in to the fact that differential responses to different feline species could also be explained by different levels of a particular urine compound or olfactory cue, e.g. felinine³⁹ or cauxin^{20, 40} (an enzyme involved in the production of felinine and regulating urinary levels of male cat pheromones⁴⁰), which could be eliciting the rat's avoidance/attraction response. As described previously, components of urine differ between different domestic and wildcat species^{18, 19}. Further to this, urine components of cats differ according to sex, age, health status and whether intact or castrated. Felinine, a putative pheromone precursor attracting females and a territorial marker²⁰, and higher in intact compared to castrated male cats⁴¹, is one candidate for being the active component in feline urine, and this may imply that male cats initiate a stronger response than female cats, through higher levels of felinine in their urine³⁹. One finding that supports this is that *T. gondii* prevalence was shown to be higher in male feral cats than females⁴², which was thought to be due to differences in prey composition and amount, but could perhaps also be explained by a stronger response of infected prey to male cat odour. This suggests that future studies would benefit from controlling for the sex of the cat the urine has been collected from, and investigating if this influences the response of infected and uninfected rats. Age and health status of the cat may also have an impact on the active components within its urine²⁰, as, for example, geriatric cats at risk of azotemia display a higher cauxin-to-creatinine concentration ratio⁴³. This therefore suggests that in future studies investigating feline response in *T. gondii* infected rats; more emphasis needs to be placed on controlling for individual cat variation. In my study I controlled for volume and mass of urine saturated paper, and where possible pooled urine to limit effects due to individual cat variation. However limitations of these feline response tests include limited control of urine concentration, and therefore the strengths of the individual olfactory cues within different urine samples. Other limitations include the fact that the four-choice feline response test showed in some instances interaction effects

between parasite strain and experiment round, which were controlled for but may explain some of the inconsistencies seen in results in this and previous studies⁴⁴. This highlights that the effects of *T. gondii* are subtle, and are subject to high variation depending on individual host, parasite, environmental and laboratory factors^{44, 45}.

The results of this study indicate that *T. gondii* is selectively manipulating the response of infected rat hosts by differentially altering activity profiles depending on the rat's proximity to feline odour. TgTH does not appear to be directly involved in the specific response to feline definitive host odour. I conclude that *T. gondii* differentially alters an infected host's response to feline odour depending on the feline species, and that this may depend on the dose of cat odour stimulus, the urine compounds and olfactory cues present in the urine, and the capacity of the feline species as the definitive host. The results from this study indicate that these feline specific responses do not involve, or at least are not exclusively dependent upon TgTH production. These findings provide further support as to the sophistication and specificity of the adaptive parasite manipulation induced by *T. gondii*.

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Chapter 4

Rats showed no preference for novel object or odour choice in novelty discrimination tests irrespective of infection status

Summary

Toxoplasma gondii has been shown in previous studies to reduce neophobia levels in rats. Opposing results have been found in mice, which have led to the suggestion that *T. gondii* may in fact be impairing novelty discrimination mechanisms, which are based on recognition memory processes. A range of non-invasive, classic behavioural assays based on recognition memory were conducted to assess the impact of the parasite on novelty discrimination and novelty seeking, as well as a spontaneous alternation test designed to assess spatial memory. The novel approach of using genetically modified parasites that overexpress *T. gondii* produced tyrosine hydroxylase (TgTH) gives us further insights into the mechanisms behind this, and whether there is involvement of the dopaminergic system. My findings did not provide evidence that *T. gondii* alters recognition memory, or novelty discrimination traits, nor of involvement of TgTH. Novelty discrimination was not convincingly demonstrated in uninfected rats in this study, therefore I am unable to ascertain the effect of *T. gondii* or TgTH on these behavioural traits. As these behaviours are not directly associated to predation risk, one could tentatively suggest that the lack of differences observed between uninfected and infected animals is in support of specificity of parasite manipulation.

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that has an indirect life cycle, of which the definitive host are members of the cat family *Felidae*¹. Natural intermediate hosts of the parasite are small mammals and birds, including rodents. The parasite also infects a wide range of secondary hosts, including humans. The potential impact of *T. gondii* infection on novelty seeking and recognition memory is highly relevant to infection in humans, as novelty seeking behaviours in humans have been linked to the dopaminergic system, in particular the D4-dopamine receptor²⁻⁴. Latent toxoplasmosis in humans has been associated with an increased risk of schizophrenia⁵, and impairments in recognition memory is a pathology observed in schizophrenia patients^{6, 7}. This has led to speculation as to whether *T. gondii* infection causes alterations of neuromodulatory pathways within the human brain⁸, and whether these alterations are involved in the increased risk of schizophrenia and the subsequent recognition memory impairment observed in some schizophrenia patients. Infection with *T. gondii* in rodents has been linked to subtle behavioural alterations⁹⁻¹⁴, which have been suggested to be products of an evolutionary adaptation by the parasite to manipulate its intermediate host, in order to increase its likelihood of transmission to the feline definitive host via predation^{13, 15}.

The ability of a rat to discriminate between a novel and a familiar object, odour, or location is primarily based on recognition memory, as the behavioural trait is dependent on the rat being able to recognise the previously encountered familiar stimulus, and on it having an intact memory of the previously encountered stimulus¹⁶. Novelty seeking behaviours in rodents are distinct from but related to novelty discrimination behaviours, as discrimination of novelty is required before the rodent chooses to approach or avoid the novel stimulus. Novelty-seeking behaviours in rodents are affected by the strain of rat or mouse, whether they are wild or laboratory bred strains, by gender and by age¹⁷⁻²².

Studies on the effect of *T. gondii* on novelty seeking behaviours have suggested discrepant results depending on the host species being tested. Studies on wild brown rats (*Rattus norvegicus*) have suggested that infection with *T. gondii* increases novelty-seeking behaviours in this intermediate host¹⁴. As novelty-seeking is linked to exploration and anxiety levels^{18, 23}, it could be that the reduced neophobia observed in rats is linked to reduced anxiety levels that may increase the likelihood of predation of the infected rat by the feline definitive host¹⁵. Conflicting results were however found in studies with laboratory mice, which indicated that infection reduces novelty-seeking behaviours^{24, 25}. One explanation for

this may be the difference in innate levels of novelty-seeking in these species. Rats are thought to be innately neophobic whilst mice and humans are innately neophilic species²³. It may be that latent toxoplasmosis in fact impairs novelty discrimination mechanisms²⁶, which may explain why the parasite appears to have opposing effects on novelty seeking in the closely related mice and rat species. This impairment in novelty discrimination mechanisms may also explain the poorer performance of infected mice in learning tests²⁶. Rats and mice are not the same in terms of their susceptibility, behaviour, morbidity and overall response to *T. gondii*, and other behaviours such as learning capacity and memory are shown to be more affected by *T. gondii* in mice than in rats^{27, 28}.

At present little is known about the mechanisms by which *T. gondii* infection alters novelty seeking and/ or recognition memory in rodents. Dopamine receptors have previously been implicated in the functioning of recognition memory in rats²⁹. The spontaneous alternation test also involves the dopaminergic system^{30, 31}, and L-DOPA has been shown to induce contralateral turning behaviour in rats^{32, 33}.

Four studies on humans have indicated a reduction in novelty-seeking behaviours in humans³⁴⁻³⁷. It is anticipated that the parasite may affect similar neural pathways within the human host as the rodent host^{13, 31}, and the opposite effect to novelty seeking profiles observed in rats may be due to differences in innate novelty seeking behaviours between uninfected rats and humans. This highlights that some caution should be taken when extrapolating findings in rodents to humans. Although the alterations in novelty seeking behaviours observed in *T. gondii* infected humans appear to be more similar to the mouse model than the rat model, the rat is considered to be a superior model in terms of both ethics and generalizability to humans, as mice display an increased potential for parasite-induced morbidity and mortality during the acute phase of infection³¹. These studies in rats will therefore help us to elucidate the effect of the parasite within the human brain, and how this may impact the development of schizophrenia^{5, 38}, other human affective disorders³⁹⁻⁴¹, and various subtle personality differences seen in *T. gondii* infected humans⁴².

A range of tests can be used to assess novelty seeking behaviours in rodents, which build on an animal's natural predispositions to certain types of behaviour. These behavioural tests enable one to further evaluate the potential impact of *T. gondii* on novelty discrimination and recognition memory. A unique component of the current study was, for the first time, to characterise the impact on these behavioural traits using genetically modified (GM) modified *T. gondii* lines that overexpress the *T. gondii* tyrosine hydroxylase (TgTH) gene in order to ascertain the effect of this gene expression on novelty discrimination behaviour. Tyrosine

hydroxylase is thought to be involved in the production of L-DOPA, a precursor of dopamine, which is of interest as the dopaminergic system is involved in novelty discrimination behaviours in rats²⁹. This will therefore give us further insight into the relationship between *T. gondii* infection, the dopaminergic system, and novelty discrimination. Based on previous findings of increased novelty seeking in infected compared to uninfected rats, I hypothesised that rats infected with parasites that overexpress the TgTH gene may display increased novelty seeking behaviours compared to wildtype infected rats. I particularly predicted to see this in the odour recognition test as both odours were food-related, and previous research indicated food-related novelty-seeking behaviours towards novel odours¹⁴. Comparison with unexposed controls will enable me to further elucidate whether the parasite, and the TgTH gene, is in fact influencing novelty seeking or novelty discrimination traits. Alterations in the spontaneous alternation test seen in wildtype *T. gondii* infected rats would therefore support my hypothesis that *T. gondii* infection involves the dopaminergic system, and enhanced levels of these alterations seen in TgTH overexpressor infected rats would further support this hypothesis as well as indicate involvement of the TgTH genes. I predicted that TgTH overexpressor parasites may increase alternation rates in the spontaneous alternation test, and increase discrimination ratios in the object recognition, odour recognition and object location tests. Analysis into neuromodulator assays provided further insights into these mechanisms. This study will help elucidate the potential links between latent toxoplasmosis, novelty seeking, recognition memory, and the involvement of the dopaminergic pathway in both rodents and humans^{35, 43-45}, as well as the potential associations with schizophrenia pathologies in humans.

Materials and Methods

Host maintenance and parasite strains

Rats were infected with wildtype *T. gondii*, *T. gondii* TgTH overexpressor strain parasites or sham inoculated with phosphate buffer solution as described previously (Chapter 2; Appendix 2). Three experiment rounds were conducted as before (Chapter 2; Appendix 2), with each experiment round(s) stated below for each behavioural test.

Behavioural assays

Novelty discrimination assays were conducted when rats were 20-24 weeks post-*T. gondii* exposure (or sham controls). Behavioural tracking was conducted with Ethovision XT (Noldus, Wageningen, Netherlands) as described previously (Chapter 2; Appendix 2)

Spontaneous alternation test^{46, 47}

The spontaneous alteration test measures the pattern of movement that emerges when a, in this case, rat, can freely alternate between two arms of a maze⁴⁸. It is thus a behavioural test aimed to quantify the willingness of rodents to explore new environments, since both rats and mice typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited⁴⁸. Many parts of the brain, including the hippocampus, septum, basal forebrain, and prefrontal cortex, are involved in this task, and spontaneous alternation is very sensitive to hippocampal dysfunction³⁰. There also appears to be involvement of the vestibular system^{30, 46}. Continuous spontaneous alternation has also been widely used as a simple measure of short-term spatial working memory, and can reflect responsiveness to novelty as well as sensory and attentional factors⁴⁹. Dopamine in the septum and hippocampus has been implicated in the exploration of novel maze arms³⁰. Spontaneous alternation has also been seen to be affected by other neurotropic diseases such as scrapie⁵⁰. As the assay involves a continuous alternation test, there is no experimenter contact during the trial, it is non-aversive and is easily automated. This may allow for the more subtle behavioural changes usually seen in *T. gondii* infection¹⁵ to be detected. Tests for hippocampal dysfunction are important in *T. gondii* research as the parasite has been shown to invade hippocampal neurons and glial cells⁵¹, and the septohippocampal pathway is involved in the innate fear of feline odour⁵². As the assay also involves the vestibular system, this may be important as *T. gondii* has been shown to cause sensorimotor deficits in mice⁵³. In terms of the association between *T. gondii* and schizophrenia, schizophrenia patients have been shown to display spatial working memory and short-term memory deficits⁵⁴⁻⁵⁶. Hippocampal



Figure 1: Spontaneous alternation test

dysfunction also plays a role in the neuropathology of schizophrenia⁵⁷. Finally, there is a speculated involvement of dopamine in spontaneous alternation behaviour³⁰. This test requires no habituation, and is based on the animal's natural exploratory behaviour of a novel environment.

Rats were placed individually within the third arm of the test, with the sliding door down, facing away from the two arms. The sliding door was then raised and the rat was allowed to freely explore all three arms of the maze for 8 minutes (Fig. 1)⁴⁷. The rats were recorded using Ethovision XT as described in Chapter 2. Variables recorded included the number of

entries into each arm, duration spent in each arm, and latency to entering each arm. The number of alternations and the number of non-alternations were scored manually by playing back the rat tracks on Ethovision XT. The maze was cleaned in between trials with Trigene, to remove any odour from the previous rat. In the first experiment round the maze was placed on the ground, in the second experiment round however I altered the test by elevating the maze to improve detection in Ethovision XT and avoid the rats occasionally escaping. This was accounted for in the statistical analysis.

Object recognition test

Object recognition task tests can be used to assess recognition memory in rats by measuring the ability of a rat to discriminate between a novel and familiar object, and thereby its recognition of the familiar object, and its intact memory of the familiar object. It can also assesses novelty-seeking behaviour¹⁶ which may help elucidate anxiety and/or exploration levels of the rats. This test can be modified to assess various forms of object recognition, such as the alteration of a particular dimension, recognition of object location, or of the environment in which an object was previously encountered. *T. gondii* alters novelty-seeking behaviours in mice, rats and humans¹⁵, potentially due, in part, to alteration of novelty discrimination mechanisms²⁶. As this test is a standardized test used in the field of learning and memory, this may provide a further understanding as to the observed link between *T. gondii* infection and schizophrenia^{5, 38}, as patient with schizophrenia often display impairments in recognition memory^{6, 7}. Dopamine receptors have been implicated as having a role in functioning of this task²⁹, which ties in with the potential link between *T. gondii* infection and alterations in the dopaminergic pathways^{58, 59}. This test does not require exposure to aversive stimuli, or food or water restriction, and has been replicated in many laboratories using both mice and rats⁶⁰. Two types of object were used, a pink plastic bowl measuring 12.5 cm in diameter, and a light brown ceramic bowl measuring 8 cm in diameter (Fig. 2).

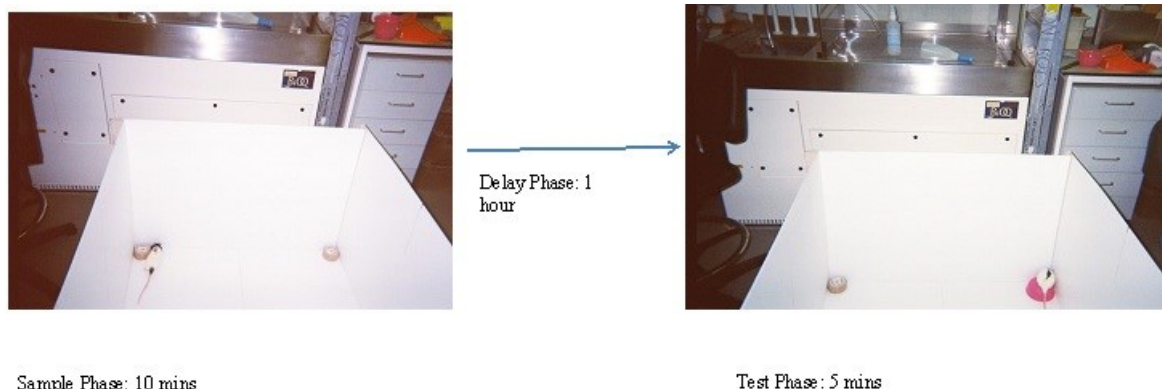


Figure 2: Object recognition test

This test has three phases – a sample phase, a delay phase and a test phase. The sample phase was 10 minutes long, with the rat placed in the arena with two samples of a particular object (which was to become the familiar object). After the 10 minutes, the rat was removed from the arena and placed back in its cage for an hour long delay phase. The rat was then placed back into the arena for a 5 minute test phase. During this phase, the rat was exposed to the familiar object experienced in the sample phase, and to the alternative, novel object. The amount of time spent in proximity to the novel object was recorded, defined as the ‘novel object zone’, i.e. the 25 x 25 cm quadrant containing the novel object in the corner. A short test phase of 5 minutes is used to avoid temporal effects, as the novel object will become more familiar with time.

Both the positioning of the novel object, and the type of object that was novel (pink bowl or brown bowl), were matched within groups to minimise bias. Both objects were smooth surfaced and secured so as not to be easily displaced. This was to ensure that both objects had similar manipulability and complexity, and thereby induced a similar degree of object interaction. Visual discernations would have been made based on shape, size and colour of the objects. Data were from the second and third experiment rounds, but unexposed control data were only available from the third experiment round. The test took place in an arena measuring 100 x 100 cm. No bedding was used in the arena in these tests, as bedding may induce other behaviours such as defensive burying and digging, which may compete with the novelty seeking behaviour of interest. All apparatus were wiped down with Trigene before and after each sample phase and each test phase to remove odours.

Odour recognition test

Odour recognition tests follow a similar principle to the object recognition test using olfactory rather than visual pathways⁶¹, which will enable us to further determine the impact of the

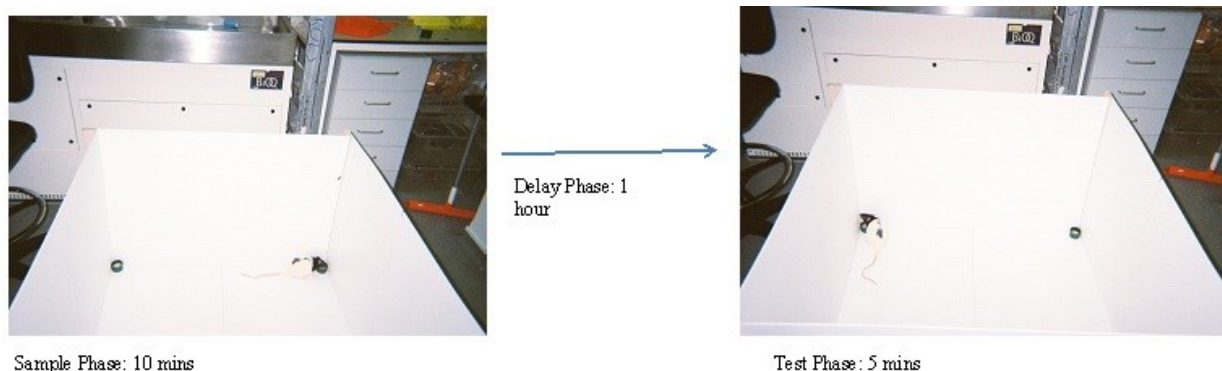


Figure 3: Odour recognition test

parasite on the olfactory system^{62, 63}. As rodents rely heavily on their olfactory abilities for predator avoidance⁶⁴, altered neophilia/neophobia in the intermediate host may alter transmission probabilities of the parasite through increasing their risk of predation by the definitive host. Odour recognition/ novel odour assays in rodents may thereby be valuable for evolutionary and mechanistic studies into parasite altered innate behaviour. In terms of the *T. gondii* and its association with schizophrenia, *T. gondii*-induced neophobia/neophilia can be ameliorated using D2 antagonists or selective dopamine uptake inhibitors, indicating involvement of the dopaminergic neuromodulatory system on exploratory behaviours^{44, 65}. Olfaction dysfunction is also frequently observed in patients with schizophrenia, with the greatest impact on odour identification⁶⁶, which may thereby present a parallel to that observed in *T. gondii*-infected rodents. This test therefore measures novelty-seeking and levels of neophilia/neophobia, as well as olfactory responses and discrimination. Two odours were used, cumin and coriander, both in the form of finely ground powder. Two small food bowls of 2 inch diameter were used. Each food bowl was filled with a small quantity of woodchips and mixed with 0.5 g of either coriander or cumin powder as required.

This test has three phases exactly as above for the object recognition test, but using two samples of one odour (the familiar odour) to start with in the 10 minute sample phase (Fig. 3). After this a delay phase of 1 hour took place during which the rat was placed back in its home cage. The rat was then placed back into the arena for a 5 minute test phase. During this phase, the rat was exposed to the familiar odour experienced in the sample phase, and to the alternative, novel odour. Both the positioning of the novel odour, and the type of odour that was novel (coriander or cumin), were matched within groups to minimise bias. As both odours were in the form of finely ground brown powder, they were mixed with woodchips and were placed in identical food bowls, this minimised visual distinction between them, to ensure that all discernations made by the rat were based on olfaction. Data from this test

were collected from the second experiment round. The arena and between phase/test procedures were as described above in the object recognition test.

Object location test

The object location task again assesses recognition memory and also spatial memory, this time assessing the rat's response to a familiar object or odour in a novel location⁶⁷⁻⁶⁹. The objects used were two pink plastic bowls measuring 12.5 cm in diameter (Fig. 4).

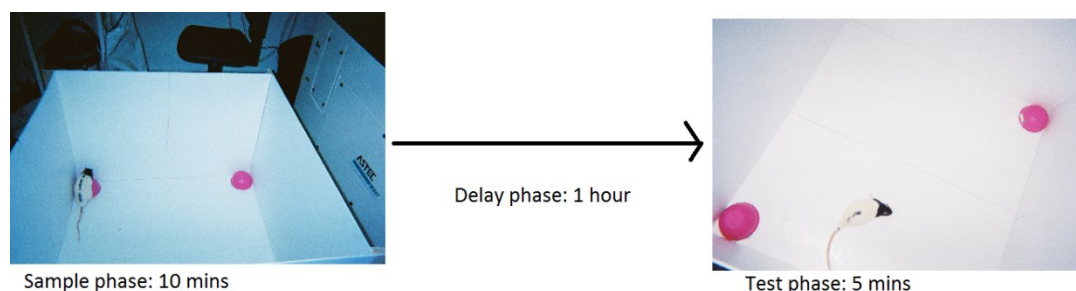


Figure 4: Object location test

This test has three phases as for the object recognition test, but instead of swapping in a new novel object in the test phase, one object was moved to a novel location. Data from this test were collected from the second experiment round. The arena and between phase/test procedures were as described in the object recognition test.

Statistical analysis

The positioning of the objects/odours during the sample phases was matched across groups to minimise positional bias. The duration of time spent in proximity to the novel object/odour or the object in the novel location were recorded. Variables recorded for each test included the animal's velocity, duration spent in each zone, frequency of entry and latency to enter each zone. STATA 11 was used to analyse each variable using multivariate regression analyses as described in Chapter 2. Analyses were used to identify effect of infection status accounting for rat sex, experiment round (Chapter 2), velocity of movement, and any interactions between infection status and these other variables.

The outcome variable used for the spontaneous alternation test was the alternation rate, calculated as:

$$\text{No. of alternations} / (\text{No. of alternations} + \text{No. of non-alternations})$$

The outcome variable used for the object recognition, odour recognition and object location task was the discrimination ratio, calculated as:

Duration in novel zone/ (Duration in novel zone + Duration in familiar zone).

If there is no difference between the rats' response to the novel and familiar zone, a discrimination ratio that does not differ significantly from 0.5 would be expected. A one-sample t-test was used to test this hypothesis. Experiment round was controlled for. The three experiment rounds are labelled as described in Chapter 2. All variables and/or their residuals were tested for normality and transformed or categorised if necessary before any parametric testing was conducted.

Results

Data from the novelty discrimination tests did not indicate any effect of *T. gondii* or TgTH on novelty discrimination or recognition memory traits. All but two exposed rats sustained infection (Chapter 5). The red reference lines indicate discrimination ratio of 0.5, expected if no difference in response to novel and familiar stimulus.

Spontaneous alternation test

The results of the spontaneous alternation test (Fig. 5) did not indicate any effect of infection status on alternation rate ($F_{4, 55} = 7.33, p > 0.10$). There was an effect of experiment round on alternation rate due to the altered experimental design ($F_{4, 55} = 7.33, p = 0.002$), which was accounted for in the analysis, but there was no interaction effect between infection status and experiment round, nor indeed any of the other variables measured (all $p > 0.10$). In the first experiment round the mean alternation rate over the 10 min time period was 0.56 (95%CI, 0.47 – 0.64) and in the second experiment round was 0.77 (95% CI, 0.73 – 0.80). No effect of rat sex was found ($p > 0.10$).

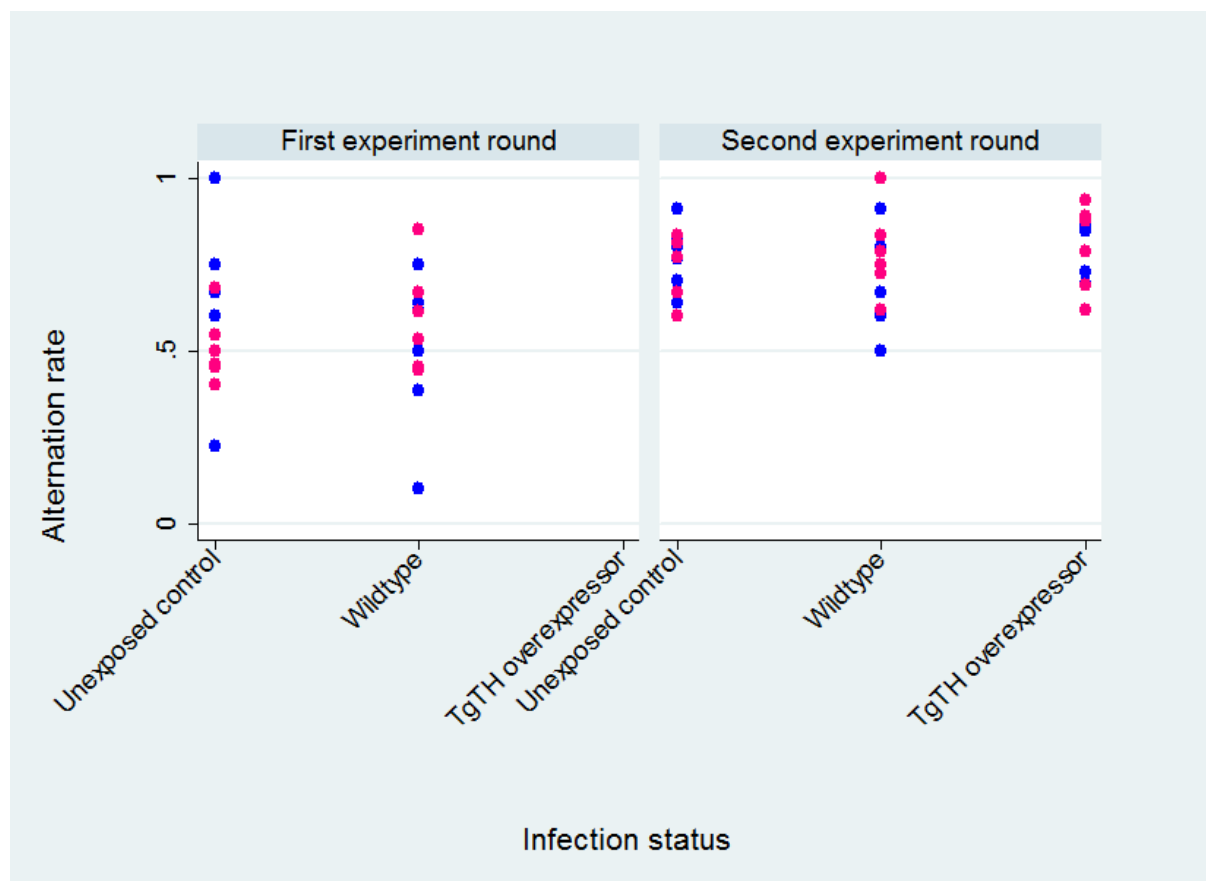


Figure 5: Alternation rate in the spontaneous alternation test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the first and second experiment rounds. There were no TgTH overexpressor infected rats in the first experiment round. Males are shown in blue and females in pink.

Object recognition test

Analysis on all 56 observations provided only weak evidence that there was a preference for the novel object zone to the familiar object zone ($t_{55} = 1.78$, $p = 0.080$). The mean discrimination ratio of all 56 observations was 0.54 (95%CI, 0.49 – 0.59). However, there was no evidence for an effect of *T. gondii* infection status on discrimination ratio ($p > 0.10$; Fig. 6).

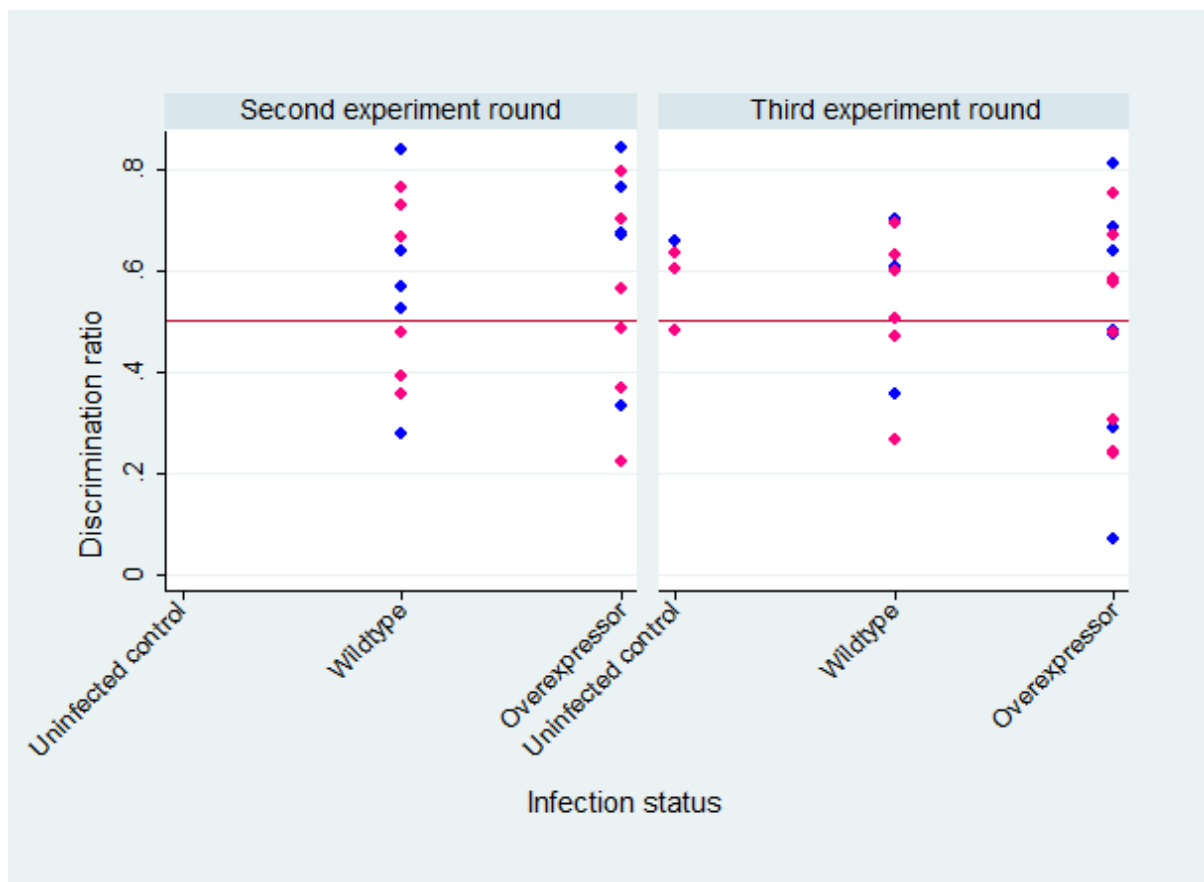


Figure 6: Discrimination ratio in the object recognition test of unexposed control (uninfected rats), *T. gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the second and third experiment rounds. There were no unexposed control rats in the second experiment round for this test. Males are shown in blue and females in pink.

Odour recognition test

The results of the odour recognition test (Fig. 7) did not indicate any effect of infection status on discrimination ratio ($p>0.10$). There was also no evidence that any group of animals, irrespective of infection status, displayed a preference for the novel odour zone as compared to the familiar odour zone ($p>0.10$). The mean discrimination ratio was 0.52 (95%CI, 0.43 – 0.61).

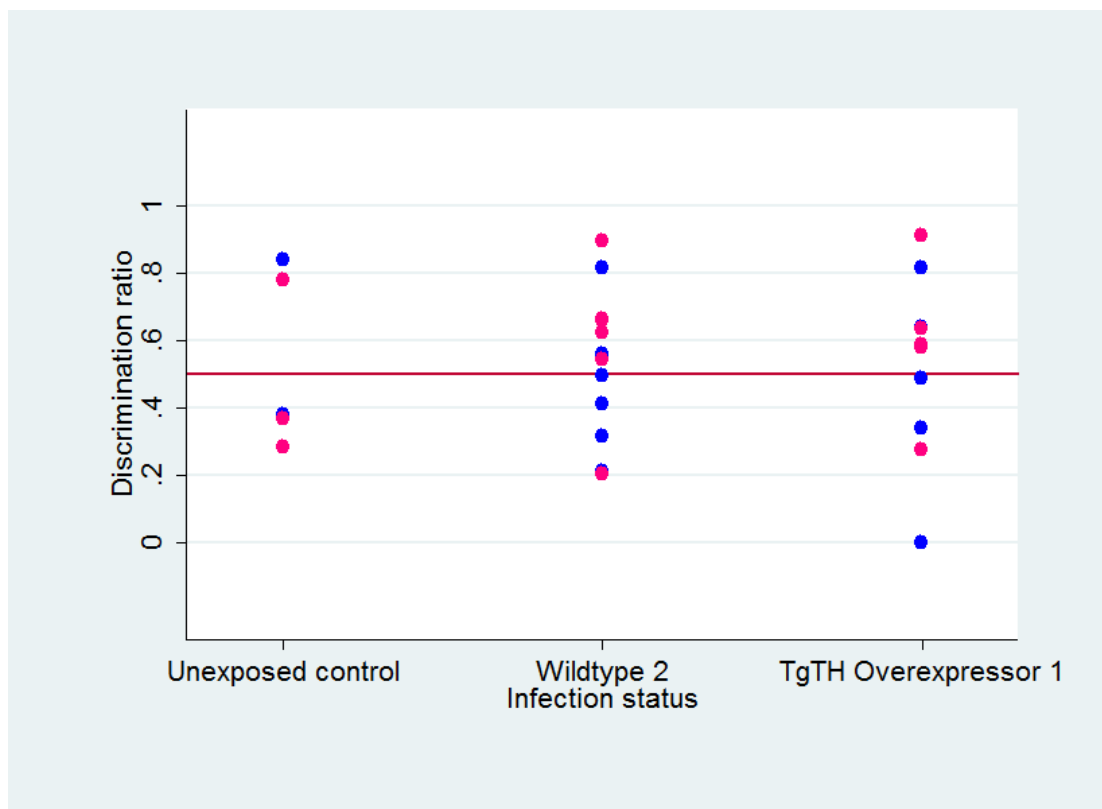


Figure 7: Discrimination ratio in the odour recognition test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data are from the second experiment round. Males are shown in blue and females in pink.

Object location test

The results of the object location test (Fig. 8) did not indicate any effect of infection status on discrimination ratio ($p>0.10$). There was also no evidence that any group of animals displayed a preference for the novel location zone as compared to the familiar location zone ($p>0.10$). The mean discrimination ratio was 0.52 (95%CI, 0.44 – 0.59).

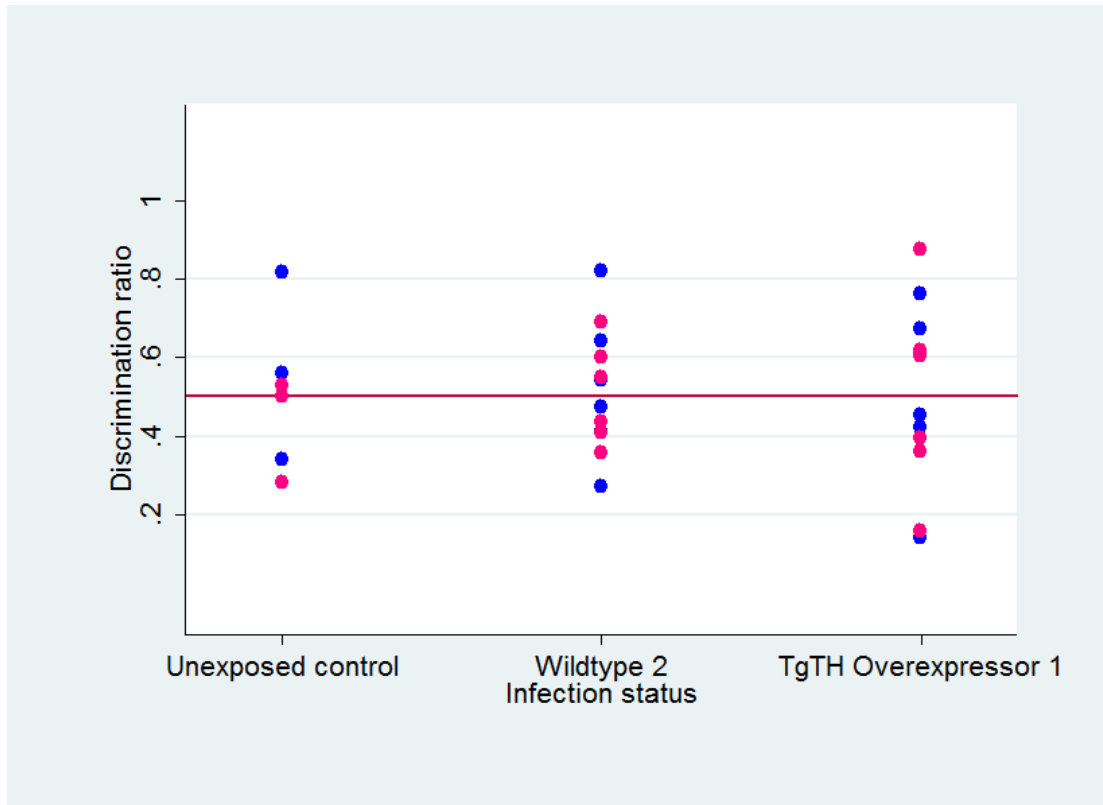


Figure 8: Discrimination ratio in the object location test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Data is from the second experiment round. Males are shown in blue and females in pink.

Discussion

It was predicted that one might see altered novelty seeking levels in these tests, either increased levels due to anxiolytic effects, or decreased levels due to impaired novelty discrimination and recognition memory. However I did not observe such behavioural alterations in rats infected with wildtype or TgTH overexpressor *T. gondii* strains compared to unexposed controls. This goes against some previous findings that *T. gondii* causes increased novelty seeking behaviours in rats¹⁴, or impaired novelty discrimination mechanisms²⁶. As no behavioural differences were found between TgTH overexpressor

infected rats and those infected with wildtype parasite or unexposed controls, there is an absence of evidence from this study that TgTH is involved in affecting novelty discrimination behaviour. These findings however may lend support to a previous study that found no effects of *T. gondii* infection in object recognition and placement tasks in mice⁶⁹, and to research which suggest that memory capacities of rodents are not affected by *T. gondii* infection^{28, 52, 69}. This is in support of the specific adaptive manipulation hypothesis of *T. gondii*, as novelty discrimination learning and memory traits are likely in the wild to mainly play a role in feeding behaviours, in particular for the purposes of taste-aversion learning and poison avoidance⁷⁰, but do not play a strong role in avoiding predation in the wild, which is innate rather than learned^{71, 72}. However the lack of evidence of novelty discrimination in general in these tests implies that caution should be taken when extrapolating from this the effects of *T. gondii* infection.

No effects of infection on alternation rate were observed in the spontaneous alternation test. This test therefore did not provide evidence for altered novelty seeking behaviour or altered spatial memory associated with *T. gondii* infection. As this test did not have the problems of habituation described below, and alternation rate was manually recorded, this test provides more evidence that *T. gondii* did not alter novelty seeking or spatial memory traits. This finding supports a previous study that suggests *T. gondii* does not affect spatial memory in mice⁶⁹. Spontaneous alternation behaviour is thought to involve several neuromodulators and brain regions of the limbic system³⁰, and it may be that the subtle alterations caused by *T. gondii* in the brain were not enough to disrupt this strong innate behaviour. It is of interest that I did not find any evidence of novelty discrimination or effects of infection in these tests, but I did find evidence of exploration and effects of infection in the generalised anxiety and feline response tests (Chapter 2, Chapter 3). This may be further evidence for specificity of behavioural manipulation by *T. gondii*, as generalised anxiety and response to feline odour are traits more associated to predation risk than novelty discrimination or recognition memory, which may be more relevant to foraging success, a trait generally shown to be unaffected by *T. gondii* infection, as implied by the unaltered body condition, weight and hunger levels of *T. gondii* infected and uninfected rats¹⁴.

A weak preference for the novel stimulus was observed in the object recognition task, but in the odour recognition and object location tests the animals, including the unexposed controls, did not appear to show preference for the novel stimulus. It may be that this is a product of habituation, as the rats had previously been exposed to the test room and arena. This could be supported by the fact that the only test where some novelty seeking was observed was in the object recognition test, which was conducted in the test arena prior to

the odour recognition and object location tests. Habituation has been shown to reduce exploratory activity in both *T. gondii* infected and control rats previously⁴⁴. Habituation in itself provides evidence of contextual memory, and therefore we could use this in itself as further evidence that *T. gondii* does not impair memory capacities⁴⁴.

Conversely, it has been suggested that rats only display novelty discrimination after repeated exposure to the test environment, therefore it may be that the animals were in fact not familiarised enough with the arena and therefore did not display novelty discrimination⁷³. Again, this would suggest novelty discrimination is not related to predation risk in the wild, where a rat would not have repeated exposure to an environment with a predation risk. Finally it may be that discriminatory behaviour was not effectively detected by the video tracking method of recording based on crossing into the novel stimulus area. This has been shown to be less reliable than the method of recording based on contacts with the novel stimulus when measuring novelty discrimination, and that being close to a stimulus should not be considered a measure of recognition unless the snout of the rat is oriented towards the novel stimulus by no more than 2 cm⁷³. Therefore in the future studies it would be advisable to manually record stimulus recognition. In future studies I would also perhaps use more complex objects in order to increase the rats' interest and exploration/ avoidance levels.

Other laboratory effects may also have been involved in the lack of novelty seeking behaviours observed in these tests. The rats had been extremely well handled for several weeks prior to these tests. Previous studies have indicated that handling can reduce novelty induced fearfulness in rats⁷⁴⁻⁷⁶, which may have impacted their behaviour in these tests. However we felt that handling was necessary for ethical reasons, and also to ensure that anxiety-related tests were not affected by stress induced from handling. Many other laboratory factors such as environmental enrichment, past exposure to aversive stimuli, method of rearing and maternal separation can also affect levels of novelty-seeking in rats^{75, 77, 78}. Some environmental enrichment was provided in cages, such as cardboard tunnels and shredded paper, as we felt this important for ethical reasons and to maintain normal behaviour in the rats over a long period of time. Rearing and maternal separation may have been influenced whilst at the animal supplier. Therefore it may be that the lack of finding from these tests is a product of the laboratory environment of the animals, and we may find different results when observing wild rats, which display far stronger avoidance behaviours than tame and/ or laboratory rats^{14, 79}. As novelty seeking behaviours vary so widely depending on both host and parasite strain^{17, 21, 79, 80}, I would hope in the future to repeat

these tests with other laboratory and wild rat strains, and with other strains and stages of the *T. gondii* parasite.

Overall, in summary, the apparent lack of behavioural differences or general novelty discrimination observed here between TgTH overexpressor infected rats, wildtype infected rats and unexposed controls do not provide us with evidence that *T. gondii* or the TgTH gene are involved in altering novelty discrimination behaviours. Further studies in which novelty discrimination is sufficiently demonstrated in uninfected animals need to be conducted before conclusions can be drawn regarding infected animals. It is possible that *T. gondii* selectively manipulates anxiety-related (Chapter 2) and feline response related (Chapter 3) behaviours, behaviours which are more associated to increased risk of predation by the feline definitive host.

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Chapter 5

***Toxoplasma gondii* appears to lower dopamine and noradrenaline levels potentially via the kynurenine pathway, which in turn is linked to behavioural outcomes**

Summary

Toxoplasma gondii has been shown in previous *in vitro* studies to increase dopamine production and in an *in vivo* mouse study to increase dopamine levels but decrease noradrenaline levels. These neuromodulators are of importance in *T. gondii* research as they may provide an understanding as to the mechanisms by which the parasite alters host behaviour. Other aspects of host physiology that may provide further insights into mechanisms of parasite-induced alterations include the immune response, which may indirectly influence neuromodulators, and levels of stress-associated steroid hormones. In this study I obtained HPLC data on neuromodulator levels from collaborators, as well as performed molecular techniques to ascertain antibody titres and steroid hormone levels myself. Physiology findings were then linked to individual rat behavioural profiles to further understand of the effects of infection on physiology and which aspects had subsequent effects on behaviour. The novel approach of using genetically modified parasites that overexpress *T. gondii* produced tyrosine hydroxylase (TgTH) gives us further insights whether and how the parasite may be influencing the dopaminergic system. My findings indicate that *T. gondii* infection reduces levels of noradrenaline and dopamine. This implies disruption of the dopaminergic pathways, but in different ways to those predicted, and may indicate the involvement of the kynurenine pathway. Antibody titres are affected by host sex and time post-infection, and TgTH overexpressors induced lower antibody titres. There is some evidence for correlations between physiological and behavioural data indicating associations between behavioural alterations and noradrenaline, and also testosterone, which may explain some of the gender-specific effects of *T. gondii* infection.

Introduction

Toxoplasma gondii is a parasitic protozoon, with felids as the definitive hosts and a wide range of endothermic intermediate and secondary hosts, including small mammals and humans respectively. The effects of *T. gondii* infections in humans are not completely understood, as although the parasite develops a latent cyst stage within the human brain that is seemingly asymptomatic in immunocompetent adults, studies have suggested that there can be, in some cases, a potential association between chronic infection with *T. gondii* and the development of schizophrenia¹. The discovery of two genes in the *T. gondii* genome that encode for tyrosine hydroxylase, an enzyme involved in the production of dopamine², the finding that *T. gondii* induced behavioural alterations in rats may be inhibited by dopamine antagonists³, and the disruptions in dopamine levels observed in many schizophrenic patients⁴, lead to the hypothesis that this association may be due to the parasite altering dopamine levels within the brain. This can then be linked with individual rat behavioural profiles; in order to gain further insights into the mechanisms behind *T. gondii* induced behavioural alterations. Rats are a natural intermediate host of the parasite, and therefore provide a useful animal model by which we can improve our understanding of the effects the parasite may be having within the human brain. *T. gondii* has been shown in several studies to alter the behavioural profile of rats⁵⁻¹¹. The mechanism by which it does this is largely unknown.

T. gondii has been shown to increase dopaminergic production in PC12 cells *in vitro*¹², and a previous *in vivo* study has indicated that *T. gondii* infection increased dopamine levels by 14% in mice¹³, as well as increasing homovanillic acid and decreasing noradrenaline levels¹³. These variations in neuromodulators may directly affect the behaviour by altering anxiety related or reward seeking behaviours^{14, 15}. Other potential mechanisms include preferential cyst location within the brain by direct physical interference with brain regions^{16, 17}, or the parasite may indirectly affect host behaviour via the immune response system and its cascade effect on neuromodulators^{16, 18}.

Whilst increased dopaminergic production has been shown to be associated with *T. gondii* infection *in vitro* and *in vivo* in the mouse study described above^{12, 13}, few studies have looked at dopaminergic production in rat *in vivo* studies, or have looked for L-DOPA levels. I have used novel genetically modified (GM) *T. gondii* strains that overexpress the genes that encode for *T. gondii* produced tyrosine hydroxylase (TgTH), in order to further investigate the function of these genes and their effect on the associated dopaminergic pathway in rats.

Wildtype strain infected and TgTH overexpressor strain infected rats were used to investigate the role of dopamine and L-DOPA in certain behaviours and how these differed to unexposed control rats, and to understand the effect of TgTH expression. Dopamine has been shown to be associated with reward seeking behaviours¹⁴, whilst L-DOPA may in fact have anxiogenic effects¹⁹. Levels of other neuromodulators are also associated with stress, e.g. noradrenaline has been associated with anxiogenic effects¹⁵, and serotonin has been associated with enhanced generalised anxiety but decreased fear in response to an aversive stimulus²⁰. Alterations in these neuromodulators were therefore thought to be key in understanding of the anxiolytic effects of *T. gondii*, both in terms of generalised anxiety and predation related response^{6, 7}. I investigated the association between levels of L-DOPA, dopamine, serotonin and noradrenaline in *T. gondii* infected rats and the association with infection induced behavioural alterations, particularly in rats infected with TgTH overexpressing parasites. For the purposes of this study, I was particularly interested in the levels of dopamine and L-DOPA found in the brain of TgTH overexpressor strain infected rats, wildtype strain infected rats, and unexposed control rats, in order to gain further insights into the function of the TgTH genes in altering these neuromodulators within the rat brain. The predictions of this study were that *T. gondii* wildtype infected rats will display elevated dopamine and/ or L-DOPA levels within the brain as compared to unexposed control rats, and TgTH overexpressor infected rats will display elevated dopamine and/ or L-DOPA levels within the brain as compared to wildtype infected rats. The chronic infection with *T. gondii* and expression of the TgTH genes are expected to lead to increased dopamine levels, as these are associated with reduced stress and increased reward and novelty-seeking behaviours^{14, 21, 22}.

Also of interest were the stress associated steroid hormones corticosterone^{23, 24} and testosterone²⁵. Corticosterone is released in response to stress, including exposure to feline odour, and induces neural changes that affect behaviour²⁶⁻²⁸. Testosterone has also been shown to be secreted in response to acute neurogenic stress²⁵. Rat sex has been shown previously to have an effect on behavioural response to both these steroid hormones^{29, 30}. Enzyme Immunoassay (EIA) techniques have been used in rat models previously to detect levels of steroid hormones from extracts of plasma and faeces³¹. I used these techniques to further understand the potential interaction of *T. gondii* infection with corticosterone and testosterone, and some of the differences between responses to infection in male and female hosts³²⁻³⁴. The novel use EIA assays in *T. gondii* research is enhanced further by linking with each rat's individual behavioural profile, both from predation specific and from generalised anxiety behavioural assays. I predicted to observe wildtype strain infection being associated with reduced levels of corticosterone and testosterone, and TgTH overexpressor

infection being associated with a further reduction of these stress hormones, as *T. gondii* infection and tyrosine hydroxylase have both been previously associated with reduced anxiety related behaviours^{7, 35, 36}.

Materials and Methods

Host maintenance and parasite strains

Eighty-seven Lister-hooded rats were handled, maintained and infected with wildtype *T. gondii*, *T. gondii* TgTH overexpressor strain parasites or sham inoculate with phosphate buffer solution as described previously (Chapter 2; Appendix 2). Three experiment rounds were conducted as before (Chapter 2; Appendix 2), with each experiment round(s) stated below for each physiological test. There were no TgTH overexpressor infected rats in the first experiment round. Exposed and unexposed animals were housed together and were not separated by cage.

Behavioural assays

Physiological data were linked to behavioural data to create a physiological and behavioural profile for each individual rat in the study, to gain further insights into the effect of *T. gondii* infection on physiology and its subsequent impact on rat behaviour. Specific behavioural assays to ascertain generalised anxiety, feline response and novelty discrimination traits were carried out as described before (Chapters 2, 3 and 4). The elevated plus maze test is described in detail in Chapter 2 and the four-choice feline response test in Chapter 3.

Faecal collection

1-2g of rat faeces were collected for each rat for later enzyme immune assay (EIA) steroid measurements to determine levels of corticosterone and testosterone³¹. To collect faeces from rats at the same time point, I separated rats into individual mouse cages cleaned with water and waited for each individual to defecate (Fig. 1).

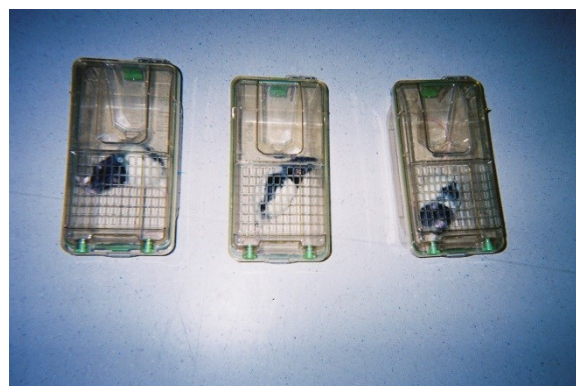


Figure 1: Faecal collection

Collection of brains and blood

Rats were anaesthetised using isofluorane gas and then sacrificed by cervical dislocation. Each batch of infected rats were euthanised at nine, five and six months post initial infection, from rounds 1,2 and 3 respectively. Blood was immediately removed from the heart by cardiac puncture using a 27 gauge needle and syringe, before coagulation could occur. These were then stored in 2.5 ml EDTA tubes. The blood was then centrifuged to separate out the serum which was then collected and aliquoted into 1.5ml eppendorf tubes.

Brains were removed by cutting open the skull with scissors and removing the skull gently with bone cutting forceps. The left hemisphere was separated into the four sections of interest: the olfactory bulb, the striatum, the hippocampus and the cerebellum. The right hemisphere of the brain was stored whole. Brain sections were snap frozen by placing in frozen isopentane and then wrapped in silver foil placed on dry ice. Samples were stored at -80°C and transported on dry ice to Leeds for HPLC testing.

High-performance liquid chromatography (HPLC)

(Conducted by collaborators at the University of Leeds)

HPLC was used to detect dopamine, serotonin, adrenaline, noradrenaline, and homovanillic acid within each of the brain regions dissected above. Dopac was used as an internal control in this catecholamine HPLC. Dopac is a metabolite of dopamine that is widely used as an internal control to calculate the concentrations of catecholamines in the brain.

Serology

Serological testing was carried out using the Toxoreagent test from Mast group Ltd. This is an indirect latex agglutination test (ILAT) for the semi-quantitative determination of anti-*T. gondii* antibodies in serum samples by the microtitration method. The serum was prepared by adding 50µl serum to 350µl buffer solution, to create a 1:8 dilution.

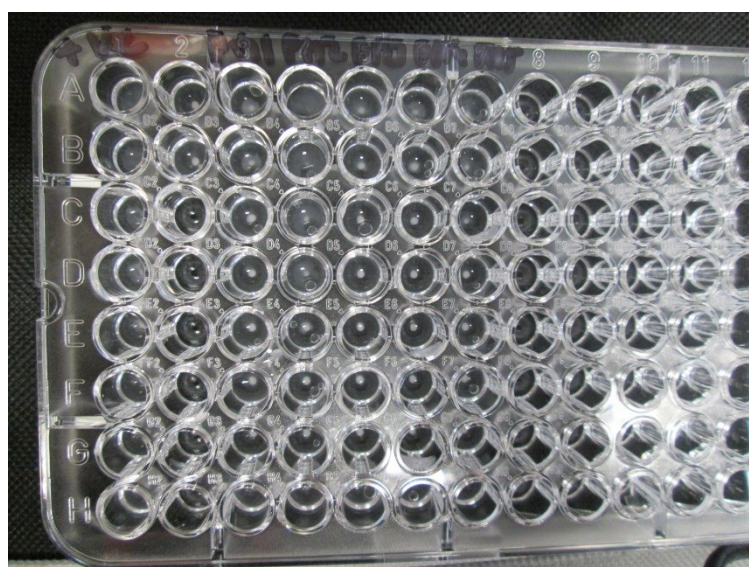


Figure 2: Micro titre plate with agglutination wells

The solution was mixed well with a pipette tip. 25µl of only buffer solution was then placed into wells 1-8 of a U-shaped bottom microtitre plate (Fig. 2). This operation is then repeated for as many columns as there are serum samples to be tested. 25µl of the 1:8 diluted serum is then transferred into the first well of a column. The contents of the well are mixed by pipetting up and down, after which 25µl of the diluted sample is transferred to the next well in the column, this was repeated until the final test well in the column, after which the final 25µl is discarded. This creates a series of doubling dilutions from 1:16 in the first well of the column to 1:2048 in the seventh well of the column. The process was repeated in a new column for each serum sample. The eighth well of every column was left without sample as a control as well as pre-diluted positive and negative controls provided by the kit used in two columns. A latex reagent is gently shaken to suspend the particles. 25µl of latex reagent is placed in each well. The plate is gently shaken and covered to prevent evaporation. It is then left for 12 hours after which the wells are read for agglutination patterns. The number of rows that displayed agglutination gave an indication of antibody titres, ranging from 1:8 (lowest) to 1: 2048 (highest). This test is capable of detecting both IgM and IgG anti-*T. gondii* antibodies.

Faecal corticosterone and testosterone extraction and preparation for enzyme immunoassay (EIA)

The EIA assays were performed on the second experiment round of wildtype and TgTH overexpressor infected rats (n=24). No data were collected for unexposed control rats in this study due to time constraints, which is a limitation that needs to be addressed in future studies. Faeces were weighed out to approx. 80 – 90mg and soaked in 1ml methanol. The faeces and methanol solvent were homogenised by placing in a beater at 6,000rpm for 30 secs with 1mm zirconia beads. The samples were then centrifuged at 13,000rpm for 10 minutes to separate the samples into a solid lower phase and a liquid upper phase. The upper phase was transferred into an eppendorf tube and stored at -20°C. This protocol was adapted from a previous protocol³¹.

A corticosterone EIA kit and a testosterone EIA kit (Cayman Chemicals, Ann Arbor, MI) was used to test for levels of corticosterone and testosterone using these faecal extract samples. Both assays were based on the competition between the steroid hormone and an acetylcholinesterase (AChE) conjugate, used as the hormone tracer. The kits arrived with a 96 well plate pre-coated with IgG and blocked with a proprietary formulation of proteins. Faecal extract samples were diluted 1:20 in Cayman EIA buffer. Wells were filled with tracer, antiserum, and the control standard or a sample, and incubated for two hours at room

temperature on an orbital shaker. The plate was then washed five times with wash buffer to remove all unbound reagents. The wells were developed with Ellman's reagent for an hour in the dark, and then placed into a plate reader to read the absorbance level. The absorbance is inversely proportional to the level of steroid hormone present.

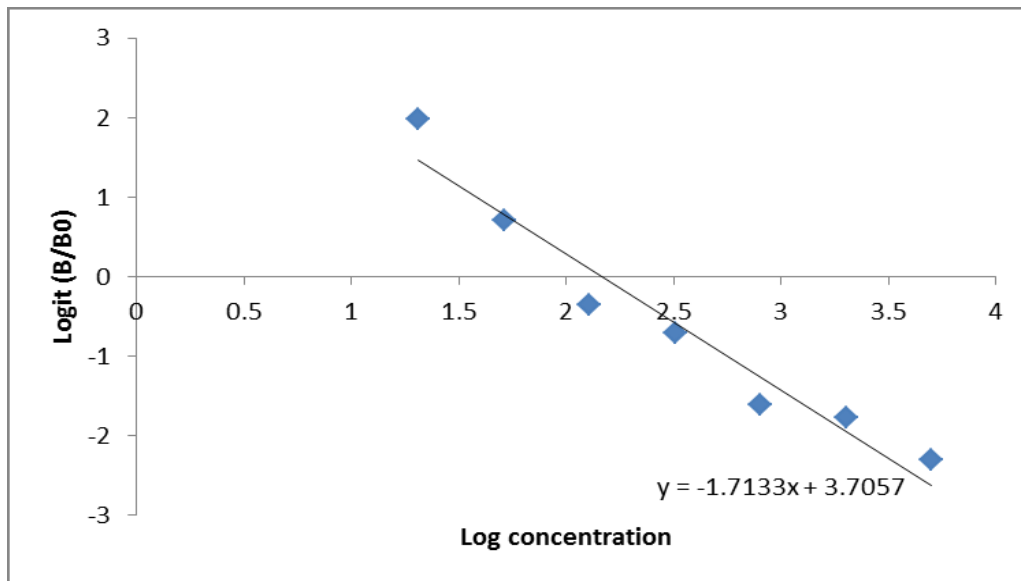
The plate also contained wells designed to measure non-specific binding (NSB), maximum binding (B_0) and total activity (TA) of the AChE-linked tracer. Standards were placed on the plate at 8 dilution levels ranging from 5000pg/ml to 8.2pg/ml for the corticosterone assay and 500pg/ml to 3.9pg/ml for the testosterone assay. Control standards were assayed in duplicate and samples in triplicate.

In order to generate the standard curves (Fig. 3), the maximum binding level (B_0) was corrected for by subtracting the mean absorbance of the non-specific binding wells. The sample or standard bound/ maximum bound (B/B_0) was calculated as:

$$(Sample\ or\ standard\ absorbance - NSB) / Corrected\ B_0$$

A linear standard curve was created by plotting logit (B/B_0) against log concentration and performing a linear regression fit:

3 a)



b)

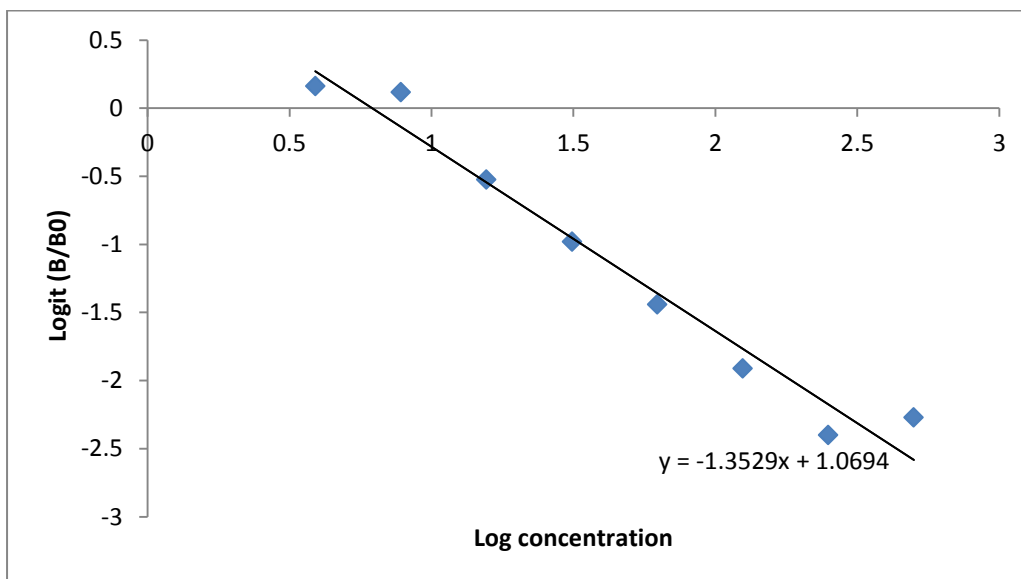


Figure 3: Standard curves for a) corticosterone and b) testosterone EIA assays

From this, the log concentration and then the concentration of the samples was calculated, after multiplying by the dilution factor of 20.

Statistical analysis

Analyses were used to identify effect of infection status accounting for rat sex, experiment round (Chapter 2), and any interactions between infection status and rat sex and experiment

round. Experiment round was controlled for. The three experiment rounds are labelled as described in Chapter 2. STATA 11 was used to analyse each variable with bootstrap repeats of multivariate regression analyses. Bootstrap repeats were employed to help analyse low sample size data. Serology and steroid hormone data were analysed using the non-parametric Kruskal-Wallis test.

Results

Serology

The serological results of the first round of infection indicated that all exposed animals did sustain infection (Table 1). The second round of infections indicated that 23/24 exposed animals sustained infection (Table 1). The third round of infections indicated that 26/27 exposed animals sustained infection (Table 1). No unexposed animals acquired infection throughout the study, indicating that even over a time period of months, transmission of infection between rats did not occur. Both exposed animals that did not sustain infection were TgTH overexpressor strain exposed male rats. A positive agglutination result for an antibody titre of 1:16 or higher was reported as positive. An antibody titre of less than 1:16 is not usually regarded as clinically significant.

Serological data indicate that overall, TgTH overexpressor infected animals displayed lower antibody titres than wildtype infected animals (chi-sq = 16.77, $p < 0.001$), females displayed higher antibody titres than males (chi-sq = 7.25, $p = 0.007$), and there were differences between experiment round (chi-sq = 14.39, $p = 0.001$; Fig. 4). There were interactions between infection status and experiment round on titre levels, indicating that results were not always consistent between experiment rounds (chi-sq = 6.41, $p = 0.01$).

Table 1: Antibody titres from ILAT test of unexposed control, *T. gondii* wildtype infected and TgTH overexpressor *T. gondii* infected rats from the three experiment rounds. The ILAT titres indicate the highest dilution where antibody detection is still possible

Experiment round	Rat ID	ILAT Titre	Parasite line
First	1	1:16	Wildtype 1
	2	1:32	
	3	1:32	
	4	1:16	
	5	1:16	
	6	1:128	
	13	1:64	
	14	1:32	
	15	1:256	
	16	1:256	
	17	1:128	
	18	1:1024	
Second	EM1	1:512	TgTH Overexpressor 1
	EM2	1:128	
	EM3	1:1024	
	EM4	0 ☹	
	EM5	1: 256	
	EM6	1:256	
	EF1	1:1024	
	EF2	1:1024	
	EF3	1:1024	
	EF4	1: 512	
	EF5	1:1024	
	EF6	1:1024	
	WM1	1:32	Wildtype 2
	WM2	1:64	
	WM3	1:1024	
	WM4	1:512	
	WM5	1:512	

	WM6	1:256	
	WF1	1:1024	
	WF2	1:1024	
	WF3	1:1024	
	WF4	1:1024	
	WF5	1:1024	
	WF6	1:32	
Third	OM1	1: 64	TgTH Overexpressor 2
	OM2	1:32	
	OM3	1:32	
	OM4	1:32	
	OM5	1:32	
	OM6	0 ☹	
	OM7	1:16	
	OM8	1:32	
	PM1	1:32	Wildtype 1
	PM2	1:256	
	PM3	1:32	
	PM4	1:32	
	PM5	1:64	
	OF1	1:64	TgTH Overexpressor 2
	OF2	1:128	
	OF3	1:256	
	OF4	1:64	
	OF5	1:128	
	OF6	1:32	
	OF7	1:64	
	OF8	1:512	
	PF1	1:1024	Wildtype 1
	PF2	1:1024	
	PF3	1: 2048	
	PF4	1:64	
	PF5	1: 128	
	PF6	1: 1024	

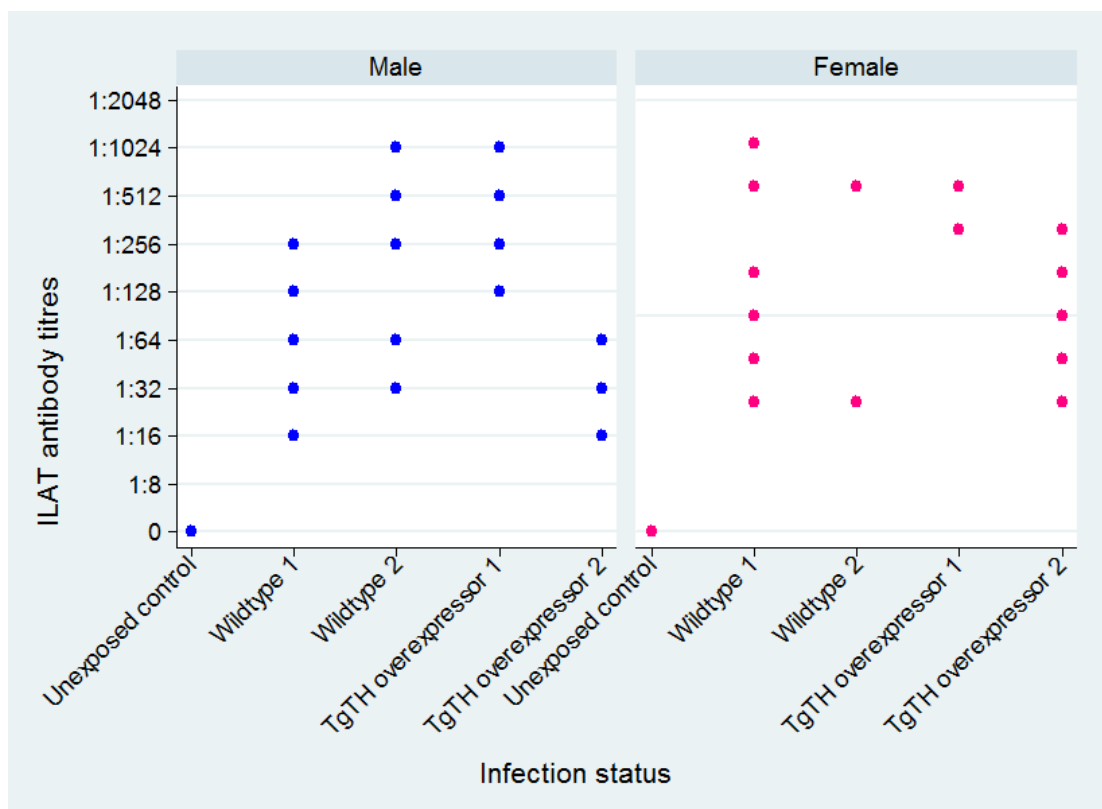


Figure 4: Antibody titres from ILAT test of unexposed control (uninfected rats), *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the three experiment rounds. Graphs are split by rat sex. There were no TgTH overexpressor infected rats in the first experiment round.

Brain dissection analysis

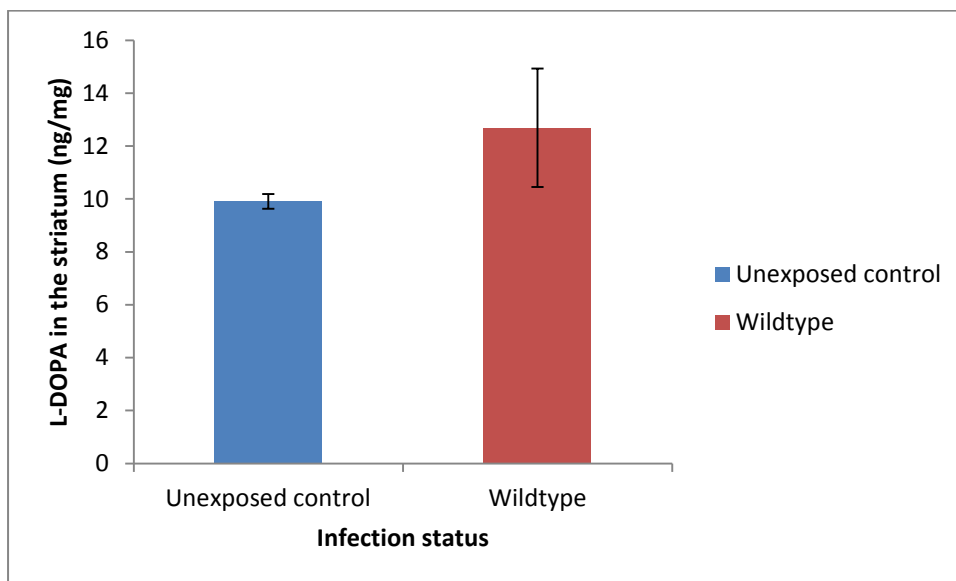
Due to technical issues at the University of Leeds, HPLC data from the first experiment round was incomplete for individual rat brains. Sample size ranges from n=4 to n=6. Data from the second experiment round was complete for individual rats (n=30). No data were able to be obtained from the olfactory bulb for any animals due to tissue damage of this small brain region during collection, preparation and testing. I have not received data from the third experiment round to date. Data are reported as ng/mg starting tissue for sample at 1µM. No significant differences between infection status were found when looking at levels of serotonin or homovanillic acid ($p>0.10$).

L-DOPA

In the first experiment round, an effect was seen of L-DOPA being higher in infected animals than in unexposed animals within the striatum (n=6, Wald chi-sq = 792.87, $p<0.001$, Fig. 5a) and the hippocampus (n=6, Wald chi-sq =11302.19, $p=0.018$, Fig. 5b). No effect was seen in the cerebellum ($p>0.10$), and there were no available data from the olfactory bulb. No such results were found in the second experiment round (n=30, $p>0.10$), between unexposed

control, wildtype or TgTH overexpressor infected rats. Rat sex is combined in the above graph as no effect was found when splitting by rat sex, due to the extremely small sample size (Males n=4, Females n=2). Mean L-DOPA levels in the hippocampus in the first experiment round were 8.80 ng/mg (Std. error 1.07, n=3) for unexposed controls and 10.71 ng/mg (Std. error 0.20, n=3) for wildtype infected rats. Mean L-DOPA levels in the striatum in the first experiment round were 9.91ng/mg (Std. error 0.28, n=3) for unexposed controls and 12.69 ng/mg (Std. error 2.24, n=3) for wildtype infected rats.

a)



b)

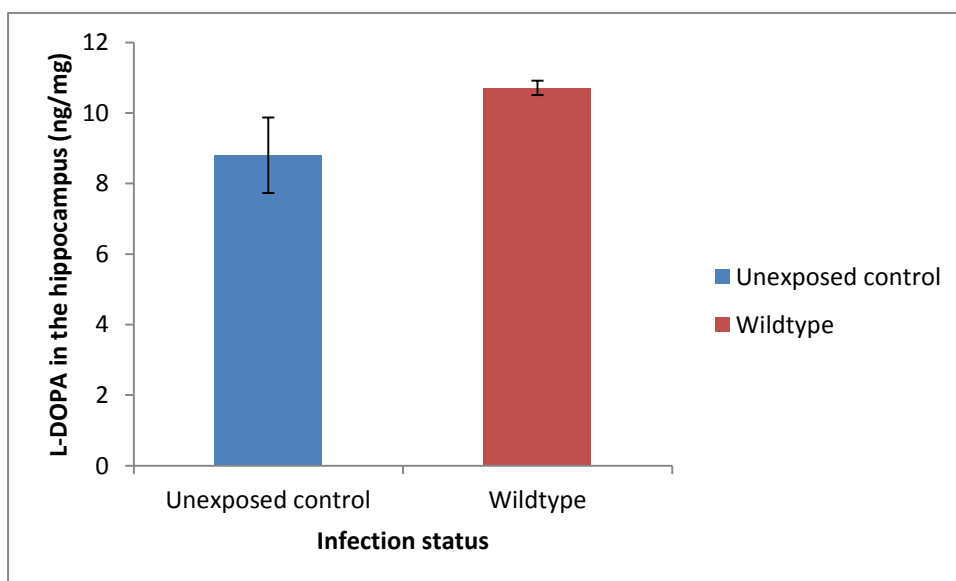


Figure 5: L-DOPA in the a) striatum b) hippocampus in wildtype *T. gondii* infected animals and unexposed controls in the first experiment round (n=6)

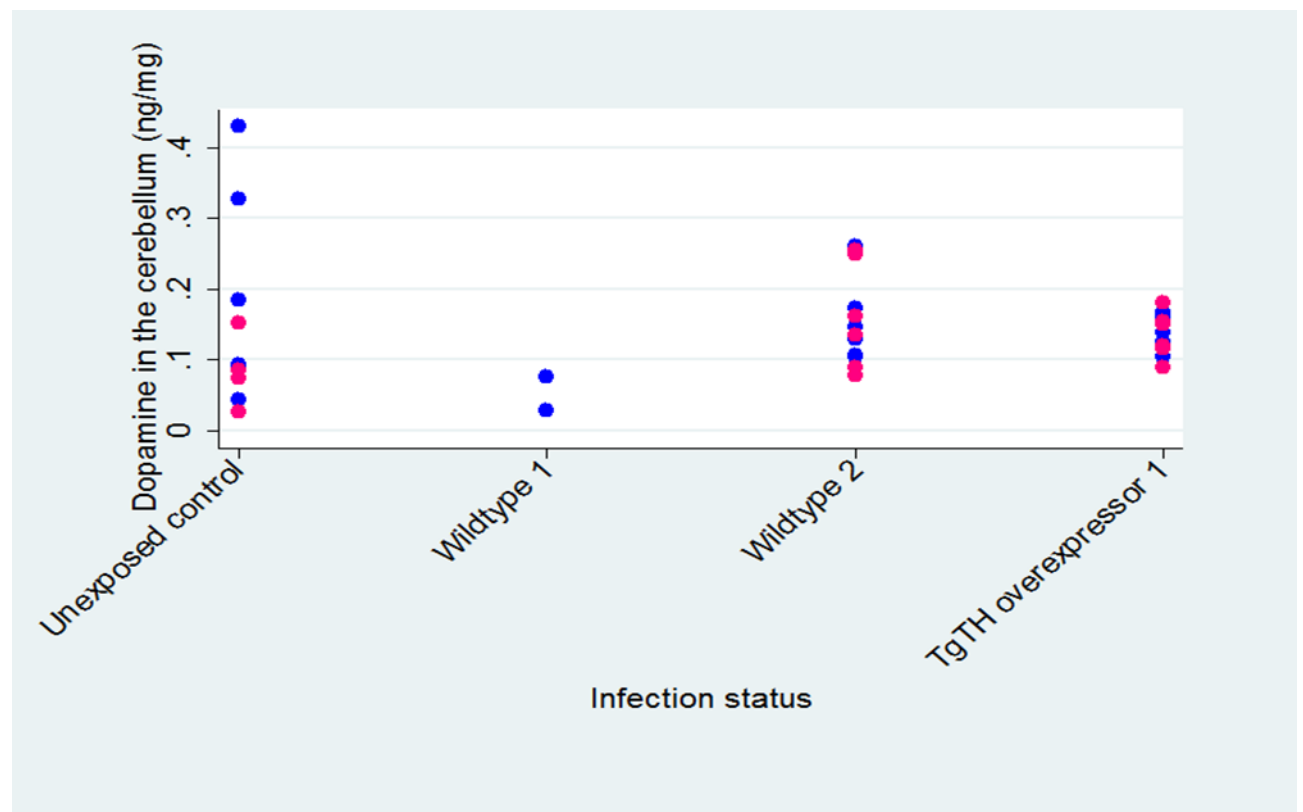
Dopamine

Within male rats, TgTH overexpressor ($p=0.006$) and Wildtype ($p=0.002$) infected animals displayed lower dopamine levels in the cerebellum than unexposed controls (Wald chi-sq = 43.65; Fig. 6a). Mean levels of dopamine in the cerebellum were 0.22 ng/mg (95% CI, 0.01 – 0.42) for unexposed control male rats, 0.13 ng/mg (95%CI, 0.07-0.19) for wildtype infected male rats and 0.14 ng/mg (95%CI, 0.12-0.17) for TgTH overexpressor infected male rats. Levels of dopamine in the cerebellum indicated an interaction effect between infection status and rat sex (chi-sq = 12.75, $p = 0.002$). No effect of infection status observed in female rats, which displayed a mean dopamine level of 0.13 ng/mg (95%CI, 0.10 – 0.16) in the cerebellum.

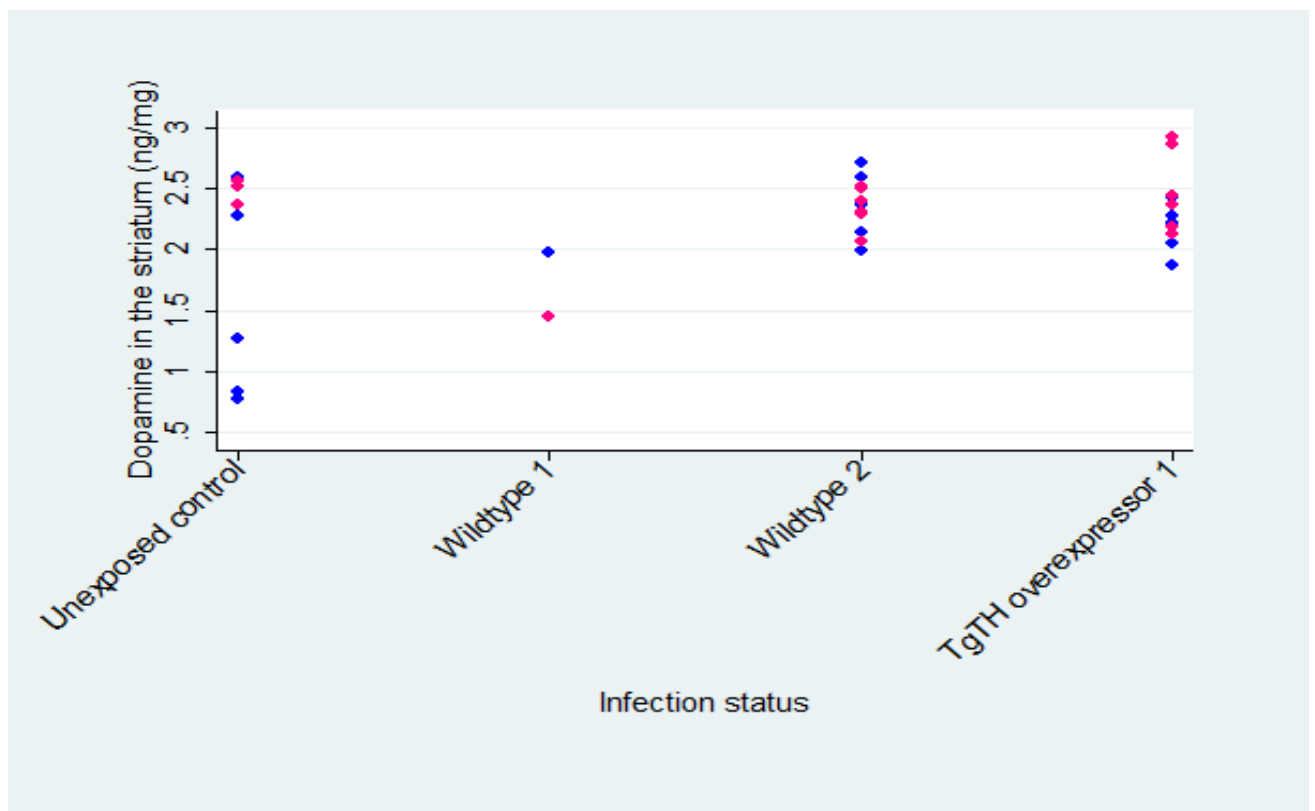
Dopamine in the striatum also displayed an interaction effect between infection status and rat sex (chi-sq =7.01, $p = 0.03$). There was weak evidence in female rats that wildtype infected animals display a lower level of dopamine than unexposed controls (Wald chi-sq =803.54, $p=0.08$; Fig. 6b). Female rats displayed mean striatum dopamine levels of in 2.48 ng/mg (95%CI, 2.23- 2.72) unexposed controls, 2.21 ng/mg (95% CI, 1.88-2.56) in wildtype infected rats and 2.48 ng/mg (95% CI, 2.13- 2.84) in TgTH overexpressor infected rats. This was not observed in male rats ($p>0.10$), which displayed a mean dopamine level of 2.06 ng/mg (95%CI, 1.78 – 2.34) in the striatum.

No differences were found between infection status' for dopamine in the hippocampus ($p>0.10$), nor interaction effects ($p>0.10$; Fig. 6c). Mean dopamine levels were for 0.26 ng/mg (95% CI, -0.5-0.6) unexposed controls, 0.11 ng/mg (0.08-0.13) for wildtype infected rats and for 0.10 ng/mg (95%CI, 0.08-0.12) TgTH overexpressor infected rats.

a)



b)



c)

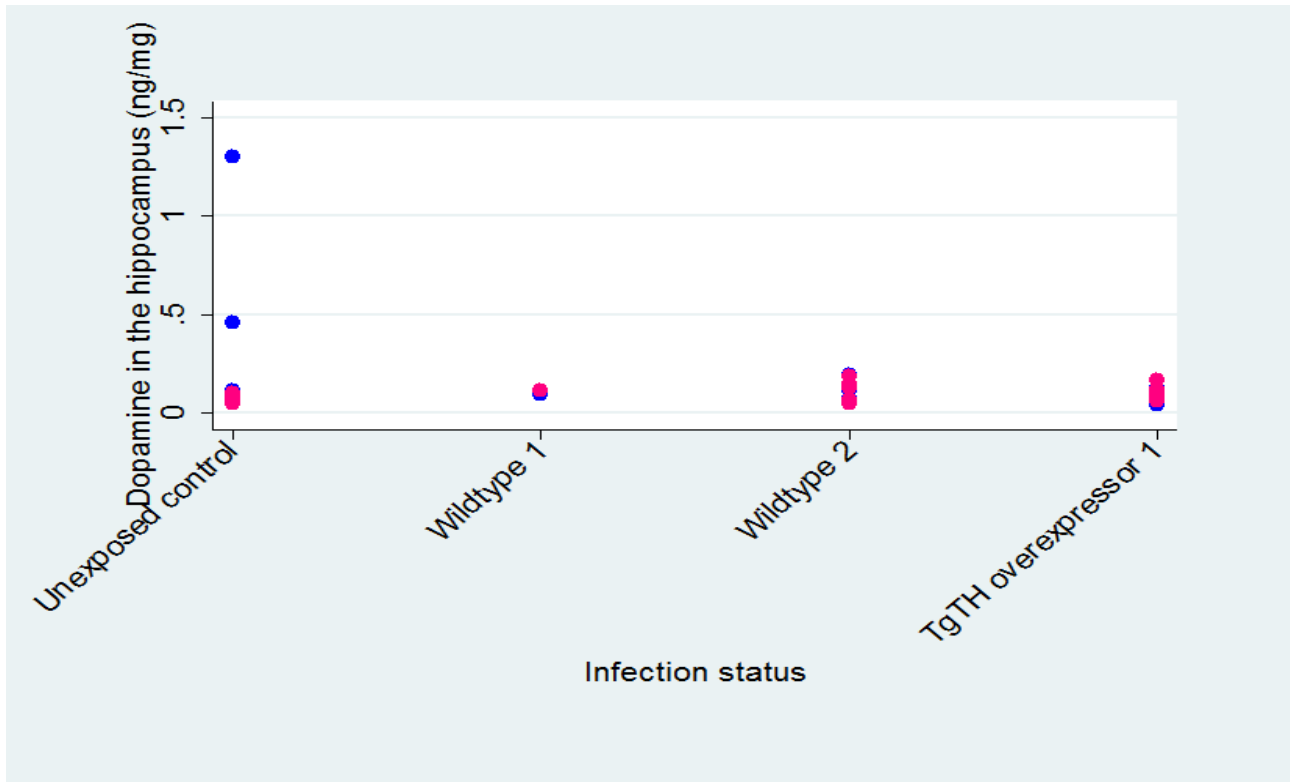


Figure 6: Dopamine levels in the a) cerebellum b) striatum and c) hippocampus of unexposed control (uninfected rats), *T. gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the first and second experiment rounds. Males are shown in blue and females in pink.

Noradrenaline

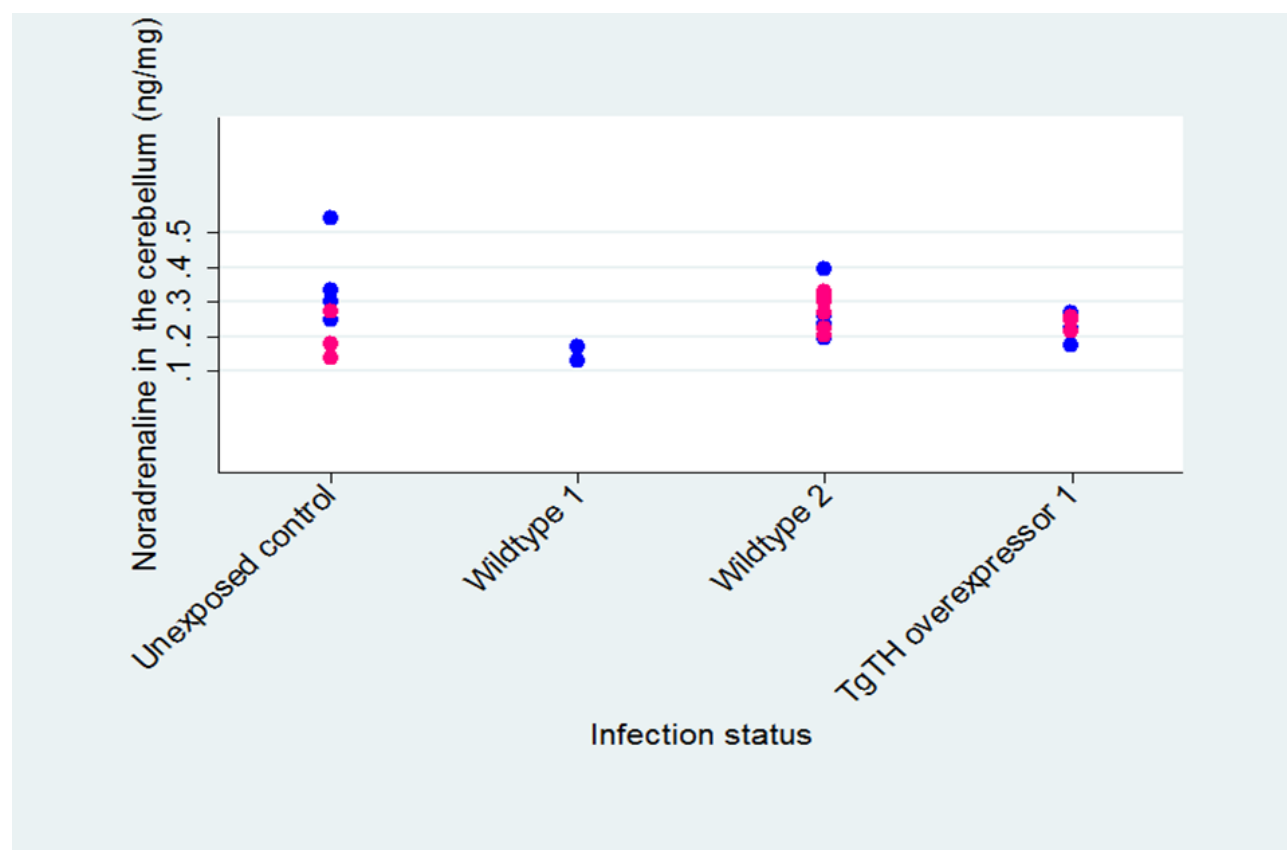
The results of noradrenaline in the cerebellum indicated an interaction effect between infection status and rat sex (chi-sq = 7.84, $p = 0.02$). Within male rats, TgTH overexpressor ($p=0.006$) and Wildtype ($p=0.04$) infected animals displayed lower dopamine levels in the cerebellum than unexposed controls (Wald chi-sq = 19.90; Fig. 7a). This effect was not observed in female rats. Overall, a weak effect was observed of TgTH overexpressor infected animals displaying lower noradrenaline levels than wildtype infected animals (Wald chi-sq = 47.14, $p = 0.09$). Mean noradrenaline levels in the cerebellum were 0.27ng/mg (95%CI, 0.17-0.36) for unexposed controls, 0.25ng/mg (95%CI, 0.21-0.30) for wildtype infected rats and 0.24ng/mg (95%CI, 0.23-0.26) for TgTH overexpressor infected rats.

The results of noradrenaline in the striatum indicate interaction effects of infection status with both rat sex (chi-sq = 24.81, $p < 0.001$) and experiment round (chi-sq = 74.98, $p < 0.001$). In the first experiment round the data suggest that wildtype infected animals may display higher

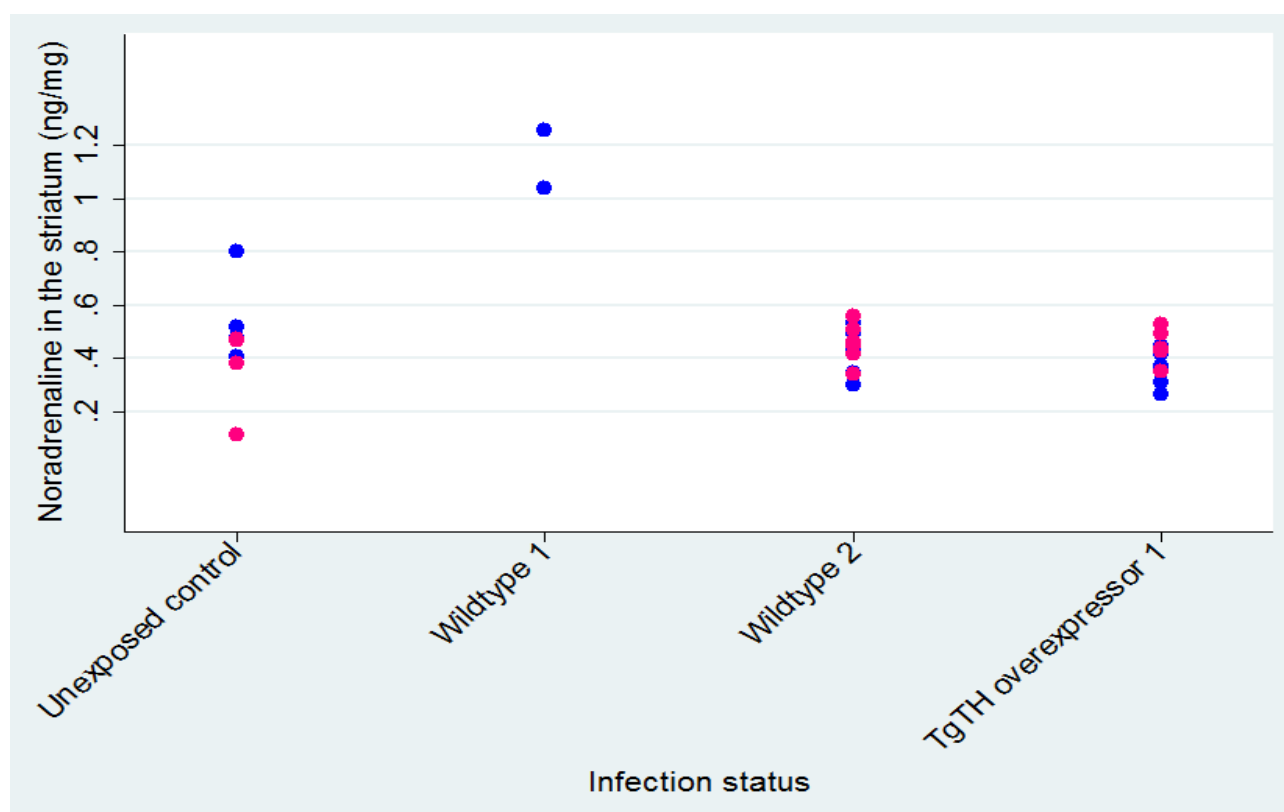
noradrenaline levels than unexposed controls ($n=4$, $p<0.001$), but not than TgTH overexpressor infected animals ($p>0.10$). However in the second experiment round, TgTH overexpressor infected males are shown to have lower noradrenaline levels than unexposed control males (Wald chi-sq = 7.01, $p = 0.008$; Fig. 7b), but not lower than wildtype infected males ($p>0.10$). Mean noradrenaline levels in the striatum in the first experiment round were 0.45 ng/mg (SEM 0.34, $n=2$) for unexposed controls and 1.14 (SEM 0.11, $n=2$) for wildtype infected animals. Mean noradrenaline levels in the striatum for the second experiment round were 0.45 ng/mg (95%CI, 0.40-0.51) for unexposed controls, 0.54 ng/mg (95%CI, 0.39-0.49) for wildtype infected rats and 0.41ng/mg (95%CI, 0.35-0.46) for TgTH overexpressor infected rats.

The results of noradrenaline in the hippocampus also indicated an interaction effect between infection status and rat sex (chi-sq =7.70, $p = 0.02$).After accounting for this interaction effect, again it appears that TgTH overexpressor infected animals ($p = 0.02$) and wildtype infected animals ($p = 0.06$) display lower noradrenaline levels than unexposed controls (Wald chi-sq = 165.17; Fig. 7c). Mean noradrenaline levels in the hippocampus were 0.46 ng/mg (95%CI, 0.29-0.64) for unexposed controls, 0.41 ng/mg (95%CI, 0.28-0.53) for wildtype infected rats and 0.33 ng/mg (95%CI, 0.26-0.42) for TgTH overexpressor infected rats.

a)



b)



c)

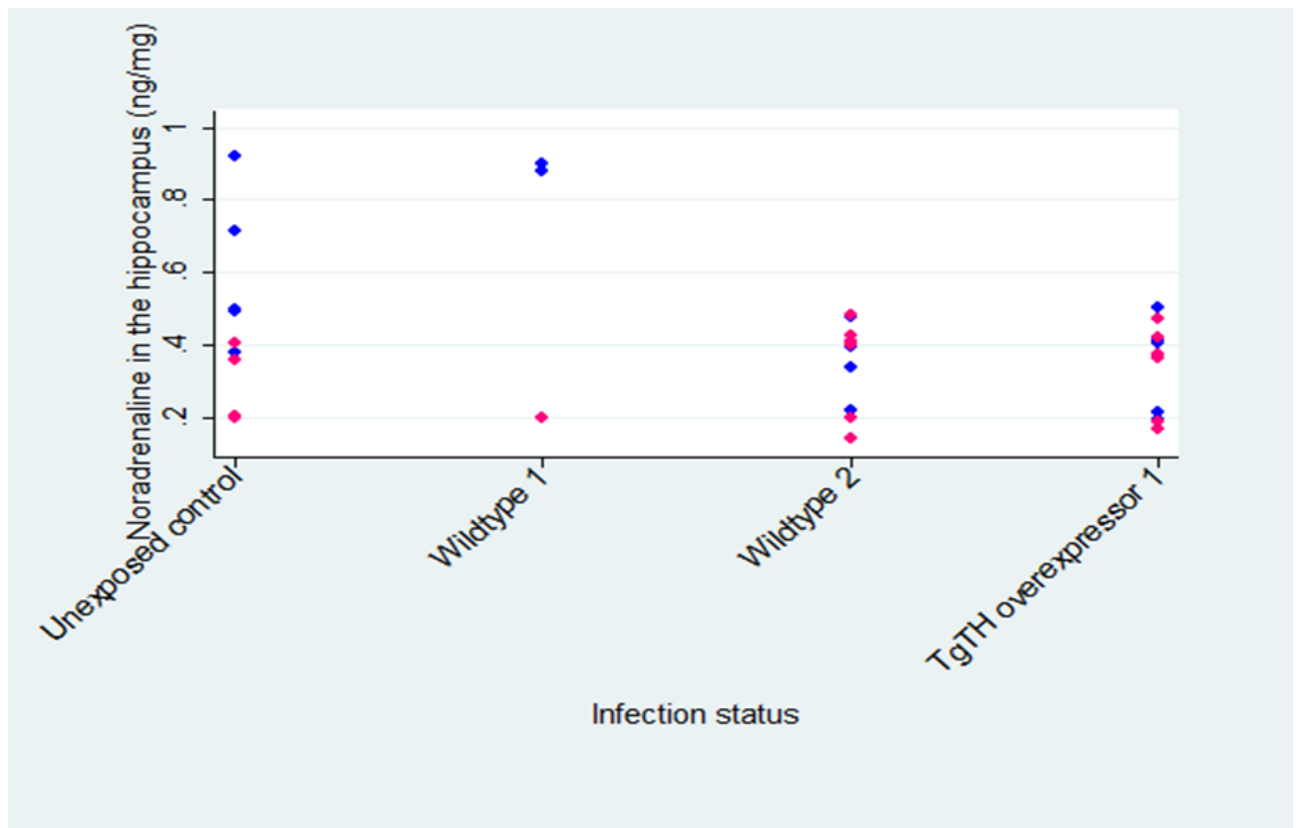


Figure 7: Noradrenaline levels in the a) cerebellum b) striatum and c) hippocampus of unexposed control (uninfected rats), *T. gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats from the first and second experiment rounds. Males are shown in blue and females in pink.

Corticosterone EIA test

Corticosterone levels from the EIA assay did not indicate any effect of infection status between wildtype and TgTH overexpressor infected rats ($p > 0.10$). Female rats displayed much higher corticosterone levels than male rats (chi-sq = 15.03, $p < 0.001$; Fig. 8). No correlations were observed between corticosterone concentrations and antibody titres ($p > 0.10$).



Figure 8: Corticosterone concentration of *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Graphs are split by rat sex. Data are from the second experiment round. No data were obtained for unexposed control rats

Testosterone EIA test

The results of this test did not indicate any effect of infection status between wildtype and TgTH overexpressor infected rats ($p > 0.10$). Female rats displayed much higher testosterone levels than male rats (chi-sq = 16.80, $p < 0.001$; Fig. 9).

No correlations were observed between testosterone concentrations and antibody titres ($p > 0.10$).

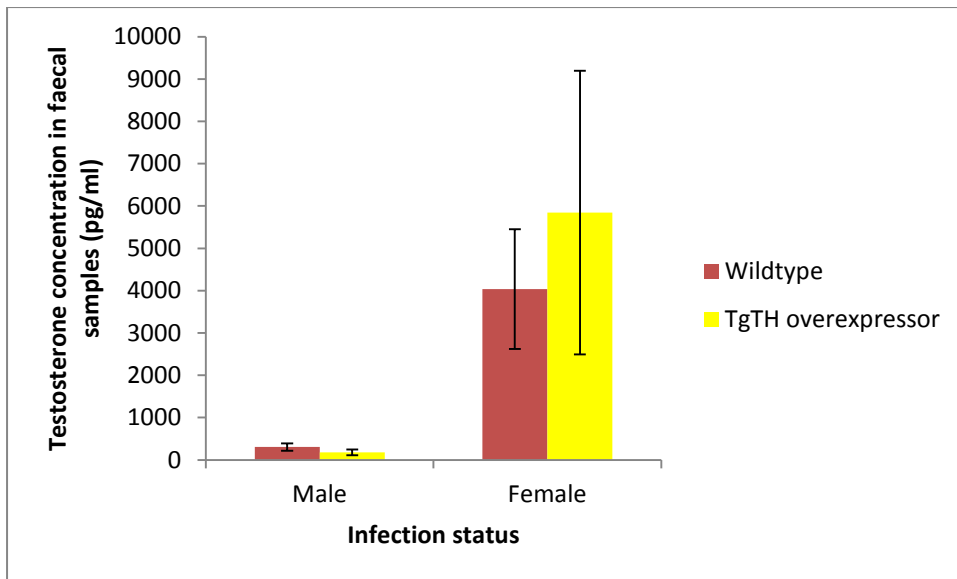


Figure 9: Testosterone concentration of *T.gondii* wildtype infected rats and TgTH overexpressor *T. gondii* infected rats. Graphs are split by rat sex. Data is from the second experiment round. No data were obtained for unexposed control rats

Correlations between physiological and behavioural data

There is some evidence that testosterone levels in female rats are linked with behavioural outcomes in the four-choice feline response test (Fig10). Testosterone concentrations in females appear to be positively correlated with the proportion of time spent in the cat zone (Wald chi-sq = 3.65, $p = 0.056^*$). This is not however significant after a Bonferroni correction.

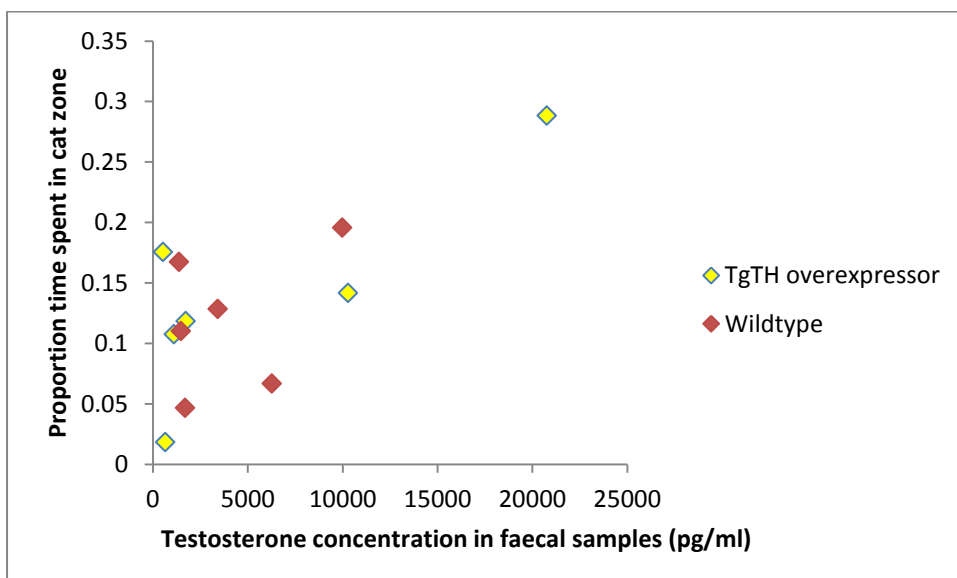


Figure 10: Proportion of time spent in cat zone from the four-choice feline response test compared against testosterone concentration in faecal samples for wildtype and TgTH overexpressor infected female rats in the second experiment round.

In the first experiment round there was weak evidence of a negative correlation between proportion time spent in the cat zone and noradrenaline levels in the hippocampus (Wald chi-sq = 4.05, $p = 0.044$; Fig. 11). There was also evidence in the second experiment round of a negative correlation between duration in open arms in the elevated plus maze and noradrenaline levels in the cerebellum (Wald chi-sq = 3.78, $p = 0.052^*$), suggesting that lower noradrenaline levels may be linked to anxiolytic behaviour in this test (Fig. 12). These are not however significant after Bonferroni corrections, and there was no effect of infection status observed ($p > 0.10$).

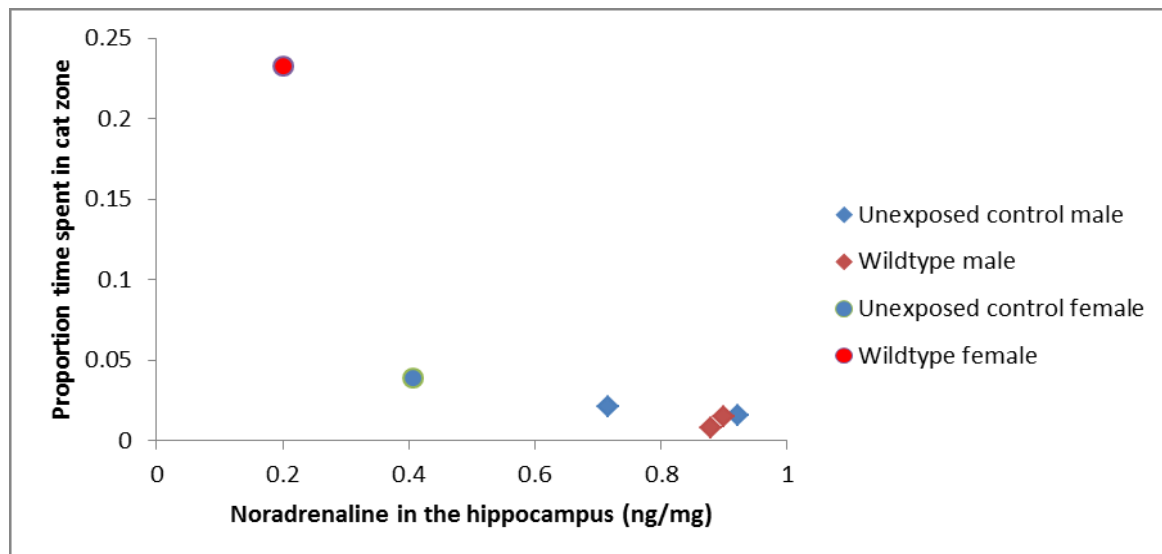


Figure 11: Proportion of time spent in cat zone from the four-choice feline response test compared against noradrenaline in the hippocampus for wildtype infected and unexposed control rats in the first experiment round.

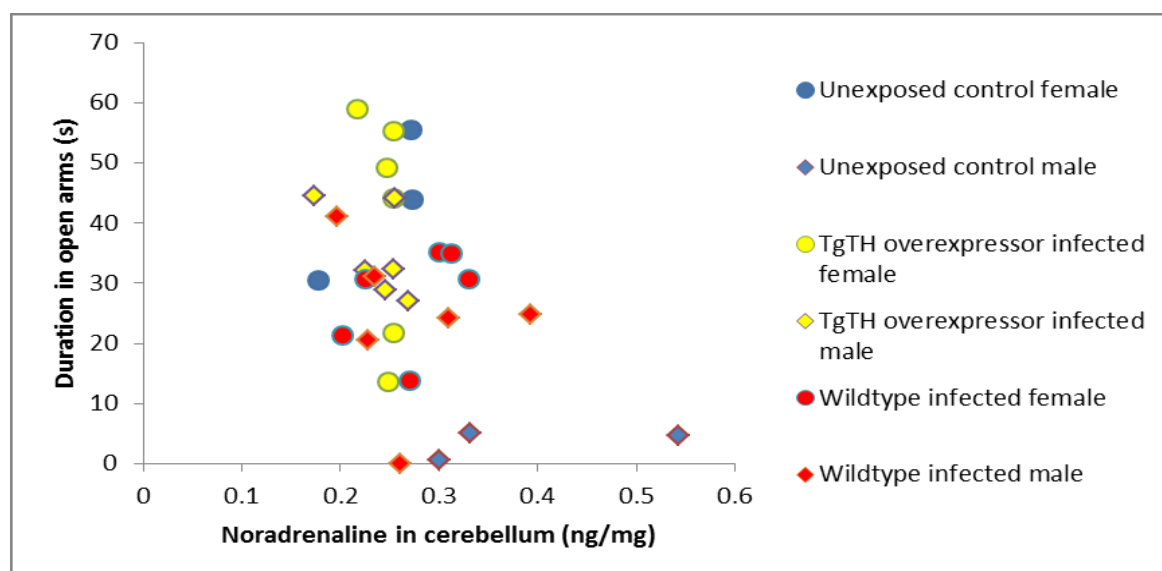


Figure 12: Duration in open arms in the elevated plus maze test compared against noradrenaline levels in the cerebellum for unexposed controls, wildtype and TgTH overexpressor infected rats in the second experiment round.

Discussion

I investigated the effect of TgTH overexpression in *T. gondii*-infected rats on the neuromodulators and hormones potentially involved in behavioural alterations in schizophrenia. The serological results indicate that most of the rats exposed to *T. gondii* sustained infections, whilst the unexposed rats did not acquire it. Antibody titre levels are expected to be associated to the parasite infection intensity and therefore may have a direct (through infection intensity) and indirect (through the immune response) effect on the observed behavioural outcomes. There was a gradient in titres, with female rats having higher antibody titres than male rats supporting previous studies in mice which have suggested that females may be more susceptible to *T. gondii* infection than males³³. Another explanation could be due to male rats being larger; therefore titre concentrations may have been lower in rats with a greater body mass, which could also explain the finding that female rats also displayed higher corticosterone and testosterone levels. This effect of sex on antibody titres, the lower titres observed in TgTH overexpressor rats as compared to wildtype infected rats, and the differences between experiment rounds, highlights the importance of measuring antibody titres. It is possible that the laboratory derived aspect of the TgTH overexpressor parasite strain lead to it being less effective at being sustained within the host. This alongside the host sex effect on antibody titres described above may be the reason why the two exposed animals that did not sustain detectable infection were TgTH overexpressor exposed male rats. The ability of GM strains to be sustained within the host could in the future be tested using a PCR assay on brain tissue. The higher levels of antibody titres observed in the second and third experiment rounds as compared to the first experiment round may have been due to the sacrifice of the first experiment round rats occurring a longer time after initial infection than the second and third experiment rounds. This was unavoidable due to time and cost constraints, although where possible a longer time was used to maximise use of animals in behavioural tests. Interaction effects observed between infection status and experiment round may be due to this and also due to the different parasite lines used. Although all parasite lines were derived from the Pru Type II strain, there is likely to have been subtle differences between them, and antibody type, level and time course of development have all been shown to be affected by parasite strain^{37, 38}. Rats' immunological responses will also always differ between individuals^{39, 40}, as can be seen here. The serology results indicate that the method of infection we used, via I.P. inoculation with tachyzoites, was effective, as all but two of the exposed rats sustained infection. The result that unexposed animals did not acquire infection post-exposure is further evidence that rats do not transmit *T. gondii* to each other via faecal-oral route⁴¹, and

exposed and unexposed animals may be housed together. Animals housed together were the same sex, so this finding does not provide further information on the suggestion that the parasite may be sexually transmitted between rats⁴².

The corticosterone and testosterone EIA assays did not indicate any effect of TgTH on levels of these steroid hormones. Future studies to ascertain effect of *T. gondii* infection would need data on unexposed controls. These findings give little information on previous studies which have indicated that *T. gondii* reduces corticosterone secretion in rats⁴³ and testosterone levels in mice⁴⁴, but are consistent with a study that indicated no effect of infection on testosterone levels in rats⁵. These results may indicate that *T. gondii* infection has an effect via alternative mechanisms to the steroid hormones, and the lack of changes in testosterone in male rats are in support of studies that suggest that this hormone along with social status and mating success are unaffected by *T. gondii*⁵. It is unclear as to why in this study females displayed higher testosterone concentrations than males, as previous studies have indicated that testosterone metabolism and concentrations are generally higher in adult males than females^{45, 46}. A possible explanation for this, as described above, may be the sex difference in body mass. Future studies using EIA assays on blood plasma samples, may give a more direct indication of steroid hormone levels within the body^{47, 48}. I opted to use faecal extractions as these can be collected non-invasively from the rat, as the more invasive blood collection techniques interfere with the rat's behaviour and stress hormone levels⁴⁸⁻⁵⁰. The lack of effect of infection status on steroid hormone levels observed may have also been an artefact of small sample size, and future research with a larger sample size may help to elucidate this. The positive correlation observed between testosterone concentration in female rats and duration spent in the cat zone in the four-choice feline response test may indicate that testosterone plays a role in some of the gender effects seen in behavioural studies, however there is an issue of multiple testing here and this was not significant subject to a Bonferroni correction. Testosterone concentrations have been shown to be altered in *T. gondii* infected individuals in previous studies with mice and humans, but evidence conflicts regarding the direction of alteration, which may depend on host sex^{32, 44, 51, 52}. Data from unexposed controls will be needed in future studies, as it is an unusual result that testosterone levels were shown to be higher in females than in males, as females would normally display lower testosterone levels⁵³. This could be due to infection, and is unlikely to be a product of the sampling method used, as faecal sampling of hormone levels has been validated as an accurate alternative to serum sampling^{47, 48}.

The effect of L-DOPA seen in our first experiment round would imply that *T. gondii* infection does indeed increase L-DOPA levels within the host brain, in support of our hypothesis.

However as this effect was only found between infected and unexposed control animals with a low sample size (n=6), and was not found in the second experiment round, we may say thus far that the results are inconclusive. It is possible that this is a strain effect as a slightly different original stock wildtype strain was used in the first experiment round, although both wildtype strains were Pru Type II strains. This highlights the importance of strain effects in laboratory experiments using *T. gondii*⁵⁴. This may also have been an artefact of detection problems of L-DOPA using HPLC, as the L-DOPA peak is situated very close to the noise peak on the HPLC trace and was therefore difficult to accurately determine.

The lower levels of dopamine and noradrenaline found in infected animals conflicts with the elevated dopamine from *T. gondii* infected dopaminergic cells seen *in vitro*¹². This finding therefore did not support our original hypothesis that *T. gondii* infection elevates dopamine levels within the brain, and in fact opposed it, suggesting that *T. gondii* infection reduces levels of these neuromodulators. One possible explanation for this is that these neuromodulators are depleted during behavioural manipulation. The neuromodulator measurements in this study occurred several months after initial infection and it is therefore not possible from this study to assess how levels of these neuromodulators were affected directly after the initial formation of the cyst stage. It is possible that infected rats were in fact displaying increased turnover, or faster breakdown of dopamine and noradrenaline as compared to unexposed controls. The disruption of dopamine and noradrenaline levels, although in the opposite direction to my predictions, nevertheless provides valuable evidence as to the involvement of the dopaminergic system in *T. gondii* induced behavioural alterations. These findings are highly potent to further elucidate the link between *T. gondii* infection and schizophrenia in humans, as schizophrenia patients have also been shown to display lowered dopamine activity in some brain regions such as the prefrontal cortex⁵⁵. The co-occurrence of high and low dopamine activity levels in schizophrenia is thought to occur as abnormally low dopamine activity in one brain region can lead to excessive dopamine activity in another region⁵⁵. Thus my findings of lowered dopamine levels associated with *T. gondii* infection have important implications for further understanding the potential association between *T. gondii* infection and the development of schizophrenia in the complex human brain.

Another possible explanation for lowered dopamine and noradrenaline levels is the kynurenic pathway activated by *T. gondii* infection¹⁸, which has been put forward as one mechanistic explanation for the link between toxoplasmosis and schizophrenia¹⁸. This theory derives from the fact that *T. gondii* infection increases astrocyte activation, which in turn leads to the synthesis of kynurenic acid (KYNA). KYNA levels are elevated in schizophrenia

patients^{56, 57}, and it is thought that KYNA may contribute to the impairment of cognitive function in these cases^{18, 58}.

KYNA has been shown to reduce levels of extra-cellular dopamine in the rat striatum^{59, 60}, but to increase phasic activity of nigral and ventral tegmental dopaminergic neurons^{61, 62}. This may explain the lower dopamine, and indeed noradrenaline, levels seen in my study, and imply that *T. gondii* could indeed be influencing the dopaminergic system, but in a different way than was hypothesised. However a conflicting result from my study was that stronger effects of infection status on dopamine reduction were observed in the cerebellum than in the striatum. The cerebellum is of particular interest in *T. gondii* research as some studies have suggested preferential cyst location in this brain region^{17, 63}, and the cerebellar lesions have been shown to disrupt behaviour both in a generalised anxiety open field test and in a feline response test, indicating that the cerebellum plays a role in the modulation of emotional and complex motivational behaviours⁶⁴.

As no differences between wildtype and TgTH overexpressor infected animals were observed when looking at dopamine levels, there is no evidence for involvement of the TgTH gene here, and therefore suggests that the TgTH gene may not be directly influencing dopamine levels. This indicates that further testing may need to be conducted on the TgTH overexpressor strains as to whether these strains do indeed result in an increase in the TgTH protein *in vivo*, and if so when and to what level. There is some weak evidence that TgTH is involved in the reduction of noradrenaline levels, as seen in the cerebellum, the importance of which is described above. These findings have implications for the behavioural effects seen in earlier chapters. Noradrenaline is of interest in my study as dopamine is further metabolised to noradrenaline in some cells⁶⁵, therefore noradrenaline is in fact further down the dopaminergic pathway. It has also been suggested that the parasite may require the use of DOPA decarboxylase produced by a host cell¹⁷. DOPA decarboxylase is produced in dopaminergic and serotonergic cells, and includes cells involved in noradrenaline synthesis¹⁷. The finding in my study of decreased noradrenaline is in fact partly consistent with the previous study looking at catecholamine levels in *T. gondii* infected mice, where noradrenaline levels were shown to be 28% lower in infected mice than in controls¹³. The weak negative correlations observed between anxiolytic behaviour and noradrenaline levels again suggest that a reduction in noradrenaline levels associated with *T. gondii* infection may also be linked with reduction in anxiety associated behaviours, although we need to apply this conclusion with caution as these do not hold subject to Bonferroni corrections.

Data from the olfactory bulb would provide further insights into the involvement of the olfactory pathway in predation related fear^{8, 16}. I tried to obtain this information within my study, however a limitation with the method of brain dissection is that the brain is a delicate organ and can be easily damaged during removal and dissection, particularly small brain regions such as the olfactory bulb. This meant that results were not available from this brain region. Damage to other brain regions was minimised by careful handling and prior practice at removing the brain from the skull, and in future studies it should be possible to collect this data.

The results of this chapter lead me to conclude that gender plays a large role in host physiology and thereby interactions with *T. gondii* infection, both in terms of antibody titres and steroid hormone levels, and this may explain the gender-specific effects observed with regards to *T. gondii* infection^{32, 34}. The neuromodulator results of this study may be explained by the kynurenine pathway induced by *T. gondii* infection¹⁸, and this suggests that benefit would be derived from further studies focussing on the pathophysiology of *T. gondii* infection, and its link to schizophrenia¹⁸. Therapeutic and medical implications could arise from this, such as the inhibition of KYNA biosynthesis in patients with schizophrenia or other disorders thought to be associated with chronic latent toxoplasmosis¹⁸.

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Chapter 6

General Discussion

The most potent findings from my thesis indicate that the behavioural changes in *Toxoplasma gondii* infected rats are specific adaptive manipulative responses, and that some of these subtle behavioural alterations may in fact be related to correlations between anxiety or predation-related fear and activity levels. Using the epidemiologically and clinically significant rat-*T. gondii* model, I used male and female Lister hooded rats, wildtype Pru (Type II) *T. gondii* strains, and genetically modified (GM) parasite strains that overexpress *T. gondii* produced tyrosine hydroxylase (TgTH), to collect behavioural and physiological data to provide further insights into the mechanisms behind *T. gondii* induced behavioural alterations. My findings suggest that *T. gondii* reduces generalised anxiety in the rat intermediate host (Chapter 2). This reduction in anxiety may be associated with activity, as more active animals appear more likely to enter and explore a risky environment, as evidenced by the positive correlation between velocity of movement and anxiolytic behaviour. There is some suggestion that *T. gondii* infection, and possibly TgTH, is involved in this anxiolytic behavioural alteration, as demonstrated by the elevated plus maze test. Some data from the light-dark test suggests that TgTH might have been to be involved in alteration of activity levels in females, although this was not consistent between experiment rounds. Olfactory pathways may also be involved, and there is some evidence for gender-specific effects of *T. gondii* infection. *T. gondii* may be selectively manipulating the response of the rat host by increasing the correlations between activity and duration of time spent in proximity to feline odour, as evidenced by the four-choice feline response test, and to differentially alter host response to feline odour depending on the feline species (Chapter 3). TgTH does not appear involved in specific responses to feline definitive host odour.

Evidence for manipulation of the intermediate host to increase predation risk by the definitive host has been observed in other heteroxenous apicomplexan protozoans, such as *Frenkelia* spp. and *Sarcocystis dispersa*, which were shown via field, laboratory and modelling techniques to increase the risk of predation of the intermediate rodent host¹. The mechanisms by which these parasites influence their host remain unclear, and may be due to altered activity profiles, lowered ability to detect predators, lowered ability to escape predation, or even altered feeding behaviours, as predation often occurs during rodent feeding¹. These parasites are likely to influence behaviour differently to *T. gondii* as their

definitive host predators are predatory birds, which display different hunting profiles to felines^{2,3}. These studies however support that apicomplexan protozoans do appear able to evolve to increase risk of predation by intermediate rodent hosts, and therefore *T. gondii* may well be another example of such manipulation, albeit likely via alternative mechanisms. Whilst many of the findings in Chapter 3 were subtle, it is worth noting that it would not necessarily require very complex neural changes to result in *T. gondii* enhancing transmission to the feline definitive host. As predation is an all-or-nothing response, only a subtle behavioural change would be needed to increase predation rates at a population level. Indeed it has been suggested that small increases in manipulative effort result in greater increases in the probability of transmission when manipulative effort is small than when manipulative effort is large⁴, suggesting that even very small alterations in activity levels or fear profiles that may not have been easily detected from my study would have an impact in the wild, and be of evolutionary importance. Even if there is no manipulative effort at all from the parasite some intermediate hosts would still be eaten at random by definitive hosts, and any investment into manipulation at this point will result in an increase in the probability of parasite transmission. However, the probability of transmission will increase with diminishing returns as manipulative effort becomes larger⁴. Therefore it may be that the optimal manipulative effort required to enhance the transmission of *T. gondii* to the definitive host is quite small, and the behavioural changes required for this transmission quite subtle. This may explain why I was not always able to observe significant differences between mean values of treatment groups, as very subtle alterations in just some individuals may in fact provide enough impetus in the wild to enhance feline predation risk. It is also worth noting that certain findings may be more evident when looking in more detail at variations in behaviour between individuals⁵. This was evident in studies where effects of *T. gondii* were found in more active rats⁶, or such as in our studies where some results could only be seen within one rat gender, and there was large individual variation in behavioural responses. Often in studies the traits of groups are described by mean values, but differences in trait frequencies and distributions may in fact be the outcome of interest. There may also be temporal variability in traits that differ between groups⁵ which may be missed when carrying out behavioural tests on a similar timeline between treatment groups or trials. Trait variance in response to infection would benefit from analyses that look at individual responses as well as group responses⁵. In population based studies, focusing the study on the extreme phenotypes, i.e. the individuals at the extremes of the behavioural outcomes, can provide more potent results than by looking at the whole population⁷. This approach was taken in previous *T. gondii* studies, where the clearest *T. gondii*-altered feline responses were observed within the most active individuals⁶. It is also important to look at animal behaviour characteristics in different contexts and situations⁸. For example in my thesis, I observed

infected rats to be displaying opposing activity profiles depending on proximity to feline odour, using the novel approach of studying activity levels in greater detail using behavioural tracking software. Behavioural correlations across situations and contexts, behavioural syndromes, and behavioural types can all provide far more detail into animal behaviour than mean or average phenotypic traits on their own⁸, and in terms of parasite manipulation of host behaviour allows us to determine in more detail what exactly is being modified by the parasite⁹. My study highlights the importance of this due to the parasite-altered behavioural correlations I observed between anxiety and predation response related behaviours and activity levels in different situations (Chapter 2, Chapter 3).

I was unable to sufficiently demonstrate preference for novel object or odour choice in novelty discrimination tests irrespective of infection status, and therefore was unable to draw conclusions as to whether the parasite or TgTH alter recognition memory or novelty discrimination traits (Chapter 4). Novelty discrimination and learning traits are likely in the wild to mainly play a strong role in feeding behaviours, in particular for the purposes of taste-aversion learning and poison avoidance¹⁰. As these behaviours are not directly associated to predation risk, which is innate rather than learned^{11, 12}, it is possible that the lack of differences observed between uninfected and infected rats here is due to specificity of parasite manipulation.

My study has provided further information into the effects of *T. gondii* within the brain, and the role of the two TgTH genes that may allow the parasite to influence host dopamine levels¹³. The reduced levels of noradrenaline and dopamine which I saw, associated with *T. gondii* infection, may indicate the involvement of the kynurenine pathway (Chapter 5). Antibody titres are affected by host sex and time post-infection, and TgTH overexpressor lines induced lower antibody titres. There is some evidence for correlations between physiological and behavioural data indicating associations between behavioural alterations and noradrenaline, and also testosterone, which may explain some of the gender-specific effects of *T. gondii* infection.

The extensive studies that have been carried out looking at the effect of *T. gondii* on the behaviour of rats^{6, 14-18} indicate that *T. gondii* affects rat behaviour making them more likely to be predated on by cats, the parasite's definitive host⁶. But what are the implications of this to human infection? Humans are, in general, thought not to represent intermediate hosts of *T. gondii*, as they are rarely predated on by cats, although it could be argued that this is a recent evolutionary phenomenon and that our primate ancestors were in fact prey to large felines¹⁹. Humans are nevertheless frequent accidental hosts of the parasite, with 23 - 33%

of the UK population and 4-92% worldwide infected²⁰. Thus any behavioural alterations observed in humans are likely to be a side effect of prior selection in intermediate hosts²¹. Behavioural alterations in latently infected humans may be predicted, as it is unlikely that the parasite would have the recognition or modulatory mechanisms necessary to restrict alterations only to the intermediate hosts who are specifically likely to be predated on by a cat. Therefore the findings above of *T. gondii* induced alterations in traits associated with anxiety, activity levels and fear may have implications for related human behavioural characteristics, perhaps particularly in people displaying anxiety-related mental illness. These findings may provide some explanation as to the growing body of evidence that *T. gondii* infection in humans is associated with outcomes such as involvement in road traffic accidents²²⁻²⁵ and suicide attempts²⁶⁻²⁸, which are indicative of greater risk taking and impulsivity^{29, 30}. Moreover, the lifespan of humans is far longer than that of the rodent intermediate host, and the effects of the parasite may therefore accumulate over a longer period of time²¹. As latent toxoplasmosis is highly prevalent in many human populations, any impact upon human behaviour may have significant clinical and therefore economic implications.

The findings within my thesis of *T. gondii* impacting anxiety-related behaviours is of great interest, due to the already established links between *T. gondii* infection and increased risk of schizophrenia³¹, and between increased dopamine levels and schizophrenia³². The dopamine hypothesis of schizophrenia is well established³³, and there have been several advances in the understanding of the genetic aetiology of schizophrenia^{34, 35}, alongside understanding of environmental risk factors^{36, 37}. These suggest that many cases of schizophrenia may be, at least in part, a result of dopamine dysfunction via a variety of interacting environmental and genetic factors. *T. gondii* infection is likely to be one, potentially strong, environmental risk factor, alongside many others such as social adversity, isolation, migration, abuse, use of cannabis, pregnancy/ obstetric complications, and pre- and perinatal factors³³. My findings may have implications for further understanding the risk factors and development of schizophrenia, and suggest that benefit may be derived from ascertaining the *T. gondii* infection status of schizophrenia patients, strain of infection, and possibly levels of TgTH within the brain. It has already been shown that several antipsychotic medications inhibit *T. gondii* replication *in vitro*³⁸, and if administered during the replicative tachyzoite stage of infection, can prevent the development of *T. gondii*- altered behaviours *in vivo*³⁹. It may be that this initial tachyzoite infection is the key point at which behavioural alterations are induced in part due to the fact that it is during the initial tachyzoite to bradyzoite conversion that TgAaaH2 is expressed¹³. This is further supported by a recent study which suggests that *T. gondii*-mediated interruption of mouse innate aversion toward

cat urine may occur during early acute infection in a permanent manner, not requiring persistence of parasite cysts or continuing brain inflammation⁴⁰.

My findings therefore further reinforce that *T. gondii* infection impacts the brain and subsequently behaviour, and therefore screening and treating for *T. gondii* infection may have diagnostic and therapeutic implications for schizophrenia patients. My findings also lend support to the more general theory that infectious agents play an important role in the development of schizophrenia. Many infectious agents other than *T. gondii* have been implicated as having associations with schizophrenia⁴¹, particularly those occurring during prenatal and perinatal stages. The association of infectious agents with the development of schizophrenia provide the means to improve both prevention and treatment efforts⁴¹.

An understanding of the impact of latent *T. gondii* infection will have implications for screening and prevention of the parasite in both humans and their sources of infection – livestock and domestic cats. Education on prevention of infection via both the tissue cyst stage and the oocyst stage may be improved⁴². At present screening policies vary widely between countries, and wide reported prevalence estimates are given, which may be affected largely by sample size, sampling method and serological techniques used²⁰. If research can provide a greater understanding of the effect of latent *T. gondii* infection on humans, more resources may be devoted towards improving screening methods and standardising screening policies between countries for this highly ubiquitous parasite.

To allow us to understand the mechanism of action of *T. gondii*, it is now, for the first time, possible to use novel genetically modified (GM) parasite models such as those that are involved in the synthesis of TgTH as in this study, and other potentially influential neuromodulators. It would be extremely useful in future work to conduct similar studies using TgTH knockout parasites, in order to directly see if this prevents the behavioural changes observed in wildtype infected animals from developing. TgTH knockout parasites would have an advantage over TgTH overexpressor parasites, as the overexpressor line does not represent an optimally co-evolved parasite strain and therefore, although it helps us to elucidate relative influences of TgTH, it could indicate that even if TgTH expression is involved, the altered behaviours may not be further maximised for predation from an additional increase in the production of this dopamine precursor enzyme. Future studies may also benefit from assessing the behaviour of rats infected with GM *T. gondii* strains that knockout or overexpress other genes involved in immunological or neuromodulatory pathways. Comparison of the behaviour of rats infected with GM parasites with those infected with wild type Pru (Type II) strain parasites provides a better understanding of the

role of particular parasite genes in manipulating host behaviour. The advancing state of parasite genomics also opens avenues to assess more questions *in vitro*, for example, using *in vitro* transcriptomics to measure changes in host or parasite gene expression when *T. gondii* infects different cell types in culture, including neuronal cells, or during tachyzoite to bradyzoite conversion in neuronal cells. Such methods may have great benefits in terms of reducing and replacing the use of animals in research, as some investigations could be conducted without the use of a living host organism. There is also much scope for further investigations that will provide us with insights into *T. gondii* and its impact in humans and animals, including metabolic profiling, functional imaging techniques, immunohistochemistry, DNA analysis, different strains and species of both animal and parasite models, and further behavioural assays to assess different aspects of behaviour (Appendix 1, Appendix 4). Future work with these techniques, and with additional knock out and overexpressor GM parasites, would enable us to create a multidisciplinary project linking the fields of parasitology, psychiatry, physiology, epidemiology and evolution.

In summary, the specificity of parasite manipulation of *T. gondii* and the potential mechanisms behind this were assessed using the novel approach of using GM *T. gondii* lines that overexpress the TgTH gene. Chapter 2 indicated effects of wildtype strain and potentially TgTH overexpressor strain in some tests on reducing generalised anxiety, and possibly inferred a role of TgTH in increased activity levels, thereby having implications for the associations between these traits. Chapter 3 indicated some further evidence of the specificity of *T. gondii* altered behaviour to the feline definitive host, indicating altered activity profiles depending on proximity to feline odour in wildtype infected animals. Chapter 3 also indicated that *T. gondii* alters rats' response to feline odour differentially depending on the feline species, and that this may mean the parasite has evolved to respond to certain urine components that differ according to species, sex and castration status of the feline definitive host. Chapter 3 suggests that the TgTH gene is not involved in specific feline responses; therefore other alternative pathways are yet to be investigated. Chapter 4 provided insufficient evidence to suggest that the parasite alters novelty discrimination or recognition memory traits, but could indicate that the behavioural changes observed were adaptive manipulation rather than the result of general pathology. Chapter 5 provided further insights into the physiological mechanisms behind these behavioural alterations, indicating that the dopaminergic pathway may be influenced by *T. gondii* in different ways to that expected from current literature, leading us to consider further indirect impacts of the parasite via the immune response, in particular the kynurenine pathway, and its subsequent influence on behaviour. Finally this study emphasises further the gender-specific effects of *T. gondii* and that this must be taken into consideration in both natural and laboratory studies involving this

parasite. Thus this study has provided further information into the behavioural effects of *T. gondii* on anxiety related behaviours, and on the specificity of altered responses to risk of predation by a definitive host species. The findings of these chapters lend support to the adaptive parasite manipulation hypothesis of *T. gondii*, and thus provide some argument against recent suggestions that behavioural changes caused by *T. gondii* are coincidental and not adaptive manipulation⁴³. These findings have profound implications both for evolutionary biology and the support of adaptive parasite manipulation hypotheses, and also in terms of chronic latent *T. gondii* infection in humans, and the potential long-term medical and psychiatric effects of this widespread yet subtle parasite.

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Appendix 1

Metabolic profiling methods

Urine collection

Urine collection from rats was attempted for metabolomics analysis. Various techniques were employed¹, such as micturition over a petri dish, the technique of expressing the bladder manually by application of gentle trans-abdominal pressure over the bladder to overcome normal urethral pressure. This proved unsuccessful. I then attempted urine collection by separating rats into individual mouse cages, watching for the expulsion of urine then collection with a pipette as soon as possible. This technique was tried both with and without aluminium foil². Without aluminium foil proved more successful, and small amounts of urine were collected from over half the rats; however expulsion did not occur from all animals, or else in such small amounts that collection with a pipette was not feasible. This technique also proved difficult due to contamination of urine with faecal matter. The final technique attempted was bladder puncture post-sacrifice, which again proved unsuccessful as the rats appeared to hold minimal amounts of urine in their bladder. There are a few techniques that have been developed to collect urine from rodents, and these vary in cost, duration for collection, volume and quality of urine obtained, ease of collection and in pain/discomfort caused to the animal¹. Unfortunately I was not able to find a technique that allowed for urine to be collected from all animals in the volume or quality required, and at appropriate cost and non-invasiveness to the animals.

Faecal extraction and preparation

Faecal samples were prepared for NMR spectroscopy. Faeces were weighed out to approx. 80 – 90mg. These were soaked with 400µl methanol, 285µl distilled water and 400µl chloroform. The faeces and solvents were homogenised by placing in a beater at 6,000rpm for 30 secs with 1mm zirconia beads. The samples were then centrifuged at 13,000rpm for 10 minutes. This process separated the samples into an aqueous phase, a solid phase and a lipid phase. The aqueous phase was transferred into an eppendorf tube which was then placed into a vacuum concentrator (SpeedVac®), to be dried for 10 hours.

The dried faecal extracts were then placed on a vortex for 1 min with 600µl buffered saline solution to dissolve the extract. They were then placed in a centrifuge for 2 minutes to ensure separation of any solid material that did not dissolve. 550µl of the prepared faecal

sample was then transferred into a 5 mm NMR tube. The samples were extracted and prepared in a random order to minimise any potential bias.

Serum sample preparation

To prepare serum samples for NMR spectroscopy, samples were placed in a centrifuge for 5 minutes to ensure that lipid and cell matter was not present in the sample being transferred. 300µl of plasma serum was mixed with 300µl of saline solution made up of 50% D₂O and 50% H₂O. If serum samples were too small to provide 300µl, the maximum amount was placed in the eppendorf and the rest of the 600µl was made up with extra saline solution. 550µl of the prepared serum sample was then transferred into a 5mm NMR tube. The samples were prepared in a random order to minimise any potential bias.

NMR Spectroscopy: Acquisition³

The NMR tube is held inside the magnet by a spinner turbine. The position of the spinner on the NMR tube is critical for sample acquisition, and this was determined by a sample depth adjustor. The sample was then lowered into the probe.

Commands on the computer allow you to define a new dataset, set the frequency, and tune the probe. It also allows you to perform “locking” to compensate for small, undesired fluctuations in the magnetic field. This is achieved by continuous monitoring of the ²H signal. It is also important to ensure that the magnetic field is as homogenous as possible for space inside the magnet occupied by your sample. The process of homogenising the magnetic field is called “shimming”. This optimises the resolution of peaks in your spectra and is essential for water suppression. Samples were placed in the spectrometer in a random order to minimise any potential bias.

NMR Spectroscopy: Spectral processing³

The acquired spectra were processed using the NMR software program Topspin 3.1 (Bruker, Karlsruhe, Germany). The main processes involved here were to perform phasing to ensure symmetry of peaks, to align the 3-trimethylsilylpropionic acid (TSP) reference peak to 0 ppm (when processing faecal samples), and to correct the baseline of the spectra. Multiple spectra were looked at simultaneously to equalise spectra properties as much as possible.

When processing serum samples, two spectra were obtained for each sample. The first was a standard spectra (NOESY), and the second (CPMG) spectra is used separately to analyse smaller molecular weight components. The reason for this is that serum samples contain lipids and high molecular weight components that are not usually observed in faecal extract samples. TSP is not used for calibration in serum samples, therefore the double peak for lactate is used for calibration, as this peak can always be found in between approx. 1.3 ppm and 1.4 ppm on the spectra, and this should be aligned to 1.33 ppm.

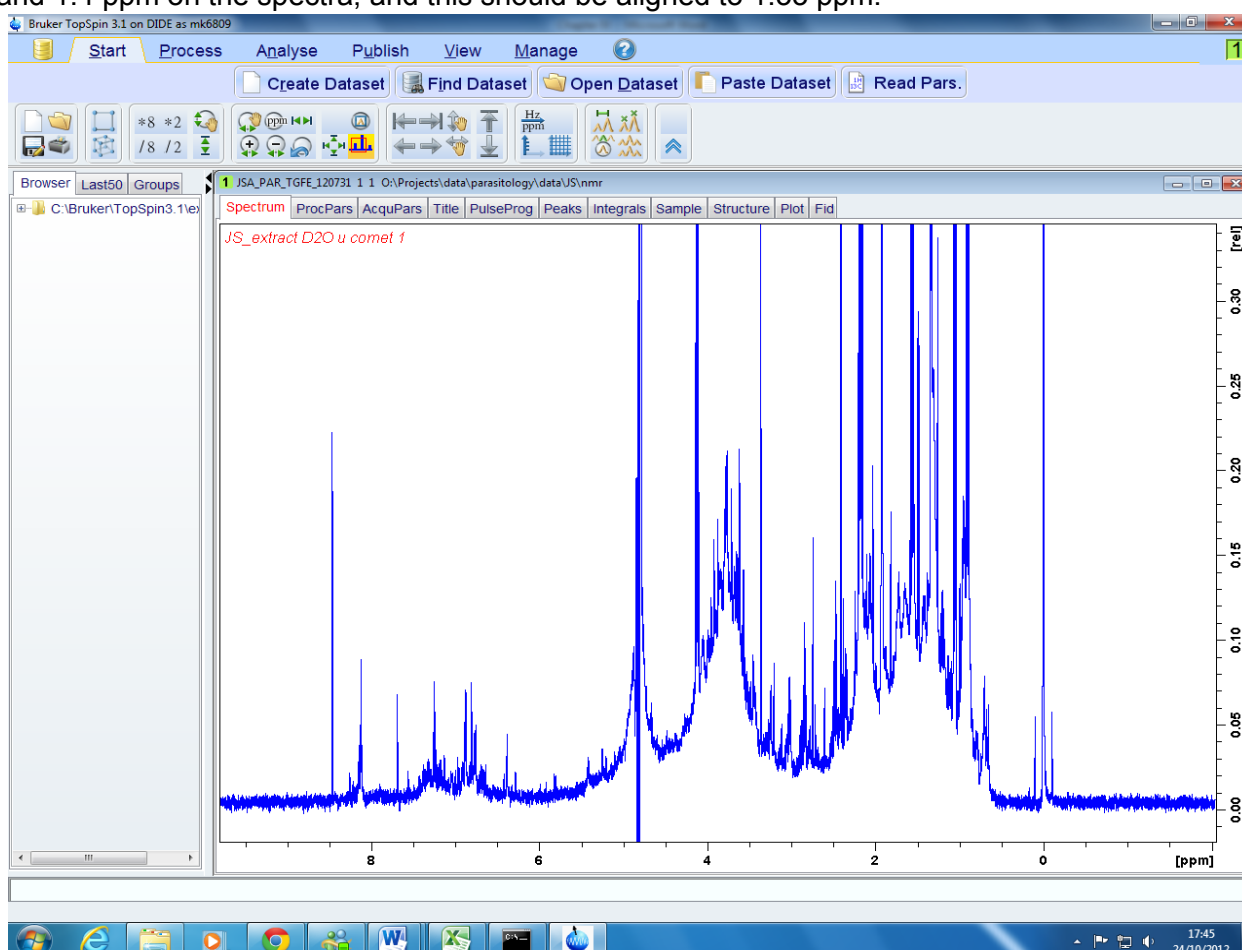


Figure 1: NMR spectral processing

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Appendix 2

Further information on materials and methods for behavioural assays

Behavioural tracking

Behavioural tracking software program Ethovision XT (Noldus, Wageningen, Netherlands) was used for many of the behavioural assays. The advantage of having automated tracking is that the experimenter can remain outside of the behavioural testing room / away from the behavioural testing equipment, so as to avoid affecting the rats' behaviour by a human observer's presence. This also helps to avoid experimenter bias and allows for blinding, as manual recording can have the disadvantage that the experimenter is aware of the animal's exposure status.

The setting up of Ethovision XT involved the set-up of the camera equipment on an autopole on the ceiling of the behavioural testing room, and adjusting of camera zoom and light settings to allow it to film the rats' movements. On the software program, arena settings must be set up before each experiment, including hidden zones where applicable and calibration settings. Trial settings and variables to be recorded are set up. Finally detection settings are created. I mainly used the differencing detection setting on Ethovision XT as by trial and error I found this to be the most effective detection method, with a contour erosion of 1 and dilation of 2. Only centre point detection was used, as nose and tail detection were found to be ineffective, perhaps due to the black and white colouring of the lister hooded strain used.

Track

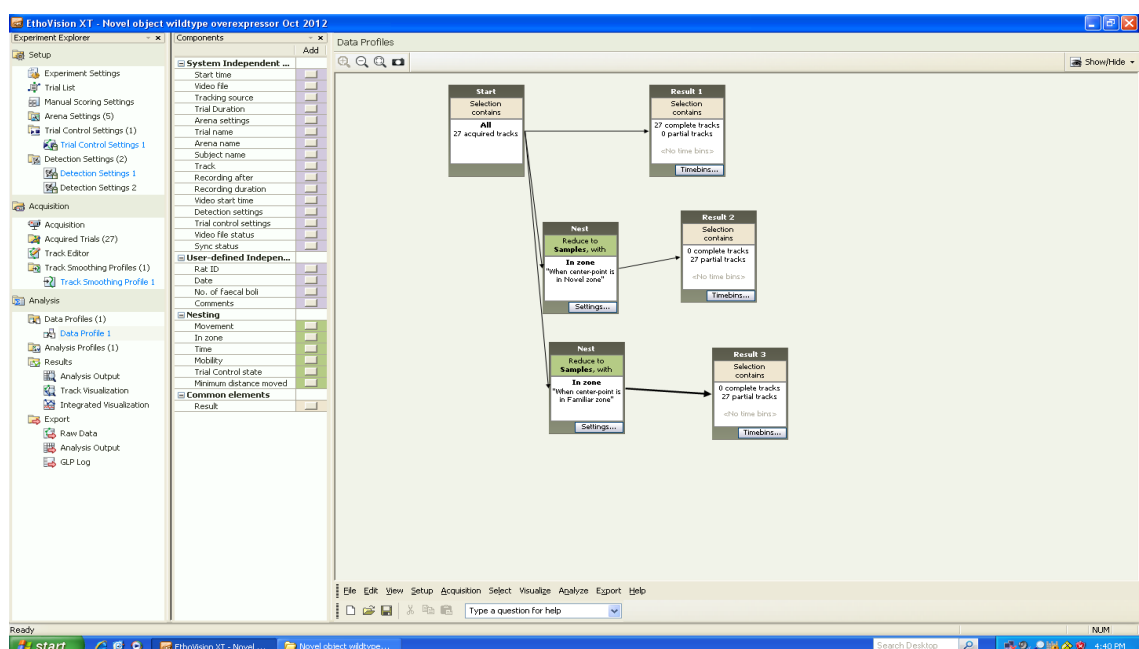


Figure 1: An example of zone nesting in Ethovision XT (Noldus)

smoothing helped to avoid detection of movements not caused by the animal itself. Variables generally recorded included duration, frequency and latency to enter particular zones, as well as velocity of movement. To record velocity of movement within a particular zone, the feature of zone nesting was used.

Sample size calculations

Sample size calculation is particularly important when using animals in biomedical research. Research using animal models is widely encouraged to follow the policy of the 3 R's – reduction, refinement and replacement^{1, 2}. Many scientific techniques would follow the general principle that a larger sample size provides more robust statistical outcomes and is therefore better for the study. However when using animals in the study, sample size generates a trade-off between higher statistical power and ethical considerations, concerning reduction of use of animals in science where possible.

I based calculation of my sample sizes around Mead's Resource equation³:

$$E = N - T$$

Where

E = the error degree of freedom. This should be in the range 10 – 20.

N = the total degrees of freedom (i.e. 1 less than the total number of animals)

T = the no. of treatments.

Therefore if for example comparing wildtype strain with uninfected controls, within each sex,

$$T = 2 (\text{sex}) \times 2 (\text{strains}) = 4$$

Therefore for our first set of experiments, $E = 23 - 4 = 19$.

When comparing an overexpressor strain, a wildtype strain and uninfected controls, within each sex,

$$T = 2 (\text{sex}) \times 3 (\text{strains}) = 6$$

Therefore for the second set of experiments, $E = 29 - 6 = 23$

For the third set of experiments, $E = 33 - 6 = 27$.

This suggests that the second and third set of experiments had a slightly higher number of animals than required. However we felt this was justified as

- a) Infection does not always sustain within the animal, and exposed rats found to be serologically negative would have to be excluded from the statistical analysis⁴. This was indeed seen as two overexpressor infected males did not sustain infection. Some animals may also sustain very low levels of infection.

- b) The overexpressor strain had never been used before in a rat model, therefore we felt it necessary to have a slightly larger sample size in case this strain was less likely to sustain infection within the animals post-inoculation, or produced unexpected virulence in an individual.
- c) Behavioural alterations caused by *T. gondii* are subtle, and may require a slightly higher sample size to achieve the statistical power needed to show these effects.

Parasite and rat strains

Data from different experiment round were not pooled to limit the influence of confounders between experiment rounds. In particular, wildtype strains used in the two rounds had some slight differences and infection occurred at different ages, therefore these data were not pooled, as some behavioural differences were seen between the two strains. All strains were derived from the Pruignard Type II strain of *T. gondii*.

Rats were chosen for use in this study rather than mice, as their increased level of resistance to *T. gondii* means that they generally display chronic, latent infection, rather than the more acute symptoms displayed in mice. As humans also display latent toxoplasmosis, rats are a more appropriate model organism for studying human infection⁵. Lister-hooded rats were chosen, as they are the most behaviourally similar to wild rats in terms of their neophobic behaviour⁶. Their natural coat markings can also aid with easy identification of individuals, and in the first experiment round identification from coat markings was used to avoid invasive tagging techniques that could impact the rat's behaviour. Both male and female rats were used as *T. gondii* has been shown in both rodent and human species to have very different physiological, immunological and behavioural effects depending on the gender of the host⁷⁻¹⁰, so it was deemed important to study the effects of infection in both genders. Before the rats could be infected, modules 1-4 home office training were completed, as well as training on the general handling of laboratory animals. I.P. injections and euthanasia were all performed by a trained and experienced member of CBS staff.

Animal welfare

Unexpected illness

During the course of this study, one male rat was observed to have a lower weight than expected. As it did not improve with a wet diet and appeared to show early signs of illness it

was culled to prevent any further pain or distress. This was deemed to be an isolated incidence and unrelated to the study, particularly as it was an uninfected control.

Re-housing of animals

Re-housing of animals was mainly avoided. In the event that re-housing with new animals was necessary, animals were placed in a new cage so as to minimise the distinctiveness of the novel cage mate. Animals were closely monitored for signs of fighting or social dominance related distress. No such problems occurred.

Time planning

Gantt charts were used to plan behavioural tests which can span over several weeks. One such example is shown here:

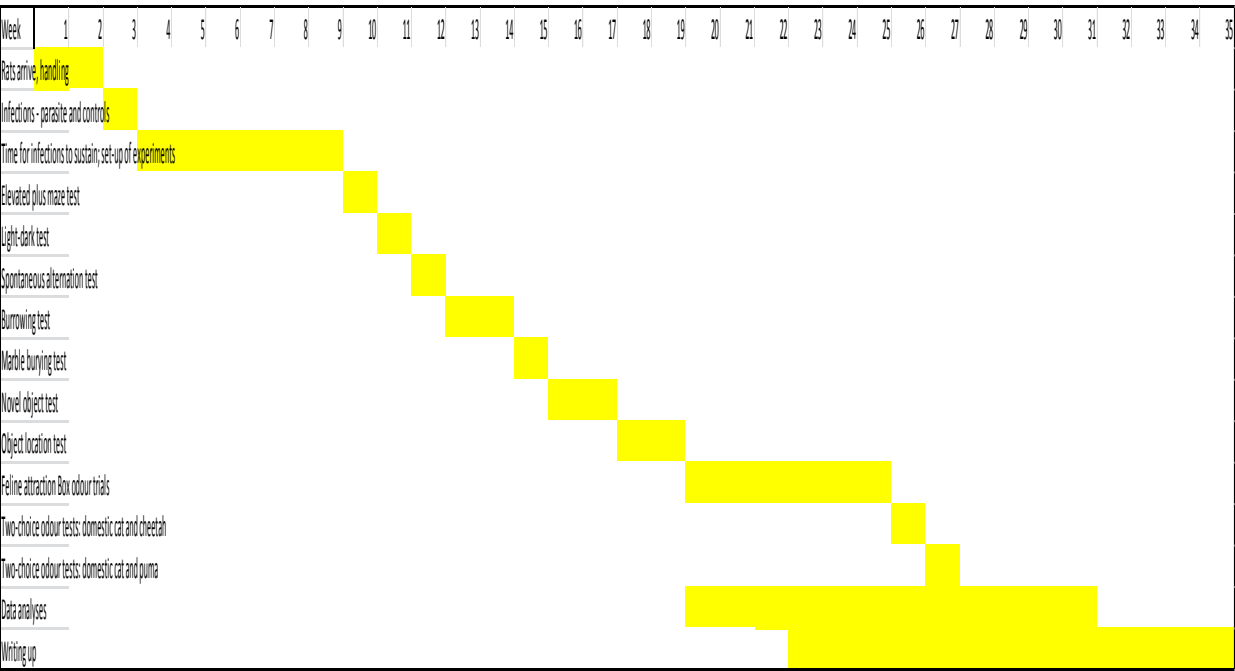


Figure 2: Gantt chart for behavioural tests

Budget planning

As with all laboratory research projects, several expenses had to be taken into account in the running of this project. These included:

- Animal costs – buying, housing and maintainence (per cage).
- Procedure room costs – behavioural assays, infection and sacrifice.
- CBS staff time costs
- Cost of behavioural testing equipment.

Cost of molecular biology test kits – ILAT test, EIA test kits.

Laboratory consumables – pipettes, pipette tips, tubes, reagents, dry ice.

Rat infection and dissection equipment – scalpel blades, bone cutting forceps, scissors, syringes, needles.

The budgeting of and ordering of these equipment were therefore a large part of this project.

General laboratory procedures and safety requirements



Figure 3: Personal protective equipment

Parasites were transported and stored at a maximum volume of 150ml. There was no risk of exposure to the parasite during behavioural tests. A small risk was present only during infection, culling and dissection of rats. To control this risk, procedures were performed within a Class II microbiological safety cabinet within a CBS procedure room according to Standard Operating Procedures (SOPs). Material transported within the laboratory was contained in petri dishes, flasks or tubes in robust sealed containers.

All tests conducted on rats were fully PPL and PIL licensed and classed as 'mild'. All rats were culled using a schedule 1 method before any pathological symptoms occurred. I.P. injections and cullings were all performed by a trained and experienced member of CBS staff.

Personal protective equipment (PPE) was worn during infection, behavioural assays, and cervical dissections. PPE consisted of blue nitrile gloves, a mob cap and blue foot covers stored in the CL2 laboratory ante-chamber and moved into the laboratory as required. Laboratory coats with elasticated cuffs were worn, stored within the procedure room. A fully fitted mask provided by occupational health was worn, and disposable face masks were also available. Face masks are required to reduce risk of animal allergens – there is no risk of aerosol transmission of *T. gondii*.

Behavioural testing equipment was either autoclaved or soaked in Trigene disinfectant before use wherever possible. Behavioural testing equipment was also wiped down with Trigene in between each individual experiment. Trigene could be made up from concentrate

at a 1:100 dilution, by adding 5ml of Trigene concentrate to 500ml water. Otherwise, pre-made Trigene wipes were available for use. Trigene is a non-selective, alcohol free biocide. All eppendorfs containing experimental material were autoclaved before use. Animals were transported to and from the procedure room within their cages within a blue transport box, designed for transport of containment level 2 (CL2) animals. The transport box was also wiped with Trigene or an alcohol based disinfectant after use.

Disposal of waste material

Liquid waste containing parasite material was decontaminated using an equal volume of 2% Virkon and left overnight (following manufactures literature and following assays confirming killed cultures for regrowth on fresh culture media performed at the University of Leeds prior to commencement of study) before disposal in drains with a large volume of water.

Sharps were disposed of in a yellow sharps bin. Woodchips and materials used during behavioural assays were disposed of in a yellow sealed bag, and animal carcasses and body parts were disposed of in a red sealed bag. These containers then underwent clinical waste disposal (incineration). All laboratory surfaces during infection, behavioural assays and dissections were regularly wiped down with Trigene. Throughout all procedures I had access to a named veterinary surgeon, a biological safety officer, and a local safety supervisor in the event of accidents or emergencies.

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Appendix 3

Preliminary trial for four-choice feline response test

Prior to infection of rats, a preliminary four-choice test was conducted with no odour and rats unexposed to infection, to test for positional bias. The experiment was conducted in the rat holding room, and under the circumstances the expected result should be that zone would not have an effect, i.e. the rat should spend equal amounts of time in each zone. However the results showed that the mean duration spent in each zone was as follows:

Table 2: Mean duration of time spent in each zone of the box.

Zone	Mean duration (s)
1	676.27
2	393.14
3	401.89
4	274.74

A multiple regression analysis showed that zone did have an effect on the rat's position ($p < 0.001$), whilst rat ID had no effect ($p = 0.651$). Zones 1 and 4 in particular had durations that deviated from the expected value of 436.51s ($p < 0.05$). This is evidence for positional bias, i.e. the presence of one or more confounding variables that affect the position of the animal in the arena irrespective of infection status or exposure to odour. Such confounding variables could include a light gradient, the presence of an experimenter or of other animals in the room, or other extraneous sound or odour stimuli. For example in this case, zone 1 was closest to the rat's home cages and zone 4 was furthest away, therefore the animals may have been responding to the sounds and smells of their home cages. This illustrates the importance of randomising the position of stimuli to reduce the effect of positional biases, and of utilising control data as a comparison. To reduce the effects of such biases, future experiments were conducted outside of the animal holding room in a separate experiment room. The experimenter spent as little time in the room as possible. Noise was kept to a minimum, and lighting was kept constant throughout the trials.

Appendix 4

Future work

1. Metabolic profiling

The expanding field of metabonomics in infection can now be used for the first time to understand the impact of *T. gondii* infection on physiology in the host^{1,2}. The methodology based on ¹H nuclear magnetic resonance (NMR) spectroscopy and multivariate statistical data modelling would enable studying effects of GM parasite strain infection to the host metabolism. The metabolic composition of urine, plasma and faeces are reflecting the systemic state of an organism and may help to understand how effects of a *T. gondii* infection in a target tissue can generate a network of molecular events throughout the host system, including the CNS²⁻⁴. The approach of combining behaviour and metabolic data is completely novel at present, and therefore presents a highly applicable new research question. This information can correlate with data obtained from behavioural studies, as well as measures of steroid hormone and neuromodulator levels. This technique enables us to increase the information we can obtain from a small number of animals, allowing us to work in accordance with the 3 R's remit of animal research. The novel use of metabolic profiling in *T. gondii* research would be enhanced further by linking with each rat's individual behavioural profile, both from predation specific and from generalised anxiety behavioural assays. Work on this has already been started during the course of this thesis. Whilst the results of this have not been analysed yet, sample collection, preparation, acquisition and processing were completed (Appendix 1).

2. Functional imaging techniques

Multi-modal imaging techniques such as PET and MRI may be employed to assess brain changes associated with *T. gondii* infection, and how this may lead to the development of psychiatric disorders such as schizophrenia^{5,6}.

3. Immunohistochemistry

Testing for *T. gondii* cysts can be done using a lectin-TRITC conjugate product. This allows detection in binding assays by fluorescence, and is often used in histochemical studies. Brains can thereby be tested for TgTH, dopamine, Dopa decarboxylase, corticosterone related enzyme localisation, and neuronal markers.

4. DNA analysis

Brain samples can be stored in sterile eppendorf tubes containing RNA*later* solution at 4°C. RNA*later* is an aqueous tissue storage reagent that stabilizes and protects cellular DNA in intact, unfrozen tissue samples. RNA*later* eliminates the need to immediately process tissue samples or to freeze samples in liquid nitrogen for later processing. Tissue pieces can be harvested and submerged in RNA*later* for storage without jeopardizing the quality or quantity of DNA obtained after subsequent DNA isolation. This preserves the genetic material for future analysis using western blotting, microarray tests, and RT-PCR (reverse transcription polymerase chain reaction).

5. Strains and species: Parasites and animal models

The effect of GM parasites on host behaviour can be combined with examining the effect of different developmental stages of the *T. gondii* life-cycle (oocyst, tachyzoite, bradyzoite) exposure on the behavioural and physiological phenotype displayed in the rat model system. Additional rat models for schizophrenia systems, including KO are currently under development. These would give me the flexibility to incorporate those when they become available to further elucidate host from parasite effects in such a gene-environment interaction system⁷⁻⁹.

6. Further behavioural assays

There are a number of other behavioural tests that may provide us with a better insight into the specific effects of *T. gondii* on host behaviour. Other useful tests that can be included are:

- Acoustic startle and prepulse inhibition (PPI)¹⁰ - The acoustic startle response is characterised by an exaggerated flinching response to unexpected auditory stimuli. This response can normally be attenuated when it is preceded by a weaker pre-stimulus - PPI. Deficits in PPI have been associated with a number of human psychiatric disorders such as schizophrenia.
- T-maze discrete alternation test¹¹ – This is similar to the spontaneous alternation that can be observed in the two-choice maze, which has already been conducted; however is a discrete trial technique rather than a continuous trial technique. Continuous trials have a lot of inter-trial interference, which is why alternation rats can be modest. Also continuous trials can fail to detect hippocampal damage, as hippocampectomised animals will often adopt a side preference, choosing only one arm repeatedly. Rewarded alternation is another test that can be performed using the T-maze. This assesses cognitive ability of the rat as well as hippocampal dysfunction.

- The five choice serial reaction time test (5-CSRTT)¹² - This normally uses visual stimuli, but could be adapted to include other stimuli such as odours. This paradigm requires subjects to detect brief stimuli presented in a pseudorandom order in one of five spatial locations over a large number of trials. The rats are trained for 30–40 daily sessions during which they gradually learn to respond in the appropriate aperture within a certain amount of time. If they fail to respond, respond in the wrong hole or at an inappropriate time, no reward is delivered. The 5-CSRTT provides the possibility to test the effect of *T. gondii* infection on discrete and somewhat independent measures of behavioural control, including accuracy of discrimination, impulsivity, perseverative responses and response latencies.

This test measures impulsivity, attentional processes, accuracy of discrimination, response control¹³. The test is sensitive to discrete brain lesions and neurotransmitter depletions. The test is in particular used to elucidate neuropsychological mechanisms that are disrupted in pathologies characterised by attentional dysfunction, such as schizophrenia, ADHD, Alzheimer's disease and Parkinson's disease¹³.

It is highly relevant for understanding the association between *T. gondii* and schizophrenia as attentional dysfunction is a pathology found in human sufferers of schizophrenia¹⁴ and other neurological disorders associated with *T. gondii* infection in humans^{15, 16}. The test has also been shown to have good translational value, as it was originally adapted from a human task, and has been successfully extended to mice and primates¹⁷. This assay provides a useful, less invasive/aversive alternative behavioural assay relevant to the aetiology of schizophrenia to that of the more widely used Prepulse inhibition (PPI) assay.

- Sensorimotor tests – These tests have been conducted in mice and have shown them to have sensorimotor deficits¹⁸. Similar tests could be conducted in rats. These include the balance beam and gait analysis.

Further tests, time permitting, could also aim to compare the behavioural profiles of rats infected with other parasites with similar life cycles to *T. gondii*, with that of *T. gondii*-infected rats. Parasites with similar life cycles include *Neospora caninum*, for which the definitive host is canids, and *Sarcocystis neurona*, for which the definitive host is the opossum. As these parasites also need to be predated on by a definitive host species, we may predict that if parasite host manipulation is observed in *T. gondii*, it may also be observed in these parasites, in a way that increases their chances of entering their definitive host. *N. caninum* is of particular interest, as a sequencing of its genome sequence revealed

an ortholog of the genes that upregulate tyrosine hydroxylase in this parasite as well¹⁹. If *N. caninum* is shown to reduce rats aversion to canine odour, this would be further evidence for the involvement of these genes in the mechanism of host manipulation.

Gambling assays - These tests would measure impulsivity, addiction, and risk-taking behaviours²⁰⁻²². Increased impulsivity and risk taking are symptoms displayed in several clinical conditions such as schizophrenia and ADHD²³. Certain drugs which target the dopaminergic (and/or serotonergic) system, in particular that of dopamine agonists, may result in, as a side-effect, increased impulsivity, risk-taking or addictive traits such as gambling in humans²⁰. *T. gondii* infection can increase risk-taking behaviours in rats^{24, 25} and in humans²⁶, potentially through related alterations of the dopaminergic system^{27, 28}.

I attempted to design a gambling test with a high risk high reward arm and a low risk low reward arm²⁹, with the reward being marshmallows. I wished to avoid an aversive stimulus and therefore the alternative to the food reward was ordinary food pellets. Tests with food rewards are often performed with food restricted rats, but I did not wish to restrict food from our animals firstly for ethical reasons and secondly as food restriction has been shown to be linked to alterations in anxiety related behaviours³⁰. However the result of this was that the animal displayed little interest in the food reward during this test. Further modifications of this test would have to be made if it is to be used in future studies relevant to *T. gondii* infection.

- Morris water maze - This measures spatial learning and reference memory. It is sensitive to hippocampal synaptic plasticity and NMDA receptor function³¹. Schizophrenia patients show dysfunction in learning and memory^{32, 33}, and therefore using a test which assesses these deficits in *T. gondii* infected rodents allows us to further understand the link between *T. gondii* infection and schizophrenia observed in humans³⁴. There is also much evidence that NMDA receptor hypofunction is involved in schizophrenia³⁵⁻³⁷, and therefore this test provides a relatively non-invasive means of testing this link in rodents.
- T-maze test for impulsivity – This test measures impulsivity, attention deficits^{38, 39}. This test is often used in studies looking at ADHD, as impulsive behaviour (i.e. the selection of small immediate gains in preference to larger delayed gains) is a symptom displayed in ADHD patients²³. As for the 5-CSRTT assay above, the test is highly relevant for the '*T. gondii*-rat-manipulation-schizophrenia model' as attentional dysfunction is a pathology found in human sufferers of schizophrenia¹⁴ and other neurological disorders associated with *T. gondii*

infection in humans^{15, 16}. The test has also been shown to differentially involve the dopaminergic and serotonergic systems in rats³⁹.

- Attentional set shifting –This assay measures impairments in executive processes. This is a characteristic symptom of certain neuropsychiatric conditions where pathophysiology of the prefrontal cortical dopaminergic system is thought to be an underlying cause⁴⁰. Impairments in attentional set shifting are observed in patients with schizophrenia and ADHD, due to alterations in dopaminergic functioning within⁴⁰. This has been seen in the Wisconsin card sorting task in humans⁴¹⁻⁴⁵. Rodent models of attentional set shifting may be used to assess mechanisms in the brain involved in such cognitive deficits.

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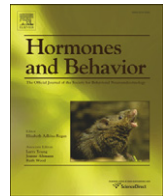
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Appendix 5

Publications and abstracts from the candidate

- 1) Webster, J. P., Kaushik, M., Bristow, G. C. and McConkey, G. A. (2013). *Toxoplasma gondii* infection, from predation to schizophrenia: can animal behaviour help us understand human behaviour? J Exp Biol **216**, 99-112.
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Review

The role of parasites and pathogens in influencing generalised anxiety and predation-related fear in the mammalian central nervous system

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ABSTRACT

This article is part of a Special Issue “Neuroendocrine-Immune Axis in Health and Disease.”

Behavioural and neurophysiological traits and responses associated with anxiety and predation-related fear have been well documented in rodent models. Certain parasites and pathogens which rely on predation for transmission appear able to manipulate these, often innate, traits to increase the likelihood of their life-cycle being completed. This can occur through a range of mechanisms, such as alteration of hormonal and neurotransmitter communication and/or direct interference with the neurons and brain regions that mediate behavioural expression. Whilst some post-infection behavioural changes may reflect ‘general sickness’ or a pathological by-product of infection, others may have a specific adaptive advantage to the parasite and be indicative of active manipulation of host behaviour. Here we review the key mechanisms by which anxiety and predation-related fears are controlled in mammals, before exploring evidence for how some infectious agents may manipulate these mechanisms. The protozoan *Toxoplasma gondii*, the causative agent of toxoplasmosis, is focused on as a prime example. Selective pressures appear to have allowed this parasite to evolve strategies to alter the behaviour in its natural intermediate rodent host. Latent infection has also been associated with a range of altered behavioural profiles, from subtle to severe, in other secondary host species including humans. In addition to enhancing our knowledge of the evolution of parasite manipulation in general, to further our understanding of how and when these potential changes to human host behaviour occur, and how we may prevent or manage them, it is imperative to elucidate the associated mechanisms involved.

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Introduction

An important strategy by which certain parasites increase their chance of transmission is by inducing behavioural changes in the infected host that can positively affect the probability of transmission to a subsequent

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host. Certain species of parasite which are transmitted via predation, where a definitive or secondary host is infected through consuming infected prey, could be expected to evolve such behavioural changes, but only where such parasites possess the appropriate mechanisms with which to achieve this. It appears that several parasite species are indeed associated with a range of behavioural alterations likely to enhance transmission through increased predation (Barber and Dingemans, 2010). Whilst the majority of these experimental and field studies concern parasites of invertebrate hosts (Moore, 1983; Pasternak et al., 1995; Poulin et al., 1992), there are a notable few of such cases occurring amongst parasites of vertebrate hosts also. Such parasites appear to achieve this either by altering the physical capacity of infected hosts to attract predation in some manner, and/or, perhaps more sophisticatedly, through altering the infected host's sensory perception of the predation risk and its subsequent fear response—hence effectively manipulating the 'mind' of the infected host. The precise mechanisms by which such parasites alter host behaviour, particularly those involving the more subtle cognitive or sensory changes of the latter group, are yet to be fully elucidated. These may, however, be plausibly predicted to involve complex interactions between the endocrine and immune systems, in combination and complement with the mammalian brain and central nervous system (CNS).

We consider here, with a focus on those, albeit rare, potential 'manipulatory' parasites and pathogens infecting the mammalian CNS, why and how parasites might achieve host manipulation. One parasite of particular interest, in terms of both its impact on the infected host and the potential mechanisms involved, is the protozoan *Toxoplasma gondii*, which frequently has a high prevalence across its intermediate (predominantly rodent, 27–35%; (Dubey, 2010; Webster, 1994a, 1994b), definitive (feline 7–74%; (Tenter et al., 2000), and a full spectrum of other mammalian secondary hosts (Tenter et al., 2000) including human (4%–92%) populations around the world (Dubey, 2010; Flegel, 2007). *T. gondii* will thus be a key, but not

exclusive, model example within this review. Our focus will be directed towards the potential mechanisms involved in the innate aversion of rodent prey to feline predators and how certain parasites may alter or even specifically manipulate this aversion (Fig. 1).

Parasitic manipulation of rodents

The manipulatory effect of parasites on the behaviour of rodents is of importance in both evolutionary and medical fields. Rodents are the main natural intermediate hosts of several potential 'manipulatory' parasites, and the rodent CNS and immune system are often used as an experimental model for humans, sharing many structural and neurochemical similarities. There are several parasitic species that appear to alter their host's anxiety levels in a manner which may enhance their transmission success. The behavioural alterations induced by this spectrum of parasites have some similarities, but the physiological mechanisms involved may be quite different. These differences depend on, among others, whether the parasite is unicellular or multicellular, is a 'microparasite' (viruses, bacteria, protozoa) or 'macroparasite' (helminths and arthropods), has a direct or indirect life-cycle, and its host range and/or route of transmission. Parasites that alter host behaviour can also be enteric or neurotropic. Certainly, whilst parasites within the CNS would appear to be situated in an ideal location with which to specifically alter or manipulate host behaviour, it must also be acknowledged that many parasitic species have a tropism for the CNS simply due to this region representing a 'privileged site', being somewhat protected via the blood–brain barrier from the full attack of the immune system. As a consequence we may predict some behavioural alterations to arise amongst CNS-infected hosts simply as a by-product of the parasite's means of immune evasion rather than indicative of specific manipulation. Likewise, when considering purely enteric parasites, even infections originating in discrete tissues or organs can trigger a cascade of consequent events throughout the host system as the host attempts to

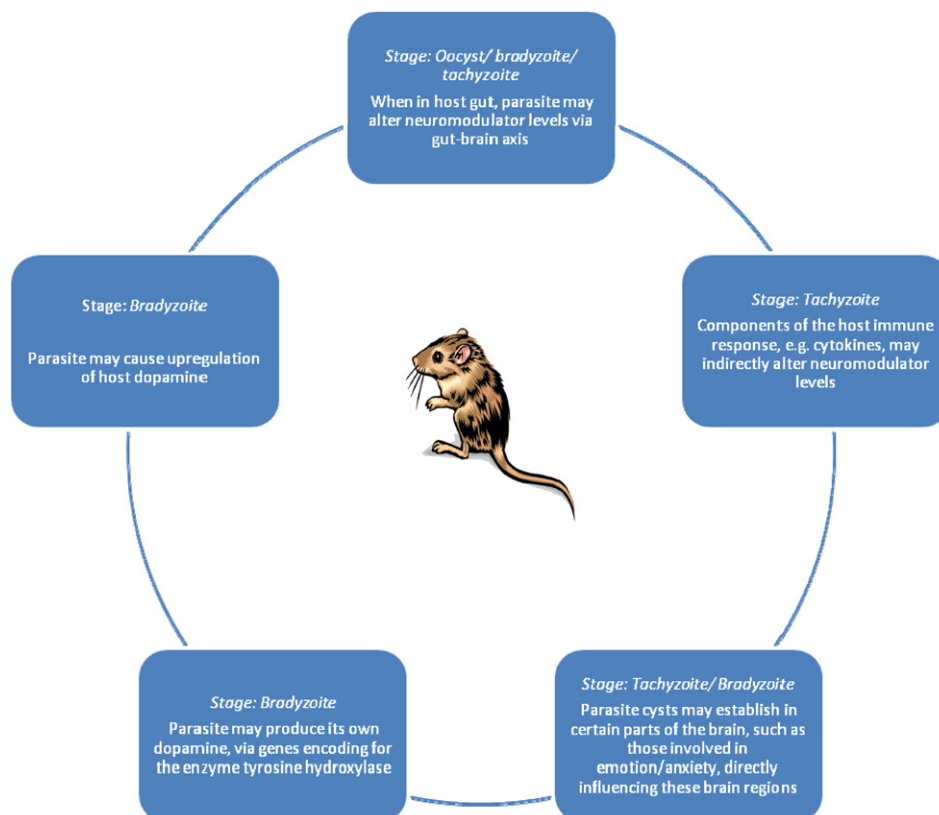


Fig. 1. Potential mechanisms utilised by the protozoan *Toxoplasma gondii* to alter intermediate host behaviour.

maintain homeostatic equilibrium (Lederberg, 2000; Nicholson et al., 2002; Saric et al., 2010a, 2010b). Thus, the effects of a parasitic infection are rarely confined to a single target tissue and instead a network of molecular events can generally be detected throughout the host system, including the CNS (Saric et al., 2010a, 2010b), all of which could plausibly be associated with subsequent behavioural alterations. When we are considering parasite 'manipulation', however, such alterations are likely (but not necessarily) to be highly specific and only predicted to evolve if there is a selective advantage to the parasite to achieve such manipulation (Webster and McConkey, 2010). Nevertheless, deciphering whether any such behavioural alterations observed amongst infected hosts result in a selective benefit to the parasite or not, and/or whether such changes are a consequence of simple generalised pathology (defined here in terms of a behavioural alteration induced in the host as a side-effect/by-product of infection not under (current) selective pressure to enhance parasite transmission nor that of a host response aimed to inhibit parasite establishment) or active parasite manipulation is not necessarily straightforward. One initial step towards such elucidation may be provided by considering the potential routes and mechanisms that may be both available and susceptible to modification by parasites of vertebrate hosts, either as a generalised or specific manipulatory response (Table 1).

Hormonal and neuromodulatory mechanisms involved in generalised anxiety in rodents

A number of neuromodulators appear to be involved in the mechanisms of anxiety within the mammalian CNS, in particular γ -aminobutyric acid (GABA) and serotonin (5-HT). GABA is an inhibitory neurotransmitter which interacts with other neurotransmitters in the brain. It acts by binding with GABA-A and GABA-B receptors in regions of the brain thought to be important in the control of fear and anxiety, such as the amygdala, the hippocampus, and the frontal cortex (Kalueff and Nutt, 2007). 5-HT cell bodies are located predominantly in the raphe nuclei of the midbrain, and the axons project to these regions of the brain (Barker, 2008). The role of 5-HT in anxiety is complex: it is thought that it may enhance conditioned fear but inhibit innate fear (Graeff et al., 1997). In the case of conditioned fear, the animal learns to fear an aversive stimulus, whilst innate fear is a genetic trait that has been selected for independent of individual experience. The serotonergic system has at least 14 5-HT receptor subtypes, e.g. 5-HT_{1A} (Barnes and Sharp, 1999). Both agonists and antagonists of the 5-HT_{1A} receptor modulate anxiety behaviour in animals (Toth, 2003). Rats exposed to the elevated plus maze test (Walf and Frye, 2007), a test for assessing generalised anxiety levels, showed decreased cortical GABA function and increased hippocampal 5-HT function (File et al., 1993a, 1993b, 1993c). Changes in uptake of 5-HT after the anxiety test were greater than changes in release, although the two were not independent of each other. Increased hippocampal 5-HT function was observed after withdrawal of benzodiazepine (an anxiolytic (anxiety reducing) drug), and after handling habituation. This suggests that an increase in hippocampal 5-HT function occurs in a variety of situations that lead to anxiogenic (anxiety increasing) responses in animal tests. Exposure to the elevated plus maze has also been shown to cause a decrease in GABA function in the cortex, via decreased basal and K⁺ stimulated release of GABA, and hence it seems that presynaptic changes in the cortex can occur rapidly in response to tests of anxiety amongst mammals (File et al., 1993a, 1993b, 1993c).

Corticotropin-releasing hormone (CRH) has also been demonstrated to have an anxiogenic effect (Barker, 2008). In mammals, the endocrine stress response is mediated through the hypothalamic–pituitary–adrenal (HPA) axis. During stress, the synthesis of CRH in the paraventricular nucleus increases. When the CRH neuropeptide reaches the anterior pituitary gland, it binds to a CRH receptor which leads to the release of other neuropeptides such as β -endorphin and adrenocorticotropin (ACTH). ACTH in turn can induce the synthesis and release of

glucocorticoids, principally the steroid compound molecule corticosterone in rats, from the adrenal cortex (Arborelius et al., 1999). Corticosterone plays an important role in fear and anxiety, although it is thought that whether, and how, behaviour is affected is dependent on the timing of its release. Furthermore, evidence suggests that corticosterone does not directly influence behaviour, but instead alters neural pathways making certain behavioural outcomes more likely in certain contexts (Korte, 2001). The activity of the noradrenergic system has also been observed to be increased during stress and anxiety in several animal species (Charney, 1995). The neuropeptide oxytocin is a single peptide hormone released from the endocrine system in response to a number of physical and psychological stresses. It is thought to have an anxiolytic effect, as central oxytocin administration reduces stress-induced corticosterone release and subsequent anxiety behaviour in rats (Windle et al., 1997). This effect is thought to be mediated by the oxytocin receptor (Bale et al., 2001).

Hormonal and neuromodulatory mechanisms involved in feline aversion in rodents

Fear and anxiety specifically related to predation are controlled by endocrine and neuromodulatory mechanisms. These effects are dependent on several factors such as the intensity of predator odour, and the species of predator (Takahashi et al., 2005). In rats, exposure to feline odour has been observed to, for example, initiate greater c-Fos expression in the medial amygdala (Dielenberg et al., 2001). c-Fos is a transcription factor that is used as a functional marker of activated neurons, and can be used to monitor transcriptional activity in the stress-related circuitry of the CNS (Kovacs, 1998). Exposure to feline odour has also been demonstrated to raise corticosterone levels in the rat (File et al., 1993a, 1993b, 1993c). Male rats exposed daily for 60 minutes over 20 days to a cat displayed higher basal plasma corticosterone levels, higher adrenal weights and lower thymus weights in comparison with unexposed controls (Blanchard et al., 1998). Conversely these rats then displayed a low corticosterone response to an acute restraint stressor, suggesting that chronic exposure to feline stimulus alters subsequent patterns of endocrine response (Blanchard et al., 1998). This chronic exposure to a cat was not shown to alter levels of testosterone, nor spleen or testes weights, suggesting that the stress response induced by feline stimulus is different from, for example, the stress induced by social subordination in male rats (Blanchard et al., 1998) where such associated reductions in fitness are observed.

Exposure to feline odour has also been shown to increase the release and decrease the uptake of the neurotransmitter GABA in both the cortex and the hippocampus (File et al., 1993a, 1993b, 1993c). Conversely levels of the neurotransmitter 5-HT and 5-Hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, have been shown to decrease in the hippocampus, but increase in the cortex following feline odour exposure (Andrews et al., 1993). As described above, the GABA and 5-HT systems act within these brain regions to modulate animal behavioural traits related to aversive environmental event responses (Miczek et al., 1995). In rats that show high avoidance of feline odour, cortical noradrenaline levels were observed to be higher after exposure to feline odour, suggesting that the cortical noradrenergic system may play a role in phobic avoidance (Andrews et al., 1993). It has been suggested that feline odour also activates the wolframin gene in the amygdaloid area (Koks et al., 2002), suggestive of multiple potential mechanisms involved in feline aversion in rodents. Activation of the wolframin gene occurs in parallel with the activation of carboxypeptidase E, indicating that wolframin may be involved in neuropeptide synthesis (Koks et al., 2002). The function of the wolframin protein is not fully understood, however, although it appears to be a multi-spanning membrane glycoprotein of the endoplasmic reticulum (Hofmann et al., 2003).

Table 1

Some protozoal and helminthic parasites that influence anxiety-related behaviours in rodents.

Parasite	Protozoa/ helminth	Enteric/ neurotropic	Life cycle	Host species	Effect on anxiety-related behaviour	Potential mechanisms involved	General or specific effect speculated?	References
<i>Trypanosoma brucei brucei</i>	Protozoa	Neurotropic	Indirect	Non-human mammals Vector: Tsetse fly	↓ Locomotor activity; ↓ Exploratory behaviour	Onset of meningo-encephalitis; altered cytokine levels and metabolic changes	General pathology	(Darsaud et al., 2003; Saric et al., 2010a, 2010b)
<i>Eimeria vermiformis</i>	Protozoa	Enteric	Direct	Mice	↓ fear of feline odour	GABA receptor mechanisms; serotonergic mechanisms	Reduction in generalised anxiety	(Kavaliers and Colwell, 1995; Kavaliers et al., 1997a, 1997b, 1999, 2000)
<i>Heligmosomoides polygyrus</i>	Helminth	Enteric	Direct	Mice	↓ fear of predator (weasel) odour	NMDA receptor mechanisms	Reduction in generalised anxiety	(Kavaliers et al., 1997a, 1997b, 1999, 2000)
<i>Toxocara canis</i>	Helminth	Neurotropic	Indirect	Definitive host: Dogs Intermediate host: Rodents and small mammals Paratenic hosts: Humans, other mammals	↓ fear of feline odour; ↑ time in illuminated and open spaces; ↓ exploratory behaviour; ↓ investigation of novel objects; ↓ and ↑ activity levels	Number and positioning of larvae in brain; neurochemical alterations	General pathology and/or Reduction in generalized anxiety	(Cox and Holland, 1998, 2001a, 2001b; Good et al., 2001; Holland and Cox, 2001)
<i>Toxoplasma gondii</i>	Protozoa	Neurotropic	Indirect	Definitive host: Cats Intermediate host: Rodents and small mammals Secondary hosts: Humans, other mammals	↓ fear of feline odour; ↑ activity; ↓ neophobia ↑ spontaneous running activity;	Production of dopamine by parasite cysts; location of cysts in brain; host immune responses	Specific reduction in feline (definitive host) aversion; possible reduction in generalised anxiety	(Berdoy et al., 1995, 2000; Lamberton et al., 2008; Prandovszky et al., 2011; Webster, 1994a, 1994b, 2001, 2007; Webster and McConkey, 2010; Webster et al., 1994, 2006)
<i>Plasmodium</i> spp. (<i>P. chaubadi</i> , <i>P. berghei</i> , <i>P. yoelii</i>)	Protozoa	Neurotropic	Indirect	Rodents Vector: Mosquito	↓ activity; ↓ anti-mosquito defensive behaviours	Accumulation and activation of macrophages leading to lesions; production of tumour necrosis factor (TNF-α)	General pathology	(Day and Edman, 1983; Grau et al., 1987)
<i>Schistosoma mansoni</i>	Helminth	Enteric	Indirect	Definitive hosts: Humans, non-human primates, rodents Intermediate host: Molluscs.	↓ nociception; ↓ exploratory behaviour; ↓ cognitive abilities	Increased cytokine levels; metabolic changes in plasma and urine; neuroinflammation; induction of brain granulomas; altered brain nerve growth factor levels	General pathology	(Aloe and Fiore, 1998; Aloe et al., 1996; Fiore et al., 1998, 2002; Saric et al., 2010a, 2010b)

In mice, data on neurotransmitters has revealed that overall brain levels of noradrenaline, dopamine, and 5-HT may not be significantly different in feline-odour-exposed mice from those of non feline-odour-exposed individuals. However, the turnover rates of noradrenaline and serotonin are increased in the hippocampus, and the turnover rate of dopamine increased in the hypothalamus and striatum, after feline odour exposure (Belzung et al., 2001). Exposure to feline odour is also thought to induce opioid analgesia in rats (Lester and Fanselow, 1985). This modulates nociceptive (pain) sensitivity in the rat, via opioid receptors. The K, Δ , and M opioid receptors are the three main types of opioid receptors found in the CNS and its periphery (Mansour et al., 1994). Endogenous opioid peptides can stimulate noradrenergic, serotonergic and enkephalinergic neurones in the brainstem (Barker, 2008), and tonic activation of K and M receptors has been shown to be involved in the modulation of the mesolimbic dopaminergic pathway (Spanagel et al., 1992).

Evidence suggests that rats respond differently to feline odour as opposed to fox odour (active component 2,3,5-Trimethyl-3-thiazoline - TMT) (McGregor et al., 2002). The anxiolytic benzodiazepine drug midazolam, for example, was shown to have opposing modulatory effects on the rat's response to cat and fox odour, suggesting that the two predator odours may be quite distinct in their anxiogenic effects within the CNS. Exposure to TMT on its own can increase dopamine metabolism in the prefrontal cortex (Morrow et al., 2000), an effect that has not been observed with exposure to feline odour. It has been suggested, however, that this effect could be due, at least in part, to the intense acrid smell of TMT, rather than it being a specific anti-predator response (McGregor et al., 2002).

There is therefore evidence to suggest that the neuromodulatory response of rats and mice to feline odour is different from any generalised anxiety responses or indeed that of responses to other predator odours. There are also differences in response to feline odour between individual rats. It has been demonstrated that rats exposed to feline odour can be divided into two categories, 'responders' and 'non-responders' (Hogg and File, 1994). Responders show a clear innate behavioural response to feline odour, whilst non-responders show no response. However, these two groups do not differ in other tests of anxiety or social interaction. This result further supports the distinction between phobic anxieties, generated by cat odour, and generalised anxiety states. This theory is supported by the different patterns of release of 5-HT and GABA observed after exposure to cat odour (File et al., 1993a, 1993b, 1993c) and after exposure to other stressors and behavioural tests (File et al., 1993a, 1993b, 1993c). The difference in behavioural and neurochemical responses to phobic anxiety and generalised anxiety is likely to be due, at least in part, to their different evolutionary paths. Generalised anxiety, measured in classic approach-avoidance tests, measures anxiety that does not vary within time, and is an enduring feature of the animal (Belzung and Griebel, 2001). Anxiety elicited by a stimulus such as predator odour is a sudden fear, accompanied by autonomic system arousal (Sullivan et al., 1999). Generalised anxiety, such as neophobia and a fear of entering unfamiliar, aversive (e.g. open/ illuminated) places, reduces the animal's risk of predation in general, whereas the phobic anxiety elicited by predator odour allows the rat to respond to a sudden change in its environment (Belzung and Griebel, 2001). The partly conditioned aspect of phobic anxiety to feline odour (Adamec, 2001; Adamec and Shallow, 1993; Zangrossi and File, 1992) may plausibly allow the rat's fear response to be more adaptable, as some rats may be in environments where they are far more likely to encounter a predator than other rats.

Manipulation by parasites and pathogens on hormonal and neuromodulatory levels in anxiety-related behaviours in rodent hosts

As discussed above, there are many neurological and hormonal pathways involved in generalised anxiety and predation-related fear in rodents. There are a variety of mechanisms by which parasites

may alter these pathways to achieve manipulation of anxiety-related behaviours in their host. Parasites that influence predation risk related behaviour, through modified anxiety of their hosts, are likely to do this either by altering the host's perception of the risk and/or by altering their fear response to this risk (Poulin, 1998).

Anxiety levels in mice infected with the neurotropic canine nematode *Toxocara canis* have been shown to be reduced, both in general anxiety tests and in relation to feline (non-definitive predator host) odour (Holland and Cox, 2001). As there are no obvious selective advantages to the parasite for enhancing predation by a dead-end feline host here, such behavioural alterations may be suspected to be indicative only of general infection-induced pathology/by-product rather than specific manipulation. Nevertheless, infected mice spend increased time in proximity to feline odour, and increased time in illuminated and open spaces in general anxiety tests in comparison with uninfected mice (Holland and Cox, 2001). Infected mice have also been observed to display decreased exploratory behaviour and decreased investigation of novel objects (Cox and Holland, 2001a, 2001b). Activity levels of mice have, however, been shown to either increase or decrease depending on the strain of the mouse (Cox and Holland, 2001a, 2001b). Altered behaviours were also observed to be dependent on intensity of infection, i.e. the number of parasite larvae within the brain (Cox and Holland, 1998). The mechanisms by which these parasites are altering host behaviour remain unclear, although it has been shown that the administration of lead in conjunction with *T. canis* infection can ameliorate the behavioural alterations caused by *T. canis* alone (Dolinsky et al., 1981), suggesting that one mechanism is, at least in part, due to neurochemical alterations rather than simply due to the physical positioning of larvae in the brain (Holland and Cox, 2001). Lead was selected for this investigation as it is a common environmental contaminant, although how it attenuates the behavioural effects of *T. canis* are unknown, particularly as it does not appear to alter viability or distribution of larvae within the CNS (Dolinsky et al., 1981).

Neurotropic positioning of parasites and pathogens need not, as mentioned above, be essential to alter neuromodulator levels and anxiety behaviours. Mice infected with the enteric protozoan *Eimeria vermiformis*, for instance, have been observed to spend a significantly greater amount of time in proximity to feline odour in comparison with their uninfected controls (Kavaliers and Colwell, 1995). However, whilst *E. vermiformis*-infected mice do not display malaise or illness, suggesting that reductions in predator response are not due to general pathological changes, the feline-attraction response in *E. vermiformis*-infected mice may also plausibly be suspected to be a by-product of a reduction in generalised anxiety rather than a specific manipulation towards 'feline attraction', particularly as felines are again not part of this parasite's life cycle, and thereby predation would result in the death of the parasite as well as the mouse host. Likewise, the observed behavioural changes do not appear to be due to any augmented opioid activity or decreased pain sensitivity, as opiate antagonists have little effect on infected mice's avoidance of cat odour (Kavaliers et al., 1997a, 1997b). Furthermore, evidence indicates that *E. vermiformis* neither non-selectively decreases olfactory sensitivity nor locomotor activity, as infection has also been shown to cause male mice to display an increased preference for the odours of oestrus females (Kavaliers et al., 1997a, 1997b). *E. vermiformis* is, however, thought to influence the behaviour of its host through neurochemical systems associated with anxiety, involving, at least in part, GABA_A receptor mechanisms (Kavaliers and Colwell, 1995). Indeed, GABA antagonists have been shown to significantly decrease the anxiety-reducing effects of the parasite infection, increasing the avoidance of predator odour by the infected mice (Kavaliers and Colwell, 1995). As stated above, exposure to feline odour has been shown to decrease GABA uptake and increase its release in the rat (File et al., 1993a, 1993b, 1993c), so there is the possibility that *E. vermiformis* is causing altered GABA activity that influence fear and anxiety in the host. As the GABA antagonists do

not completely block the effects of parasite infection, it is likely that other neurochemical mechanisms are also involved, in particular the serotonergic mechanisms that have been implicated in the mediation of predator-controlled anxiety and analgesia (Andrews et al., 1994; File et al., 1993a, 1993b, 1993c).

The enteric murine nematode *Heligmosomoides polygyrus* has also been shown to reduce the fear response of mice to predator odour (Kavaliers et al., 1997a, 1997b), although this again is likely to be explicable as a generalised anxiety alteration response, since predation of an infected rodent by a cat would result in death of both host and parasite. Infection appears to remove the non-opioid analgesia displayed by uninfected mice in response to predator odour, significantly lowering levels of opioid analgesia (Kavaliers et al., 1997a, 1997b). It has been suggested that *N*-methyl-D-aspartate (NMDA) receptor mechanisms are involved in the expression of predator-exposure induced analgesia, and the evidence indicates that *H. polygyrus* alters this mechanism to cause the observed behavioural changes (Kavaliers et al., 1997a, 1997b).

Seoul virus, a hantavirus, has been shown to increase aggressive behaviours and wounding in male rats. This is another example of how a general alteration in aggressive behaviour can facilitate transmission of a pathogen, in this case potentially via contact of wounds and virus present in saliva or excrement. Here, whilst there appears to be no viral antigen present in the brain, it is present in the gonadal and adrenal glands, suggesting that levels of hormones such as testosterone or corticosterone may be altered by the virus with a subsequent impact on host behaviour (Hinson et al., 2004; Klein et al., 2004).

Finally, a recent study has demonstrated that the colonisation of gut microbiota impacts mammalian brain development and subsequent adult host behaviour (Heijtz et al., 2011). When germ free (GF) mice were compared with specific pathogen free (SPF) mice with a normal gut microbiota, it was observed that the GF mice displayed increased motor activity, reduced anxiety, elevated turnover of noradrenaline, dopamine and serotonin in the striatum, and altered expression of synaptic plasticity-related genes (Heijtz et al., 2011). Another study indicated that the commensal bacteria *Bifidobacterium infantis* could modulate tryptophan metabolism in rats, and thereby influence the precursor pool for 5-HT (Desbonnet et al., 2008). *Campylobacter jejuni* is another bacterial microbe that inhabits the intestines of humans. This has been shown to activate visceral sensory nuclei in the brainstem, in the absence of any measurable systemic immune responses in mice (Gaykema et al., 2004). *C. jejuni* can alter anxiety behaviours in mice (Lyte et al., 1998), demonstrating further that parasites and other micro-organisms have the capability of altering neural pathways and impacting the CNS even without any direct positioning or even cytokine response from the host. The gut–brain communication that these organisms are utilising may be via signals through the vagal nerve, via modulation of stress hormones, or via modulation of transmitters within the gut itself, although mechanisms are yet to be fully elucidated (Heijtz et al., 2011).

Parasitic impact on the human CNS and behaviour

Parasites and pathogens well known for affecting the human CNS include viruses of the genus *Lyssavirus*, the causative agents of rabies, an acute encephalitis. Rabies causes severe symptoms in humans, ending in an acute neurological phase, which can present either as encephalitis or as paralysis and is almost invariably fatal if post-exposure prophylaxis is not administered before the onset of severe symptoms (Hemachudha et al., 2002). Changes in behaviour observed in infected individuals include hyperactivity, phobic spasms and severe agitation. There are a few mechanisms by which these changes in behaviour could be induced. Infection of the CNS by the rabies virus leads to the production of cytokines and nitric oxide. These can modify limbic system functions, the HPA-axis, and serotonin metabolism. The cytokines may also activate the p55 TNF α receptor, resulting in the

recruitment of T and B cells, which can then provoke another amplification of the cytokine cascade, exaggerating the disturbance of the limbic and sympathetic nervous systems (Hemachudha et al., 2002). Humans are not natural hosts of *Lyssavirus* spp., and therefore these behavioural changes are likely to be by-products of the virus' mechanisms in the brain, that in the wild/appropriate hosts (dogs, bats etc.) would increase its chance of transmission. Dogs with furious rabies show higher levels of aggression and biting (Kaplan, 1986), increasing the chance of transmission via infected saliva to the susceptible hosts blood and/or body tissues (Rupprecht et al., 2002).

The bacterial spirochete *Treponema pallidum* is well known historically for its role in neurosyphilis, one of the later stages of the sexually transmitted infection. Neurosyphilis has various manifestations, potentially due to the pathology caused by the bacteria in the CNS. *T. pallidum* invades the CNS early in infection and reaches the meninges, most commonly, during the secondary stage of the disease (O'Donnell and Emery, 2005). Early pathological findings in patients with chronic syphilitic meningitis consist of collections of lymphocytes, plasmacytes and occasional polymorphonuclear leukocytes in meningeal spaces (Hotson, 1981). The behavioural symptoms of neurosyphilis, however, become apparent during the tertiary stages of the disease. General paresis, which occurs 10–20 years after infection, includes deterioration of cognitive function and concentration, irritability and dementia. This is thought to be attributed to a progressive neuronal loss in the cerebral cortex, with most cortical atrophy occurring in the frontal and temporal lobes (O'Donnell and Emery, 2005). As such behavioural changes caused by *T. pallidum* occur long after the period of highest infectivity, and hence are unlikely to be under strong selective pressures at this stage, it appears that these behavioural alternations are likely to be non-adaptive, and hence due to general pathology caused by infection rather than any specific form of parasite manipulation.

Trypanosoma brucei gambiense and *T. b. rhodesiense*, *brucei* are protozoans that can cause sleeping sickness in humans (and *T. b. brucei* causes nagana disease in livestock, as well as additional behavioural changes in various animal species) (Darsaud et al., 2003). Trypanosomes penetrate the CNS and can be found in the cerebrospinal fluid. Infection impairs locomotor activity and exploratory behaviour in laboratory rats, potentially due to the onset of meningo-encephalitis (Darsaud et al., 2003). Although a general pathological effect, it could be perhaps argued that this is indicative of parasite manipulation aimed to increase the chance of transmission from the vertebrate host to the tsetse fly vector. A decrease in host energy and activity increases the likelihood of being bitten by an insect vector (in contrast to the benefits of increased activity in predation-transmitted parasites) via an increase in host landing rate and a reduction in fly swatting behaviours (Ewald, 1994; Holmstad et al., 2006).

In contrast to the severe pathological changes seen in humans with rabies, neurosyphilis or sleeping-sickness, latent *T. gondii* infection may cause much more subtle behavioural changes in humans, and indeed until recently was thought to be asymptomatic in immunocompetent adults. Rodents, as well as other small homeothermic animals predated upon by felines, are the natural intermediate hosts of the parasite, and here we discuss, in detail, how this parasite may alter behaviour in both its natural intermediate hosts (potentially indicative of specific manipulation) and in secondary, dead end human hosts (potentially indicative of a by-product of infection).

Case study: *Toxoplasma gondii* and altered mammalian host behaviour

T. gondii is an indirectly transmitted parasite. The sexual stage of the parasite's life cycle occurs in the feline intestine, from which oocysts are excreted with the faeces. Natural intermediate hosts of the parasite include rodents and birds, which can then ingest these oocysts when foraging. Intermediate hosts may also acquire the parasite via ingesting infected meat, via congenital transmission, and even potentially via sexual transmission (Dass et al., 2011; Dubey, 2010). In the intermediate

or secondary host the parasite forms a fast-replicating tachyzoite stage, and subsequently a slow-replicating bradyzoite (cyst) stage. These cysts are able to persist in the CNS, placing the parasite in a prime position to manipulate behaviour (Webster and McConkey, 2010). The intermediate host is then predated upon by the definitive feline host to complete the life cycle. Altering an infected intermediate host's behaviour to increase the chance of predation and successful transmission to the definitive feline host could have strong evolutionary benefits for such a parasite, particularly as sexual recombination can only occur within the feline host.

Specificity of behavioural manipulation by *T. gondii*

A potentially important factor of parasite manipulation is the specificity of the behavioural change it causes. An alteration that specifically increases the contact rate of the infected host with the subsequent predatory host strongly supports the likelihood that the behavioural change has been selected for this adaptive advantage. Whilst many parasites can cause gross pathological changes that reduce activity levels or social interaction in their host simply through a general reduction in the host's fitness, in the case of *T. gondii*, behavioural changes in the infected rat host have been shown to be highly specific to increase the chance of predation by the definitive feline host (Hay et al., 1983a, 1983b, 1984; Hutchison et al., 1969, 1980a, 1980b; Jackson et al., 1986; Webster, 2001; Webster and McConkey, 2010). Infected rats have been observed to approach feline odour more readily (Berdoy et al., 2000; Vyas et al., 2007), demonstrate increased activity (Webster, 1994a, 1994b), increased 'trappability', and decreased neophobia (Webster et al., 1994) and predator vigilance activities (Webster et al., 2006) relative to their uninfected counterparts. Furthermore, infected rats been observed to spend more time specifically within proximity to feline odours and not other non-predatory mammal (rabbit) odours (Berdoy et al., 2000; Webster et al., 2006), or even alternative predatory mammals such as mink (Lamberton et al., 2008) or dog (Kannan, 2010). Moreover, it has been suggested that the loss of fear amongst *T. gondii*-infected rodents is restricted to feline odour and that infection does not affect other learned fears, olfaction and non-aversive learning (Vyas et al., 2007). Previous studies have, however, conflicting indications on the effect of *T. gondii* infection in rodents in general anxiety tests (Gonzalez et al., 2001, 2007; Piekarski, 1981; Vyas et al., 2007). There is some evidence that infection may reduce fear of open spaces in the elevated plus maze (Gonzalez et al., 2007), whilst other studies have indicated no effect in the open field test (designed to test the same type of response of a fear of open spaces) (Vyas et al., 2007), and there has been evidence both for and against food-related neophobia (Vyas et al., 2007; Webster et al., 1994). The conflicting results from these studies may be due to the different strains, dosage and stages of the parasite used, the species, strain, or sex of the rodent host and/or, in particular, the test and experimental (laboratory versus 'semi-naturalistic') set-up used. For instance, it has been suggested that infection may reduce neophobia in naturally neophobic rats, but reduce neophilia in naturally neophilic mice (Hodkova et al., 2007).

Potential mechanisms of action of *T. gondii*

The route/s by which *T. gondii* uses to influence host behaviour remain to be fully elucidated, although recent discoveries have indicated a few clues towards potential mechanisms of action (Webster and McConkey, 2010; Webster et al., 2012). There is, for instance, a possibility that *T. gondii* may in part alter host behaviour via the preferential localisation of cysts within the brain (McConkey et al., 2012). Various studies have suggested different regions of the brain as predominant cyst locations, such as the olfactory bulbs, amygdala, nucleus accumbens, cerebral cortex, cerebellum, medulla oblongata, basal ganglia, and hippocampal regions (McConkey et al., 2012). However, although it appears that cysts are able to locate in most regions of the

brain, and are found in a non-random manner, this may plausibly be due to the relative accessibility of particular brain structures, rather than to any targeted tropism (Berenreiterova et al., 2011; McConkey et al., 2012). Therefore it is unlikely that this is the sole mechanism by which the parasite causes specific behavioural alterations (Webster and McConkey, 2010).

There is, however, stronger evidence that *T. gondii* may achieve such manipulation through, at least in part, altering neuromodulator levels in the brain (Webster and McConkey, 2010). Chronic *T. gondii* infection in mice has been associated with elevated dopamine levels of 14% (Stibbs, 1985), and acute infections were associated with elevated levels of homovanillic acid and reduced levels of noradrenaline (Stibbs, 1985). The *T. gondii* genome also, rather uniquely amongst such Apicomplexan parasites (with the notable exception, amongst those parasites examined to date, also of the indirectly-transmitted related species *Neospora caninum*) contains two genes that encode for the enzyme tyrosine hydroxylase (Gaskell et al., 2009). This enzyme is involved in the synthesis of L-DOPA, a precursor of dopamine. A recent study showed that *T. gondii* infection increases dopamine metabolism in the mammalian host (Prandovszky et al., 2011). In dopaminergic cells, the parasite increases the K⁺-induced release of dopamine more than three-fold. High levels of dopamine were also found within *T. gondii* cysts in brain tissue. Furthermore, the enzyme tyrosine hydroxylase detected within intracellular *T. gondii* cysts was demonstrated to be encoded for by the parasite itself, rather than by the mammalian host (Prandovszky et al., 2011).

The *T. gondii* parasites may also be altering anxiety-like behaviours indirectly, via the host's immune response. Cytokine production plays an important role in sustaining the latent *T. gondii* infection of the cysts (Carruthers and Suzuki, 2007). Cytokines are produced by microglia, astrocytes and neurons, which promote or suppress inflammatory responses. Interferon- γ (IFN- γ) is one of the most important cytokines involved in the cell-mediated immune response to *T. gondii*, and is produced in response to the proliferation of tachyzoites, leading to the development of the chronic latent cyst stages in the brain (Carruthers and Suzuki, 2007). Cytokines involved in the rodent inflammation process have been shown to directly influence the level and turnover of many neuromodulator levels, including dopamine (Novotna et al., 2005). Interleukin 2, for example, has been shown to potentiate dopamine release and to alter behaviours known to be mediated by forebrain dopamine pathways (Petitto et al., 1997). IFN- γ is involved in the expression of indoleamine 2,3-dioxygenase (IDO), which results in strong toxoplasmostatic effects (inhibition of parasite replication) through the depletion of intracellular pools of tryptophan (Carruthers and Suzuki, 2007). Elevated IFN- γ and IDO have both been associated with altered behaviour in mice (Moreau et al., 2008; O'Connor et al., 2009). IDO degrades tryptophan along the kynurenine pathway, which generates compounds such as kynurenine acid, and an increase in kynurenine acid has been shown to alter dopaminergic activity in the rat brain (Erhardt and Engberg, 2002; Miranda et al., 1997; Rassoulpour et al., 2005). In this way, the host immune response to the proliferation of tachyzoites in the brain may further, albeit indirectly, result in alterations of dopamine and other neuromodulator levels and hence subsequently host behaviour. Such indirect effects of the host immune response may be predicted to interact with the direct route of the parasite increasing dopaminergic activity via production of tyrosine hydroxylase (Prandovszky et al., 2011), and future research should aim to disentangle the relative, and/or potential facilitatory, influence of the two routes on dopamine levels and manipulation of host behaviour.

Finally, it should also be perhaps acknowledged that the potential effects of enteric parasites and pathogens on host behaviour may also apply to *T. gondii*, as this parasite has an intestinal stage both within the feline definitive host and when first ingested by the intermediate or secondary host. This initial enteric infection may be likely to initiate a cascade of changes that lead to further alterations in the

CNS, as described above, although, to the authors' knowledge, this has yet to be investigated.

Implications of latent toxoplasmosis in humans

Whilst *T. gondii* appears to specifically manipulate the behaviour of its rodent intermediate hosts to enhance predation to the feline definitive host, similar behavioural alterations, from subtle to severe, have also been reported in other host species. Within the definitive host itself, for instance, whilst CNS toxoplasmosis is uncommon, some neurological signs have been reported, such as circling, head bobbing, atypical crying, and increased affectionate behaviour (Bowman, 2002; Dubey and Carpenter, 1993). In terms of alternative potential secondary (though not intermediate) animal host species, California sea otters with moderate to severe toxoplasmic encephalitis have been observed to be 3.7 times more likely to be attacked by sharks than otters without encephalitis (Miller et al., 2004), suggesting that they may exhibit aberrant behaviour, similar to findings in infected rodents. Likewise, behavioural alterations observed in *T. gondii*-infected humans may reflect a non-specific by-product of the adaptive changes seen in the rodent intermediate host. It seems unlikely that the effects of *T. gondii* infection on humans are specifically manipulative, as humans are not a natural intermediate host of the parasite's life cycle, and therefore the parasite is unlikely to have come under a strong selective pressure to cause specific manipulations in humans. Furthermore, the effects of the parasite on behaviour are likely retained in humans, and other secondary mammalian hosts, due to the phylogenetically primitive structures of mammalian brains and neurochemical systems (Klein, 2003). Therefore, as latent human toxoplasmosis is highly prevalent around the world, the implications for knowledge on the effect of this parasite in the brain are highly relevant, particularly with recent advances in neuroscience and mental health research.

Studies carried out in human populations have indicated that there is a link between *T. gondii* infection and observed personality differences in humans. For example, *T. gondii* infected men were found to have lower superego strength (be more likely to disregard rules) and higher protension (related to suspiciousness and jealousy). Infected women score higher in other personality factors, e.g. affectothymia (warm-hearted and outgoing traits) and alaxia (trusting and tolerant traits) (Flegr et al., 1996, 2000, 2003). A recent finding has even suggested that the feline fatal attraction phenomenon seen in rats (Berdoy et al., 2000) may also be observed in humans, where *T. gondii*-infected humans showed altered questionnaire responses to the odours of the domestic cat (and of the brown hyena) (Flegr et al., 2011). Research by the same group also reported that *T. gondii* infection increases testosterone levels in men, and decreases testosterone levels in women (Flegr et al., 2008), and that latent toxoplasmosis may cause differential immunomodulatory effects in men and women (Flegr and Striz, 2011). Indeed a range of human behavioural studies have indicated that gender interacts strongly with *T. gondii* infection in determining different outcomes (Flegr, 2007). The importance of gender and how it may be specifically interacting with infection remains little understood, and highlights the importance of testing both sexes when looking at both humans and animal models of infection. There is also evidence to suggest that the Rhesus D (RhD) protein molecule, found in individuals with a Rh positive blood group phenotype, may confer a protective effect on latent toxoplasmosis in humans, negating the effects of infection on reaction times (Novotna et al., 2008) and personality changes (Flegr et al., 2010). Future *T. gondii* studies in human cohorts would therefore benefit from knowledge on the Rh phenotype.

An epidemiological link has also been established between *T. gondii* infection and the increased risk of development of schizophrenia (Torrey and Yolken, 2007; Torrey et al., 2007), suggesting that *T. gondii* may be an environmental risk factor for this disease, alongside other environmental, genetic and social risk factors (Allen et al., 2008;

Howes and Kapur, 2009; McDonald and Murray, 2000). The association between toxoplasmosis and schizophrenia appears to be strong, having a higher odds ratio than for any one human gene in a genome-wide linkage analysis (Purcell et al., 2009). It has also been shown that schizophrenia patients with *T. gondii* infection are more likely to be women than men (Dickerson et al., 2007a). The suggestion of elevated dopamine levels in the brain caused by infection with *T. gondii* provide further understanding of this link, supporting the dopamine hypothesis of schizophrenia (Howes and Kapur, 2009; Snyder, 1976). It has also been suggested that *T. gondii* may impact brain morphology in schizophrenia patients, with a reduction in gray matter volume observed in *T. gondii*-positive patients compared with *T. gondii*-negative patients (Horacek et al., 2011).

Several studies performed on military personnel and blood donors implicated dopamine as a link between *T. gondii* infection and schizophrenia, and were based on evidence suggesting that the level of *T. gondii* antibodies was inversely correlated with novelty seeking in men (Flegr et al., 2003, 2010; Novotna et al., 2005; Skallova et al., 2005). Novelty seeking is thought to negatively correlate with dopamine concentration in the ventral midbrain, and to be associated with certain alleles of dopamine transporter and receptors. Therefore the personality change observed in men has been proposed to imply an increase in dopaminergic activity in infected individuals (Flegr et al., 2003; Novotna et al., 2005).

Another study directly looking at schizophrenia patients suggested that infection with *T. gondii* may recurrently induce a response from proinflammatory T-helper lymphocyte (T_H1) cells (Hinze-Selch et al., 2007). This in turn may modulate the dopaminergic and serotonergic systems. High *T. gondii* titres were associated with increased C-reactive protein (CRP) and leukocyte values (Hinze-Selch et al., 2007). Alterations in T-helper cell responses and CRP levels are known to be found in schizophrenia patients (Dickerson et al., 2007b; Fan et al., 2007; Schwarz et al., 2001), and therefore it may be that *T. gondii* is involved in inducing a proinflammatory response that leads to dysregulated dopaminergic and serotonergic neurotransmitter systems, which in turn may lead to psychiatric symptoms and the precipitation of schizophrenia in vulnerable human subjects (Hinze-Selch et al., 2007).

There are a few other human neurological disorders that latent toxoplasmosis has been implicated in. These include obsessive-compulsive disorder (OCD) (Miman et al., 2010b), Parkinson's disease (Miman et al., 2010a), Alzheimer's disease (Kusbeci et al., 2011), autism (Prandota, 2010a, 2010b), and a history of suicide attempts (Arling et al., 2009; Ling et al., 2011; Yagmur et al., 2010). It is possible that *T. gondii* infection is also linked with bipolar disorder, as it has been shown that drugs used in the treatment of bipolar disorder inhibit replication of *T. gondii* *in vitro* (Jones-Brando et al., 2003). Furthermore, as dopamine has been also shown to play a role in OCD, bipolar disorder, suicide attempts, and other such mood disorders (Berk et al., 2007; Denys et al., 2004; Diehl and Gershon, 1992; Roy et al., 1992), these findings may suggest that related dopaminergic mechanisms are at work, if these relationships are indeed causal.

Overall conclusions and implications

Parasite-induced alterations in host behaviour have been reported in a wide range of protozoan, metazoan, bacterial and viral agents, most of which have complex life-cycles. Some of these behavioural alterations may be non-adaptive and non-specific, and are simply by-products of gross pathology caused by the parasite. Some behavioural changes may be a by-product of another behavioural phenotype that increases the parasite's chance of transmission in a different host, or even a consequence of simple infection-induced malaise. Other behavioural changes may, however, have specific adaptive advantages for the parasite. There are likely to be many, often interacting, mechanisms by which parasites may manipulate host behaviour. These

include behavioural changes after infection that are mediated by the host immune response, and manipulations caused by the parasite itself infecting neurons, causing CNS inflammation, and altering hormonal and neuromodulator communication. Behavioural alterations caused by parasite manipulation to specifically facilitate transmission may even be, in certain cases, caused by the parasite infecting specific cells or regions of the brain, or by altering specific neuromodulatory or hormonal pathways. The role of the host immune response in mediating behavioural changes following infection has in the past mainly been studied with regards to general sickness pathologies, and more research needs to be conducted as to whether parasites can specifically exploit host immunological pathways. Behavioural outcomes of infection are also likely to be affected by several host factors, including age, gender and immune status. An understanding of how parasites may impact hormone and neurotransmitter levels and beyond within the mammalian host is, nevertheless, vital to elucidate the mechanisms behind parasite manipulation of host behaviour, the evolutionary implications of these and the subsequent impact upon health, including implications for the complex fields of mental health and psychology.

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REVIEW

Toxoplasma gondii infection, from predation to schizophrenia: can animal behaviour help us understand human behaviour?

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Summary

We examine the role of the protozoan *Toxoplasma gondii* as a manipulatory parasite and question what role study of infections in its natural intermediate rodent hosts and other secondary hosts, including humans, may elucidate in terms of the epidemiology, evolution and clinical applications of infection. In particular, we focus on the potential association between *T. gondii* and schizophrenia. We introduce the novel term '*T. gondii*–rat manipulation–schizophrenia model' and propose how future behavioural research on this model should be performed from a biological, clinical and ethically appropriate perspective.

Key words: *Toxoplasma gondii*, rat, mouse, behaviour, manipulation, by-product, pathology, schizophrenia, intermediate host, secondary host, 3Rs.

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Introduction

The ability of parasites to alter host cognition and behaviour captivates the interest of both the scientific and lay communities, partly because it raises novel questions about longstanding philosophical issues such as the existence of free will. 'If the mind is a machine, then anything can control it – anything, that is, that understands the code and has access to the machinery' (Adamo, 2013). Certain parasites, including a notable minority of vertebrates, appear to possess the mechanisms required to manipulate the central nervous system (CNS) of their hosts. Whereas some of the behavioural changes associated with infection are quite general, such as depressed feeding or general malaise/sickness, others appear remarkable in their specificity, subtly altering only a limited repertoire of host behavioural traits for apparent selective benefit to the parasite.

What happens, however, when a 'manipulatory' parasite has a broad host range, able to infect species both part of and independent to its own life cycle, relevant or not to its own transmission potential? Altering host behaviour in 'inappropriate' hosts is plausibly an unavoidable consequence of parasite-altered behaviour. This raises questions as to whether one would predict similar behavioural alterations to be mirrored across contrasting host organisms, such as that between a rat and a human, despite often markedly divergent life histories, behavioural repertoires, infection exposure histories and natural longevities. If we are to fully elucidate the range of behavioural alterations evoked and the mechanisms employed, between and within species and life stages, how should, therefore, future studies test for these in the most biologically and ethically appropriate manner?

Here, we examine the role of the protozoan *Toxoplasma gondii* as a manipulatory parasite and question what role study of infections in its natural intermediate rodent hosts and other secondary hosts, including humans, may elucidate in terms of the epidemiology, evolution and clinical applications of infection. In particular, we question whether studying the impact of infection on

rodent behaviour may help us predict what behavioural changes may be observed amongst infected humans, from subtle to severe. We focus on the potential association between *T. gondii* and schizophrenia, and how future animal behaviour studies should be performed from a biological, clinical and ethically appropriate perspective.

We define and categorize behavioural alterations in terms of whether the behavioural changes observed in an infected host are indicative of: (1) active manipulation for the selective benefit of the parasite; (2) active manipulation for the selective benefit of the host to ameliorate the impact of infection; (3) a generalized pathological response in the host of no obvious selective benefit to the parasite or host; or, finally, a subtle distinction of the latter grouping we refer to here as (4) 'by-product pathology' as a consequence of accidental infection with a parasite selected to manipulate behaviour in an alternative host species and/or life-cycle stage. Furthermore, as this perspective article assesses the applicability of studying *T. gondii* in rat (and/or mice) intermediate hosts as a model for helping us understand both the evolution of and mechanisms underpinning parasite-altered behaviour (ranging from rodent predation to some cases of human schizophrenia), we introduce, for the first time here, the novel term of the '*T. gondii*–rat manipulation–schizophrenia model' (see Appendix 1).

Toxoplasma gondii

Until relatively recently, latent adult-acquired toxoplasmosis in immunocompetent humans and animals was generally considered to be asymptomatic. In contrast, the causative agent, *Toxoplasma gondii*, represents perhaps one of the most convincing examples of a manipulative parasite of vertebrates. *T. gondii* is a highly successful apicomplexan protozoan capable of infecting all warm-blooded animals worldwide, often at extremely high prevalence levels. Members of the cat family (Felidae) are the only definitive hosts, within which the parasite undergoes full gametogenesis and mating within the intestinal epithelium, culminating in the

generation of oocysts containing sporozoites that are shed in the cat's faeces (Hutchison et al., 1969). Infection of intermediate (such as rodents and birds) or other secondary (such as humans and domestic livestock) hosts can occur following ingestion of oocysts (*via* contaminated soil, water or food) or tissue cysts (through raw/undercooked infected meat, including *via* cannibalism), congenital transmission and also potentially, under certain conditions, by sexual transmission (Vyas, 2013). Within intermediate secondary hosts, the parasite undergoes asexual reproduction, characterized by rapidly dividing tachyzoites and the more slowly dividing bradyzoites. Bradyzoites encyst in the brain, heart and other tissues, where they remain potentially for the host's lifetime. Transmission to the feline definitive host occurs when an immunologically naive cat ingests an infected intermediate host through predation (and/or consumes contaminated meat). Since sexual reproduction of *T. gondii* can be accomplished only in felines, there are likely to be strong selective pressures on the parasite to evolve mechanisms to enhance transmission from the intermediate host to the definitive feline host.

The predilection of *T. gondii* for the CNS places it in a privileged position to manipulate host behaviour. Potential mechanisms of action have recently been described elsewhere (Kaushik et al., 2012; McConkey et al., 2013; Vyas and Sapolsky, 2010; Webster and McConkey, 2010) and thus need not be repeated in detail here. However, as similar mechanisms are likely to be involved in both rodent hosts as in latently infected human hosts, irrespective of the manipulatory or pathological outcomes (Appendix 1), and as this raises important implications for the direction of future research in relation to the *T. gondii*–rat manipulation–schizophrenia model (Appendix 1), we will briefly focus here on the proposed mechanistic role of dopamine on the expression of altered host behaviour.

Raised or disrupted dopamine levels have been reported in both rodent and human *T. gondii* infection and within human patients with schizophrenia (Howes and Kapur, 2009; Prandovszky et al., 2011; Stibbs, 1985; Torrey and Yolken, 2003), together with other affective disorders such as obsessive compulsive disorder (OCD) and bipolar disorder and amongst those with suicide attempts (Berk et al., 2007; Denys et al., 2004; Diehl and Gershon, 1992; Roy et al., 1992). Furthermore, recent research indicates that the parasite itself may actually be a source of this dopamine (Gaskell et al., 2009; Prandovszky et al., 2011). Dopamine is synthesised in two steps from its precursor amino acid tyrosine: (1) tyrosine hydroxylase metabolism to produce L-DOPA; then (2) decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase to dopamine. In some cells, dopamine is further metabolized to norepinephrine by dopamine β -hydroxylase. *T. gondii* was recently found to encode a protein with high homology and showing similar catalytic properties to the tyrosine hydroxylases found in mammals. This *T. gondii* ortholog synthesises L-DOPA, precursor to dopamine, as well as tyrosine, and has been demonstrated to result in increased dopamine levels associated with *T. gondii* cysts in the rodent brain (Prandovszky et al., 2011). These mechanistic studies thereby provide both a potential explanation for previous, and a driving force for further, empirical research into behavioural manipulation in intermediate hosts as well as a plausible explanation for by-product pathological changes in behaviour in all infected secondary hosts.

Current evidence for *T. gondii* manipulation of intermediate host behaviour

Though behavioural studies of wild animals with naturally occurring infections remain rare, wild brown rats (*Rattus*

norvegicus) on farmlands exhibit higher activity levels and, at least under certain situations, an increased propensity to be trapped in cages, amongst *T. gondii*-infected individuals relative to their uninfected counterparts (Webster, 1994; Webster et al., 1994). Laboratory and/or experimentally controlled naturalistic studies on rats and mice have also demonstrated that *T. gondii* infection is associated with a range of subtle behavioural alterations, many of which would facilitate parasite transmission from the infected intermediate host to the feline definitive host [detailed reviews of which may be found elsewhere (Webster, 2001; Webster, 2007)]. For example, *T. gondii*-infected rodents exhibit an increase in activity and a decrease in predator vigilance behavioural traits (Berdoy et al., 1995; Hay et al., 1983; Hay et al., 1984; Hutchison et al., 1980a; Hutchison et al., 1980b; Lamberton et al., 2008; Webster, 1994; Webster, 2001; Webster, 2007; Webster et al., 1994; Webster et al., 2006). Moreover, whilst uninfected rats show a strong innate aversion to predator odour, *T. gondii* infection appears to subtly alter the rats' cognitive perception of cat predation risk, turning their innate aversion into a 'suicidal' 'fatal feline attraction' (Berdoy et al., 2000; Vyas et al., 2007c; Webster et al., 2006). Such fatal feline attraction appears specific towards a response to cat (urine) odour, with no difference observed between infected and uninfected rats in their responses to odours of non-predatory mammals such as rabbit (Berdoy et al., 2000; Vyas et al., 2007c; Webster et al., 2006) nor contrasting potential predatory species odours such as mink (Lamberton et al., 2008) or dog (Kannan et al., 2010). Furthermore, other key health and behavioural traits, such as social status and mating success, remain intact and indistinguishable between infected and uninfected rats (Berdoy et al., 1995) (but see Vyas, 2013). Overall, such rodent studies are consistent with the hypothesis that *T. gondii* specifically manipulates the behaviour of its rodent (rat at least) intermediate host rather than simply causing a broad pathology or destruction of particular behavioural traits (Appendix 1).

Current evidence for *T. gondii* 'general pathology' and 'by-product' alterations of secondary host behaviour

Although empirical studies beyond those focused upon rodents are rare, *T. gondii* appears to cause a range of behavioural alterations across host species. Within the feline definitive host itself, some potential 'general pathology' (Appendix 1) neurological signs have been reported, such as circling, head bobbing, atypical crying and increased affectionate behaviour, even though CNS toxoplasmosis in felines is uncommon (Bowman, 2002; Dubey and Carpenter, 1993). California sea otters with moderate to severe toxoplasmic encephalitis have been observed to be 3.7 times more likely to be attacked by sharks than their uninfected counterparts (Miller et al., 2004), suggesting that they may exhibit aberrant behaviour similar to that displayed in infected intermediate host rodents, although as a 'by-product' (Appendix 1) of infection of no current adaptive advantage to the parasite in this host–predator combination. Physical and behavioural changes in *T. gondii*-infected human secondary hosts may also be viewed as either 'general pathology' or 'by-product' (Appendix 1) and/or a potential spectrum between the two, depending on the particular characteristics, severity and trait altered. For instance, one may suspect that *T. gondii*-induced meningoencephalitis [including within immunocompetent adult hosts (Kaushik et al., 2005)] and brain cancer (Thomas et al., 2012) may be indicative of 'general pathology', perhaps simply as a consequence of the extended duration of infection within the CNS of the relatively longer-lived human host as compared with the short lifespans of rodent or bird intermediate hosts. Others,

however, appear more indicative of ‘by-product’ pathology, where we see similar physiological or behavioural alterations in human secondary hosts to that observed in rodent intermediate hosts, even if humans are not (currently) under selective pressure to influence transmission potential. Indeed, human studies have revealed a range of subtle behavioural alterations associated with *T. gondii* infection, many of which may be comparable to those observed amongst infected rodent intermediate hosts – such as increased activity, decreased reaction times and altered personality profiles (Flegr, 2007; Flegr, 2013; Flegr et al., 2002; Flegr and Hrdy, 1994; Flegr et al., 2003; Webster, 2001). Even an equivalent of the fatal feline attraction phenomenon observed in infected rats (Berdoy et al., 2000) has been identified in humans, where *T. gondii*-positive humans showed altered questionnaire responses to the odours of the domestic cat (and of the brown hyena) (Flegr et al., 2011). Such subtle changes in human behaviour and personality in response to latent *T. gondii* infection at the individual level have even been proposed to alter the human aggregate personality at the population level (Lafferty, 2006).

Latent *T. gondii* infection in the human host may, furthermore, in a small number of cases, have substantial health implications. Consistent with a possible impairment in psychomotor performance and/or enhanced risk-taking personality profiles, individuals with latent toxoplasmosis have been reported to be at a 2.65 times increased risk to be involved in a traffic accident relative to the general population (Flegr et al., 2002), a result subsequently replicated by other groups (Flegr et al., 2009; Kocazeybek et al., 2009; Yereli et al., 2006). Another recent study, albeit significant only in a subset with lower socioeconomic status, linked *T. gondii* seropositivity with workplace accidents (Alvarado-Esquivel et al., 2012). There is also the ever growing and convincing body of evidence concerning a potential relationship linking *T. gondii* with that of some forms of affective and neurological disorders in humans. Correlations have been found for OCD (Miman et al., 2010b), Parkinson’s disease (Miman et al., 2010a), Alzheimer’s disease (Kusbeci et al., 2011), suicide (Arling et al., 2009) and bipolar disorder (Pearce et al., 2012). The most substantial body of empirical evidence gathered to date relates to the potential association between *T. gondii* and some cases of schizophrenia in humans. *T. gondii* seroprevalence has been associated with schizophrenia in at least 38 studies to date (Mortensen et al., 2007; Torrey et al., 2007; Torrey et al., 2012; Torrey et al., 2000; Torrey and Yolken, 2003; Yolken and Torrey, 2008). Recent meta-analyses assessing potential associations between different infectious agents and schizophrenia found a highly significant association with *T. gondii* (OR=2.70; CI 95%: 1.34–4.42; $P=0.005$) (Arias et al., 2012) and a stronger association between schizophrenia and detection of *T. gondii* antibodies (combined odds ratio 2.73) than for any human gene in a genome-wide linkage analysis study (OR≤1.40) (Purcell et al., 2009). There are similarities in the epidemiology of schizophrenia (Cichon et al., 2009) and toxoplasmosis (Johnson et al., 2002), where, for instance, both have been demonstrated to have strong familial associations, affecting multiple members of the same family. Further support for an association includes analyses of serum samples obtained from mothers shortly before or after giving birth that revealed a significantly raised proportion of immunoglobulin M (IgM) antibodies to *T. gondii* in those whose children developed schizophrenia in later life (Torrey and Yolken, 2003), and individuals suffering from first-episode schizophrenia have significantly elevated levels of IgG, IgM and/or IgA class antibodies to *T. gondii*, within both serum and cerebral spinal fluid

(CSF), compared with uninfected control subjects (Yolken et al., 2001). Likewise, in a study of military personnel from whom serum specimens were available from periods of up to 11 years prior to the onset of their schizophrenia (180 individuals with schizophrenia and 532 matched controls), significantly increased levels of IgG antibodies to *T. gondii* were observed prior to the onset of illness (hazard ratio=1.24, $P<0.01$), with a peak in the six months prior to onset but seen as early as three years prior to the onset (Niebuhr et al., 2008). Studies have even demonstrated that *T. gondii* antibodies in patients with schizophrenia treated with antipsychotic drugs are intermediate between those of patients never treated and those of control groups, with a significant reduction in those patients undergoing current drug treatment, thereby suggestive that antipsychotic treatment may affect *T. gondii* infection levels (Leweke et al., 2004). Indeed, antipsychotic drugs used in the treatment of schizophrenia have been observed to inhibit the replication of *T. gondii* tachyzoites in cell culture (Goodwin et al., 2011; Jones-Brando et al., 2003). Likewise, *T. gondii*-infected/exposed rats treated with the same key antipsychotic or mood stabiliser drugs during the tachyzoite replicative stage of infection, in particular that of the dopamine D2 antagonist haloperidol (Webster et al., 2006) or the dopamine selective uptake inhibitor GBR 12909 {1-[2-[bis(4-fluorophenyl)methoxy]-ethyl]-4-[3-phenylpropyl]piperazin} (Skalova et al., 2006), did not develop the potentially suicidal feline attraction or other predation-specific altered behavioural profiles displayed by their untreated but infected counterparts, nor was there the same level of parasite establishment within the brains of these drug-treated infected rats relative to their untreated infected counterparts (Webster et al., 2006). Such results therefore raise the hypothesis that the antipsychotic and mood-stabilizing activity of some medications may at least be augmented through their inhibition of *T. gondii* replication, invasion and/or subsequent modulatory impact in infected individuals.

What animal models should future research incorporate to elucidate the impact of *T. gondii* on host behaviour?

T. gondii can be successfully maintained *in vitro* and hence many studies on the parasite, such as those examining gene expression, can be performed without the need for animal infections. However, to fully elucidate the impact upon behaviour, and the mechanisms involved, future animal (and human) studies, across a range of different host species and individuals, will remain essential. Host factors undoubtedly contribute to differences in behavioural and clinical outcomes of infection, even when the parasite genotypes remain constant. For example, inherent differences in genetic predisposition at the genera, species and individual level, the state of the immune system, the time of *T. gondii* exposure (e.g. prenatal pregnancy trimester, neonate, juvenile or adult), the duration of infection (e.g. humans live longer than the average rodent intermediate host) and past behavioural and parasitic histories are all likely to be important. Host gender is also extremely important in determining the behavioural outcome of *T. gondii* infection in both humans and rodents (Flegr, 2007; Webster, 2007). For instance, recent studies have revealed sex-specific changes in gene expression and behaviour induced by chronic *T. gondii* infection in mice (Xiao et al., 2012). Similarly, *T. gondii* has been reported to increase testosterone levels in men but decrease levels in women (Flegr et al., 2008).

In terms of which host species to examine to further understand the evolution and epidemiology of this parasite, it seems intuitive that studies should ideally incorporate its major intermediate host

species, especially those maintained under natural or semi-naturalistic conditions. Likewise, correlations between apparent altered behaviour and *T. gondii* infection amongst other wildlife secondary hosts with ‘naturally’ occurring infections, such as seen for the sea otters (Miller et al., 2004), are also extremely useful. Such studies, however, remain rare, and further investigations are warranted. For instance, minimally invasive studies on wild bird populations could be fascinating, particularly as birds, common in the diet of predatory felines, are plausible major intermediate hosts and hence likely to remain under strong selective pressures. Furthermore, as birds detect and subsequently avoid predatory cats primarily *via* their visual system (Cuthill et al., 2000), in contrast to olfactory perception by rodent intermediate hosts (Dielenberg et al., 2001), this may be pertinent to further elucidating mechanism(s) of action, particularly since *T. gondii* is known to frequently infect and interfere with its host’s visual system. Moreover, the avian archistriatum is believed to be homologous to parts of the mammalian amygdala, with a role in assigning an emotional value to new stimuli by means of multimodal association of temporally coincidental stimuli and to elaborate complex responses (Medina and Reiner, 2000; Puelles et al., 2000). However, whilst there are reports on the prevalence of *T. gondii* infection amongst wild bird populations [e.g. wild fowl and pigeons (Tenter et al., 2000)], there are, unfortunately, to the authors knowledge, no *T. gondii*–bird behavioural studies published. (One study did attempt to associate *T. gondii* infection status with potential predation risk in 115 wild nesting mountain bluebirds (*Sialia currucoides*) – however, the majority of birds sampled were juveniles and all but two individuals had *T. gondii* titres of $\geq 1:32$, and thus no significant association could be detected; J.P.W. and A. Moehrensclager, unpublished observations.)

Rats and/or mice are the obvious continued model system for *T. gondii* research. In addition to being key intermediate host species, we know a great deal about the behaviour and physiology of these creatures. The rodent CNS and immune system, sharing many structural and neurochemical similarities, are often used as an experimental model for humans, they are generally straightforward to maintain, and specific genetic models are being developed. Nevertheless, rather than simply replicate earlier research, future studies should consider carefully what precise animal models should be used and why, particularly within the ethical remit of today’s Replacement, Refinement and Reduction of animals in research (3Rs) environment. Furthermore, it must be emphasized that rats and mice are not the same in terms of their susceptibility, behaviour, morbidity and overall response to *T. gondii*. For instance, whilst some *T. gondii*-altered behavioural traits, such as increased activity levels (Hay et al., 1984; Webster, 1994; Webster, 2001), appear to be similar in rats and mice, other behavioural traits appear to show contrasting results. One example is the observed decreased ability of *T. gondii*-infected hosts to recognize and/or respond to novel stimuli; this can result in either a decrease of neophobia in neophobic hosts such as rats (Webster et al., 1994) or an increase of neophobia in neophilic hosts such as mice or indeed humans (Hodková et al., 2007). Likewise, early studies found that whilst learning capacity was reduced in some laboratory rats, this was much milder and rarer than that observed for laboratory mice (Piekarski et al., 1978; Witting, 1979). Potential explanations for these differences related to the higher infection rate of *T. gondii* in the brains of mice than rats during latent toxoplasmosis and the former’s increased potential for severe morbidity during the acute phase of infection. Indeed, whilst the general health and behaviour of laboratory rats usually appears unaffected by infection,

laboratory mice often show high parasite-induced mortality, even with Type II *T. gondii* strains, and/or signs of acute infection such as running in circles with their heads bent to one side (Piekarski et al., 1978; Witting, 1979). (Indeed, it may be noted that, in contrast, in all the rat studies performed by the current authors over the years, not a single rat has demonstrated any such overt pathology or mortality following *T. gondii* exposure; J.P.W., unpublished observations.) It is thus strongly proposed that experiments with more resistant animals, such as rats, provide a far superior model in which to study the behaviour changes induced by *T. gondii*, from both an ethical and biological perspective in terms of their generalizability to humans, relative to the mouse model (Hrda et al., 2000; Webster, 2007).

Even within rat species, the strain or line of rat to be used may have important implications in terms of understanding this host–parasite interaction. For instance, Lister-hooded rats are believed to be the most behaviourally similar to wild rats, at least in terms of food-related neophobia (Barnett, 1958; Webster, 2007), whilst Fawn-hooded rats have been shown to exhibit more stress-induced fear (freezing) than Wistar rats and have higher levels of corticotropin-releasing factor (CRF) mRNA in their central amygdala (Altemus et al., 1995). One could perhaps also propose that future *T. gondii* studies could incorporate the spontaneously hypertensive rat (SHR) model of anxiety, to help further elucidate host from parasite associations in relation to generalized anxiety profiles.

In terms of future research into the ‘*T. gondii*–rat manipulation–schizophrenia’ model, a number of potentially useful rat *in vivo* models are already available that could plausibly provide valuable experimental tools to further test the hypothesis of causality. Prenatal exposure to various infectious agents has been linked to increased risk of neurodevelopmental brain disorders, and specifically those associated with altered dopaminergic development. For example, there is recent evidence that rats born to mothers exposed to the viral mimic polyriboinosinic-polyribocytidylic acid (poly-I:C) in pregnancy provide a useful rodent model for human affective disorders such as schizophrenia (Vuillermot et al., 2012). Admittedly, whilst previous studies found no significant difference in the hyperactivity profiles between adult-acquired and congenitally acquired *T. gondii* infections amongst rats (Webster, 1994), it may be fascinating to examine the impact of *T. gondii* on the resulting developmental, behavioural and neurophysiological phenotype amongst such poly-I:C offspring – hence a ‘two-step environmental–environmental (i.e. infection:infection) hit’. Likewise, although there are no rodent models available yet for hallucinations (see below), *N*-methyl- δ -aspartate receptor (NMDAR) antagonists, such as MK-801, can induce schizophrenia-like psychoses, together with other ‘positive symptoms’, in rats (Brigman et al., 2010). The mechanism involves hyperactivity of mesolimbic dopamine neurons, although multiple brain regions and multiple neurotransmitter systems are affected (Seeman, 1987). Hence, one may predict that future studies incorporating the additional exposure of *T. gondii* in such rats may further provide valuable (environmental–environmental interaction) insights into the mechanisms involved and phenotypic outcomes.

Genetically engineered and naturally occurring mutant rodent models can also be particularly useful in providing requisite information about the neurodevelopmental, behavioural and molecular consequences of dysregulation in specific genes, and hence the potential gene–environmental interactions of infection with *T. gondii* here on the subsequent outcome. At present, there

are several useful and relevant mouse models available that may be particularly useful for future *T. gondii* research, in particular those using knock-out (KO) mice involving dopamine (dopamine synthesis and/or regulation genes) (Kirby et al., 2010). Unfortunately, there are few KO rat models yet available, although several are underway and their development is likely to prove extremely valuable for examining gene–environmental interactions. In particular, we may predict that, for example, the combination of such genetic (KO line) and environmental (*T. gondii* infection) factors not only exerts additive effects on behavioural traits such as locomotor hyperactivity and fatal feline attraction but also produces synergistic effects in the development of, for instance, impaired attentional shifting and sustained attention. Furthermore, we may predict that the combination of both these gene–environmental factors is necessary to trigger maldevelopment of the host dopamine system in these aetio-pathological processes.

What parasite strains should future research use and why?

The choice of animal host to study – down to species, strain, gender and past parasitic and behavioural histories – clearly will have an effect on the outcome of any behavioural assay used and, in doing so, should provide further key information regarding the evolution, mechanisms involved and behavioural outcome of infection. In a similar manner, so will that of the parasite used within such studies. Key parasite-associated factors may plausibly relate to the route of infection, such as oocyst or tissue cyst (Webster, 2001; Webster and McConkey, 2010) or perhaps even a sexually transmitted route (Vyas, 2013). Timing of initial infection may also be important, whether congenital (and at which trimester), neonatal or adult acquired (Webster, 1994; Webster, 2001). The strain of *T. gondii* is also undoubtedly of paramount importance in terms of the clinical, behavioural and ethical outcome of infection. *T. gondii* is composed of three major genotypes, Types I, II and III (previously estimated to encompass 94% of all isolates), which have emerged as the dominant strains worldwide (Howe et al., 1997). Whilst Type I strains tend to be fatal in mice, Type II and III parasites are relatively avirulent and more readily form cysts and thereby establish chronic infections (Howe et al., 1996; Sibley and Boothroyd, 1992; Sibley et al., 2002), which makes the latter two strains more suitable for the study of behavioural changes with chronic infection. Type II and III strains also show higher expression of the parasite's tyrosine hydroxylase genes, proposed to be involved in behavioural changes, relative to Type I (Gaskell et al., 2009; Prandovszky et al., 2011). Infection with Type II also accounts for most human cases (60–80%) in Europe and North America (Ajzenberg et al., 2002; Ajzenberg et al., 2009; Peyron et al., 2006), although both Types I and III are found in Colombia (Peyron et al., 2006). However, it is critical to clarify that sampling has been largely biased towards parasites recovered from symptomatic humans and domestic animals, and hence relatively little is known about the majority of wild animal infections or, potentially, even those human infections with no apparent disease. A role for atypical genotypes in cases with severe host morbidity has, however, been indicated by the Californian sea otter populations suffering increased mortality – whilst 40% were infected with the common zoonotic Type II strain, 60% were infected with a genotype that possessed novel alleles at three genetic loci different from the alleles found in Types I–III (Miller et al., 2004). Furthermore, recent research suggests that such atypical strains, previously referred to as A and X, may designate together as 'Type 12' (Khan et al., 2011), and this Type 12 lineage

may actually account for 46.7% (79/169) of isolates and dominate amongst wildlife of North America (Dubey et al., 2011). One could thus perhaps postulate that future molecular typing studies could reveal different clinical and behavioural outcomes in human or other *T. gondii* infections in relation to whether the zoonotic infection route may be wildlife or domestic, typical or atypical genotype. Even within *T. gondii* type, however, different clinical and behavioural outcomes may be predicted. Within Type II alone, variation in host cell gene expression (Xiao et al., 2011; Hill et al., 2012) and development (Diana et al., 2004), host immune and encephalitic response (Araujo and Slifer, 2003; Hill et al., 2012), parasite dissemination, reactivation and recrudescence (Saeij et al., 2005), and impact on host behaviour (Kannan et al., 2010) have all been reported between different strains. For example, whilst both Prugniald (Pru) and ME49 have been reported to increase attraction to cat odour in mice at 2 months post-infection, in at least one study this behaviour was no longer present with the ME49 strain at 7 months (Kannan et al., 2010). Pru-infected mice in this study were also reported to have greater hyperactivity than their ME49-infected counterparts, whilst only the ME49-infected group showed impaired spatial working memory (Kannan et al., 2010).

Future studies aimed at elucidating mechanisms of action may also benefit from recent advancements in mutant *T. gondii* strain development. Within Type I, for instance, new conditional mutant RH lines have recently been constructed *via* promoter replacement strategies that target genes encoding proteins that are conserved and unique to these apicomplexan parasites (Sheiner et al., 2011). Whilst no longer photosynthetic, the apicoplast, a red algal endosymbiont, is a centre of metabolic activity harbouring several major anabolic pathways, and these studies have revealed new apicoplast proteins, currently with no assigned function (Sheiner et al., 2011) and which, hypothetically at least, could perhaps be relevant to behavioural alterations.

RHΔ $hxgprt$ and PruΔ $hxgprt$ strains, for example, have also been produced to provide parasites in which the selectable marker HXGPRT could be used for gene insertion/replacement (Donald et al., 1996), although targeting specific *T. gondii* genes for knockout has previously proved difficult due to a high frequency of nonhomologous recombination in the parasite. The production of RHΔ $ku80$ Δ $hxgprt$ (Fox et al., 2009) and also notably the Type II PruΔ $ku80$ Δ $hxgprt$ (Fox et al., 2011) has, however, greatly improved the efficiency of gene replacement by homologous recombination in *T. gondii* strains, thereby increasing the opportunity to investigate the contributions of individual *T. gondii* genes to behavioural modifications of the host. Indeed, within Type II, and hence those lines particularly applicable for the '*T. gondii*–rat manipulation–schizophrenia' model, key areas of interest for the '*T. gondii*–rat manipulation–schizophrenia' model will be parasites such as PRU lines with differential levels of expression and/or KO of the tyrosine hydroxylase gene shown to be associated with dopamine metabolism (Gaskell et al., 2009; Prandovszky et al., 2011; Webster and McConkey, 2010).

What behavioural assays should future research incorporate to assess the impact of *T. gondii* on animal host behaviour?

In addition to replicating prior observations, novel approaches and tests are required, and there is no doubt that animal models can play an essential role in furthering this theoretical and applied area of research. However, if such animal studies are to be performed, it is imperative that they are performed appropriately, testing biologically and evolutionary-applicable hypotheses, and, equally importantly, in the most ethical and non-invasive manner possible,

particularly considering today's 3Rs environment. Once again, this relates to the precise hypotheses to be tested. We know, to date, that a range of behavioural alterations are observed in animals, particularly rodents, infected with *T. gondii*. Further studies deciding which tests to use should thus consider whether the aim is to simply further elucidate the behavioural repertoire impacted by this parasite – which may serve the purpose of helping us understand further the potential evolution, transmission potential and mechanisms involved directly, particularly as new molecular tools and techniques are developed. If the aim is to specifically test hypotheses relating to the role of *T. gondii* in human affective disorders such as schizophrenia, however, the behavioural assays to be used are not necessarily the same as those for either studying schizophrenia alone or the impact of *T. gondii* on host behaviour alone. Furthermore, when choosing such assays, one must consider that, in the absence of any true 'schizophrenia rodent model', the behavioural repertoire of a rodent infected with *T. gondii* may well be altered but not necessarily in the same way as that of a human with schizophrenia. For instance, if considering selective benefits to the parasite of *T. gondii* in rodents, one should focus on behavioural traits specifically associated with enhanced predation rate (such as altered activity, feline attraction and altered neophobia profiles). On the other hand, if explicitly testing for the spectrum of traits relevant to schizophrenia, a different set of behavioural assays (such as working memory, selective attention, set shifting, social interaction and psychophysiological measures) may be more appropriate. Nevertheless, there may well be certain circumstances where specific behavioural assays may be applicable to both and hence highly suitable for further study on the *T. gondii*–rat manipulation–schizophrenia model.

There are generally considered to be three groups of major symptoms of schizophrenia in humans: positive, negative and cognitive/executive (Carter et al., 2008). 'Positive symptoms' are so-called because they add to the normal behavioural repertoire. While there are no rodent models available yet for hallucinations, other positive symptoms, such as psychomotor agitation and hyper-responsivity to psychotomimetic drugs, are modelled in rodents by testing locomotor responses and hyperactivity-inducing effects of psychostimulants (e.g. amphetamine) and other psychotomimetics [e.g. the NMDAR antagonists dizocilpine/MK-801 or PCP (Brigman et al., 2010)]. Thus, one could perhaps propose that the increased activity observed in *T. gondii*-infected rat studies (Webster, 1994; Webster, 2001b) is also useful as a 'positive symptom' assay. Likewise, as the fatal feline attraction invoked by this parasite is not simply a reduction or removal of a behavioural trait, but instead a positive reversal of an innate behaviour, an apparent 'alteration of the mind of the rat in the face of predation' (Berdoy et al., 2000; Webster et al., 2006), one could perhaps also propose that altered fatal feline attraction behavioural assays may be another valuable behavioural assay relevant to both *T. gondii* epidemiology and evolution but also as a potential 'positive symptom' indicator for further '*T. gondii*–rat manipulation–schizophrenia' models (Appendix 2).

Negative symptoms of schizophrenia are so-called because they subtract from the normal behavioural repertoire, and include blunted affect, social withdrawal and loss of pleasure in normally rewarding activities (anhedonia). Various rodent assays for social behaviour and anhedonia have been typically used to model other disorders such as anxiety (File and Seth, 2003), autism (Crawley, 2004) and depression (Strekalova et al., 2004), but also lend themselves well to the study of abnormalities in these behaviours in models of schizophrenia. However, whilst *T. gondii*-infected

mice may suffer severe morbidity and hence may show equivalent 'negative symptoms' as a result of generalized pathology (Appendix 1), *T. gondii* does not generally induce any specific negative symptoms in rats, as can be illustrated by the normal social behaviour and mating success between infected and uninfected rats maintained under naturalistic conditions (Berdoy et al., 1995). Nevertheless, the fact that the 'pleasure/reward' system does appear to be altered in *T. gondii*-infected rodents, even if in perhaps an opposite direction to those related to schizophrenia in humans – i.e. some evidence of increased pleasure/fatal feline attraction through increased sex drive (enhanced pleasure rather than loss of pleasure, again potentially associated with increased dopamine levels) – there is an argument for use of these behavioural assays when examining the *T. gondii*–rat manipulation–schizophrenia model. For instance, in relation to modelling 'negative symptoms', one could perhaps test for alterations between infected and uninfected rodents in terms of their preference or motivation to obtain rewarding substances, such as sucrose, even if the direction of response predicted may be different between rats and humans (Appendix 2).

Abnormalities in cognition and executive functions are also a prominent feature of schizophrenia in humans and range from deficits in episodic memory, impaired attention and sensorimotor gating to impaired reversal learning and set-shifting. Learning and memory can certainly be, and frequently are, assessed in rodents using a range of standard behavioural assays, such as that of the reference memory version of the Morris water maze (see Appendix 2). However, although impaired episodic memory is one of the strongest features of the cognitive profile of schizophrenia (Ranganath et al., 2008), rodent models of this disease have generally not relied upon such measures – and indeed do not distinguish a model of schizophrenia from other conditions that are also characterized by memory deficits, e.g. Alzheimer's disease (Brigman et al., 2010). Furthermore, one could perhaps particularly question their utility here for any *T. gondii*–rat manipulation–schizophrenia model, and perhaps even the potential applicability of such cognition, learning and/or memory behavioural assays in the *T. gondii*–rodent system in general. This may be explained as, if *T. gondii* is selectively altering intermediate host behaviour to alter predation rate, one may predict there to be no selective advantage for this parasite to alter such traits – as with a cat, the change of avoiding predation on encounter is likely to be all or none – and hence such assays are less relevant to understanding the epidemiology and evolution of such manipulation. Indeed, although there are some conflicting results, particularly in the early literature, indicating potential cognitive deficits in mice after infection with *T. gondii* (Piekarski et al., 1978; Witting, 1979), our own earlier behavioural assays of short- and long-term memory, as assessed using different maze combinations (J.P.W. and P. H. L. Lamberton, unpublished observations) using Lister-hooded laboratory rats with adult-acquired infections with the avirulent (Beverly) strain, found no difference between infected and uninfected individuals. Likewise, recent research using C57BL/6 mice chronically infected with the avirulent *T. gondii* (ME49, a Type II strain) has found no impact of *T. gondii* infection on cognition (Gulinello et al., 2010).

Prepulse inhibition (PPI) of the startle response provides a measure of selective attention, as well as sensorimotor gating, and has been widely used in schizophrenia research (Swerdlow et al., 2008; Van den Buuse et al., 2003; Weiss and Feldon, 2001). Whilst one could thus propose incorporation of this test into *T. gondii*-altered behavioural studies to allow direct comparison with pharmacological and genetic studies, this assay may not, however, be sufficiently sensitive to assess the very subtle behavioural

alterations observed between *T. gondii*-infected and uninfected rats. Indeed, recently it was found that general olfaction and sensorimotor gating as assessed using the PPI acoustic startle were normal in both male and female *T. gondii*-infected mice (Xiao et al., 2012). Furthermore, as the acoustic/auditory startle or foot shock often used in the PPI test may be stressful for the animal, it may be a less than ethically ideal assay for this particular system, and more biologically and ethically appropriate alternative behavioural assays are available. These include, for example, the five-choice serial reaction time task (5-CSRTT), which also tests for the attentional dysfunction observed in human schizophrenia [as well as attention deficit hyperactivity disorder (ADHD), Alzheimer's, Parkinson's, aging and addiction] (Bari et al., 2008). The T-maze test of impulsivity also tests for attention deficit and involves the dopaminergic and serotonergic systems (Denk et al., 2005) (Appendix 2).

Another potential behavioural assay for both *T. gondii* and schizophrenia concerns assays incorporating odour detection. Studies to date suggest that rodents infected with *T. gondii* do not appear to have any general disruption of their odour/olfaction system – as they can, for instance, discriminate between feline and other predator and non-predator odours and even between different concentrations of odour presented – and hence it appears that their perception of cat odour is affected (Berdy et al., 2000; Kannan et al., 2010; Lamberton et al., 2008; Vyas et al., 2007b). Olfaction dysfunction is frequently observed in patients with schizophrenia, with the greatest impact on odour identification (Cohen et al., 2012), which may thereby present a parallel to that observed in *T. gondii*-infected rodents. Thus, further examination of the subtle changes in odour-specific threshold and identification deficits observed is warranted as part of the *T. gondii*–rat manipulation–schizophrenia model, and a range of standardized behavioural assays are available (Appendix 2).

Cognitive flexibility is a critical executive function that can be broadly defined as the ability to adapt behaviours in response to changes in the environment. There are, furthermore, potentially useful non-invasive behavioural assays for these deficits in rodents available that could well be highly applicable for a *T. gondii*–rat manipulation–schizophrenia model. These include, amongst others, intra-dimensional/extra-dimensional digging tasks. During such assays, rodents are trained to dig for food reward using either olfactory (digging medium odour) or tactile (digging medium texture) cues. The rewarded cue in the same dimension is switched to test for intra-dimensional shifting. The rewarded cue is changed to the different dimension to test for extra-dimensional shifting. Likewise, the Wisconsin Card Sorting Task (WCST) (Grant and Berg, 1948) has been one of the more commonly employed assays for impaired cognitive flexibility in schizophrenic patients, and analogous versions have been developed for use in rodents. In essence, these tasks involve the subject selecting between stimuli, which vary from one another in more than one perceptual dimension, and being reinforced for choosing a stimulus based upon one specific dimension alone, e.g. odour. During an 'intra-dimensional shift' (IDS), the form of the dimension the subject must choose is changed by the experimenter, e.g. from cinnamon to chocolate odour. In an 'extra-dimensional shift' (EDS), the correct dimension is changed altogether, such that choices must be guided by the new dimension (texture) while ignoring the previously rewarded dimension. In a rodent IDS/EDS analog of the WCST, rats (Birrell and Brown, 2000) dig in sand to make choices based on the dimension of texture or smell, thereby providing another example of a potentially useful, biologically and ethically behavioural assay available and able to be

incorporated into future research in this field (a non-exclusive list being provided in Appendix 2).

General discussion

Can *T. gondii* infections in animal models really help us understand behavioural changes in humans?

The very characteristics of precisely those parasites able to manipulate host behaviour, rather than parasitism *per se*, may plausibly make them the prime candidates to be associated with clinical disorders in infected humans, whether it be as a direct or indirect consequence, from specific manipulation, or 'side-effect/by-product' of infection (Appendix 1). Indeed, despite such parasite's often apparently sophisticated mechanisms aimed to achieve manipulation, it seems unlikely that they would also possess, at high cost, yet more sophisticated mechanisms able to distinguish and discriminate against expressing such manipulation to within only those 'correct' hosts intended. Indeed, there would not be selective pressure for this specificity. This may be particularly relevant to those multiple-host parasites transmitted in the food chain, as these may be particularly likely to infect the 'wrong host'. *T. gondii* may well therefore be under, or have been under, strong selective pressures to specifically manipulate its rodent intermediate host in order to enhance predation to the feline definitive host, but it is also a ubiquitous parasite capable of infecting, through a range of transmission routes, all warm-blooded animals (Beverley, 1976). Moreover, several of these species, including humans, have considerably longer life-spans than a rodent, and hence one could reasonably propose may be more susceptible to developing 'unselected' pathological behavioural changes simple as a by-product of their extended durations of infections. One of these pathologies may thereby include the potential association between *T. gondii* and schizophrenia in some humans. Can thus studying the behaviour of small intermediate hosts with natural or experimentally exposed infections really help us understand behavioural changes in *T. gondii*-infected humans? There seems little doubt that the subtle behavioural changes observed in rodents infected with *T. gondii* will be reflected by similar subtle behavioural changes in humans, and a convincing body of empirical evidence now exists in support of this (Flegr, 2013). However, when it comes to considering animal infection models for human severe behavioural alterations, as occur within schizophrenia, the case is undoubtedly more complex. There is no doubt that animal, in particular rodent, models have been and continue to be useful in helping us understand aspects of schizophrenia – such as in terms of elucidating how current and potential future antipsychotic drugs work. Indeed, it was an understanding of the interaction of certain neuroleptics with dopamine receptors that was an instigator in formulating the still-maintained theory that schizophrenia involves some form of dysregulation of brain dopamine function (Creese et al., 1976; Seeman, 1987; Seeman et al., 1976). However, we are unlikely to ever be able to reproduce the full phenotypic spectrum of a human psychiatric disorder such as schizophrenia in a rat or mouse (Arguello and Gogos, 2006). Schizophrenia is a highly heterogeneous disorder of myriad symptoms. The presentation of different symptoms and their severity varies considerably across patients. Nevertheless, while this complexity cannot be fully recapitulated in the rodent models, specific symptom categories can be behaviourally modelled. A constructive starting point has been to demarcate schizophrenia-related phenotypes into the clinical categories of positive, negative and cognitive/executive symptoms (Brigman et al., 2010). Of course, an essential step in clarifying the

aetiology of schizophrenia is understanding the gene–environment interactions contributing to this and associated disorders. In this context in particular too, animal models will have a central and indispensable role in the process of helping elucidate the mechanisms involved and perhaps the epidemiology of certain causes of psychiatric disorders (Arguello and Gogos, 2006). Where such animal models are to be used, it is of course imperative that these must only be performed in a biologically appropriate and ethical manner. It is important to emphasize, however, that mechanistic insights into the nature of the deficit under investigation cannot be achieved by behavioural assessment alone. Rather, an interdisciplinary approach that begins at the behavioural level whilst combining the cellular and molecular levels is essential (Arguello and Gogos, 2006). The hope is that future research will allow us to make some truly novel insights into the epidemiology and mechanisms of *T. gondii*-altered behaviour and, potentially, into the pathophysiology and ultimate prevention or treatment of certain neuropsychiatric diseases such as schizophrenia.

Appendix 1. Glossary of terms relating to parasite-altered behavioural alterations

Sickness behaviour

Morbidity in the host, often displayed as general malaise or ‘sickness’, that may facilitate transmission of the parasite. Often observed with vector-borne diseases, where a decrease in host energy and activity levels can increase the likelihood of being bitten by an insect vector, *via* an increase in host landing rate and a reduction in fly swatting behaviours (Ewald, 1994; Holmstad et al., 2006). Examples of this can be seen in rodents infected with *Trypanosoma brucei brucei* (Darsaud et al., 2003) and various *Plasmodium* spp. (Day and Edman, 1983; Grau et al., 1987). Another example may be sneezing and coughing in humans, which facilitates the transmission of aerosolised pathogens, *via* increasing the volume and distance travelled of aerosolised particles containing the pathogen (Gralton et al., 2011).

General pathology

Morbidity (or mortality) in the host of no obvious selective benefit to host or parasite. This may be the case with neurosyphilis caused by *Treponema pallidum*, which is associated with cognitive deterioration and neuronal loss long after the period of infectivity (O’Donnell and Emery, 2005). *Schistosoma mansoni* infection has also been associated with reduced exploratory behaviours, cognitive abilities and nociception in the human host (Aloe and Fiore, 1998; Aloe et al., 1996; Fiore et al., 1998; Fiore et al., 2002) that is of no apparent selective benefit to the parasite, which relies on contact with freshwater snails for its transmission.

Parasite manipulation

Behavioural alterations of the host with a specific selective benefit to the parasite. This is the case with *T. gondii*, where a reduction in fear of feline odour and increased activity are thought to be specific selective advantages to the parasite, increasing its chance of transmission by predation *via* the feline definitive host (Berdoy et al., 2000; Webster, 2001). Another example may be the Seoul virus, which, similar to the rabies virus in dogs, is thought to increase aggressive behaviours in rats, where the resulting wounding may facilitate transmission *via* contact with virus present in saliva or excrement (Hinson et al., 2004; Klein et al., 2004). Other heteroxenous parasites that appear to cause their intermediate hosts to be predated upon more than expected by their definitive hosts include *Plagiorynchus cylindraceus* in isopods (Moore, 1983),

Sarcocystis cernae in voles (Hoogenboom and Dijkstra, 1987) and *Euhaplorchis californiensis* in fish (Lafferty and Morris, 1996) (and see Moore, 2013).

By-product

Behavioural alterations displayed in accidental/dead-end hosts as a side-effect of selective adaptations in the parasite’s natural host species. This appears to be the case in *T. gondii* infection of humans, where adaptations designed to facilitate transmission in the rodent intermediate host are also observed in humans: such as lowered reaction times (Novotna et al., 2008), subtle personality alterations and reduced novelty-seeking (Flegr, 2007). This can also be seen in the case of *T. gondii*-infected sea otters showing abnormal behaviour and increased predation by sharks (Miller et al., 2004), despite sharks being a dead-end host to the parasite. Another example of this is perhaps *Lyssavirus* spp., causative agents of rabies. In wild hosts such as dogs and bats, the virus’ mechanisms within the central nervous system may facilitate transmission; for example, dogs with furious rabies show higher levels of aggression and biting (Kaplan, 1986) that may increase chance of transmission *via* infected saliva to susceptible hosts’ blood and body tissues (Rupprecht et al., 2002). However, in humans, a dead-end host to the virus causes hyperactivity, phobic spasms and severe agitation (Hemachudha et al., 2002) that is of no selective benefit to the virus.

T. gondii–rat manipulation–schizophrenia model

The novel term, first used here, relating to studies of *T. gondii* infection in rats as a model system for examining the evolution and mechanisms of *T. gondii*-altered behaviour in its natural intermediate host species to examine both ‘parasite manipulation’ and its ‘by-product’ physiological and behavioural changes in accidental secondary host species such as humans. Specific focus is placed upon the use of *T. gondii* in rats as a biologically and ethically appropriate model for understanding the aetiology, symptoms and mechanisms of action underpinning (some cases of) human schizophrenia and related affective disorders.

Appendix 2. Examples of non-invasive behavioural tests for assessing the impact of *Toxoplasma gondii* on rodent behaviour

Elevated plus maze

What it measures

Generalised anxiety behaviour. This tests a rat’s unconditioned fear response to heights/open spaces, as compared to a darker enclosed space, and is based on the classic approach–avoidance theory. The elevated plus maze has been used as a model of state, unconditioned anxiety for over two decades and is well documented and reviewed (Carobrez and Bertoglio, 2005; Hogg, 1996; Walf and Frye, 2007).

Relevance for *T. gondii* research

Widely used behavioural assay for anxiety-related behaviours in rodents and is therefore replicable and comparable with other related tests. It is an easy to use and easily automated assay. Unconditioned response based on natural behaviours seen in wild rats (Barnett, 1958). Non-aversive and can detect subtle changes such as those predicted to arise from *T. gondii* infection (Webster, 2001).

Light–dark Suok test

What it measures

Based on classic approach–avoidance theory, using an unstable and novel arena, this assay assesses balance and anxiety phenotypes

simultaneously, as well as potential vestibular defects (Kalueff et al., 2008).

Relevance for *T. gondii* research

Certain neural circuits are shared by pathways that mediate autonomic control, vestibular control and anxiety (Balaban, 2002). These are modulated by the monoaminergic system, which directly links balance disorders with emotional dysregulation. As *T. gondii* may influence anxiety behaviours in rats (Kaushik et al., 2012) and has been shown to cause sensorimotor deficits in mice (Gulinello et al., 2010), this test allows for both aspects of behavioural alteration to be studied. Dopamine is involved in the modulation of vestibular pathways (de Waele et al., 1995) and in anxiety-related behaviour (Mallo et al., 2007), and *T. gondii* has been shown to alter host dopamine levels (Prandovszky et al., 2011; Webster and McConkey, 2010).

'Fatal feline attraction' four-choice test

What it measures

Innate physiological and behavioural aversion to cat (urine) odour. Infected and uninfected rats' responses are assessed through the number of entrances into and/or duration spent in zones with different odours – feline odour, own (rat) odour, neutral (water) odour and non-predatory mammal (rabbit) odour (Berdoy et al., 2000; Webster et al., 2006).

Relevance for *T. gondii* research

The innate aversion to cat odour amongst rodents provides a profound obstacle for the parasite against successful predation by the feline definitive host. This assay measures the parasite's ability to manipulate such innate traits. Through providing the test rodent with a number of other odours, this assay may be comparable to a situation in the wild where a rat would encounter a number of other mammalian odours, including its own. This allows for the specificity of the difference in response to feline odour as compared to other odours to be shown. The test is also conducted over a long period of time (2.5–12 h) and therefore assesses a rat's response over time rather than just its initial response (thereby further minimizing risk of Type I or II errors). This again may be more relevant to a situation in the wild where short time constraints may not apply. In terms of the *T. gondii*–rat manipulation–schizophrenia model, as the fatal feline attraction invoked by *T. gondii* is not simply a reduction or removal of a behavioural trait, but instead a positive reversal of an innate behaviour, an apparent 'alteration of the mind of the rat in the face of predation' (Berdoy et al., 2000; Webster et al., 2006), one could propose that this assay may serve as a potential 'schizophrenia positive symptom' indicator.

Activity/velocity

What it measures

Velocity of movement, duration of time spent moving, duration of time spent stationary, frequency of entry into a zone, latency to enter a zone of interest. These data can now be easily obtained simultaneously with other behavioural assays using automated behavioural tracking software.

Relevance for *T. gondii* research

There is a convincing body of evidence that *T. gondii* increases activity levels in both rats (Webster, 1994) and mice (Hutchison et al., 1980; Hodkova et al., 2007), which may be predicted to increase the chance of predation by the feline definitive host (Webster,

2001). In terms of the *T. gondii*–rat manipulation–schizophrenia model, positive symptoms of schizophrenia, such as psychomotor agitation and hyper-responsivity to psychotomimetic drugs, are modelled in rodents by testing locomotor responses and hyperactivity (Brigman et al., 2010). Thus, one could perhaps propose that the increased activity observed in *T. gondii*-infected rat studies is also useful as a 'positive symptom' assay.

Two-choice test

What it measures

Entrance into and/or time spent in zones with different odours, e.g. feline vs rabbit, or domestic cat vs puma, or domestic cat vs cheetah (Lamberton et al., 2008).

Relevance for *T. gondii* research

Related to the 'fatal feline attraction' and 'novel odour assays', this specifically allows preference for, and/or discrimination between, two odours (such as two contrasting predatory odours alone) to directly be tested. This may be particularly pertinent for measuring between *T. gondii* definitive host vs non-definitive host predator odours or even between different predatory feline species with different definitive host capacities.

Continuous spontaneous alternation

What it measures

Spontaneous alternation is very sensitive to hippocampal dysfunction. Alternation reflects the animal's motivation to explore its environment. There also appears to be involvement of the vestibular system (Deacon and Rawlins, 2006; Lalonde, 2002). Continuous spontaneous alternation has been widely used as a simple measure of short-term spatial working memory and can reflect responsiveness to novelty as well as sensory and attentional factors (Hughes, 2004). Dopamine in the septum and hippocampus has been implicated in the exploration of novel maze arms (Lalonde, 2002).

Relevance for *T. gondii* research

This test requires no habituation and is based on the animal's natural exploratory behaviour of a novel environment. This may tie in with previous studies that show that *T. gondii*-infected rodents show altered exploration of novel environments (Berdoy et al., 1995). As the assay involves a continuous alternation test, there is no experimenter contact during the trial, the test is non aversive and it is easily automated. This may allow for the more subtle behavioural changes usually seen in *T. gondii* infection (Webster, 2001) to be detected. Tests for hippocampal dysfunction are important in *T. gondii* research as the parasite has been shown to invade hippocampal neurons and glial cells (Creuzet et al., 1998), and the septohippocampal pathway is involved in the innate fear of feline odour (Vyas et al., 2007a). The assay also involves the vestibular system; this may be important as *T. gondii* has been shown to cause sensorimotor deficits in mice (Gulinello et al., 2010). In terms of the *T. gondii*–rat manipulation–schizophrenia model, schizophrenia patients have been shown to display spatial working memory and short-term memory deficits (Aleman et al., 1999; Barch et al., 2002; Park and Holzman, 1992). Spontaneous alternation has also been seen to be affected by other neurotropic diseases such as scrapie (Guenther et al., 2001). Hippocampal dysfunction also plays a role in the neuropathology of schizophrenia (Harrison, 2004). Finally, there is a speculated involvement of dopamine in spontaneous alternation behaviour (Lalonde, 2002).

Burrowing

What it measures

Can be used to sensitively measure a wide range of behavioural abnormalities (Deacon, 2006a). An advantage of this test is that it allows digging behaviour to be directly measured by manual observation and can give a more detailed profile than marble burying.

Relevance for *T. gondii* research

The test is very sensitive and therefore able to detect very subtle behavioural changes. The test is non-aversive and examines a natural behavioural trait whilst mimicking an environmental situation encountered by wild rats, therefore making it more applicable to the natural intermediate host of the parasite in the wild. Burrowing behaviour may, in part, display elements of defensive burying (Deacon, 2006a; Dellomo et al., 1994; Fanselow et al., 1987; Pinel and Treit, 1978), which could be of particular interest for *T. gondii* research if associated to predation-related defence mechanisms. In terms of the *T. gondii*–rat manipulation–schizophrenia model, burrowing is decreased by selective serotonin reuptake inhibitors and by changes in interleukin levels in rats (Deacon, 2006a). The burrowing test has been shown to be a sensitive assay for other neurotropic diseases such as scrapie (Deacon et al., 2001).

Marble burying

What it measures

Marble burying is dependent on hippocampal function and, as a result, is disrupted by agents that affect hippocampal function. Marble burying is inhibited by many compounds that are active on the serotonergic system, many of which attenuate anxiety, depression or obsessive–compulsive disorder (OCD) (Deacon, 2006b).

Relevance for *T. gondii* research

As for burrowing above, marble burying behaviour may, in part, display elements of defensive burying (Dellomo et al., 1994; Fanselow et al., 1987; Pinel and Treit, 1978), which is of particular interest for *T. gondii* as this can be a predation-related defence mechanism. The test is non-aversive and mimics an environmental situation encountered by wild rats, therefore making it more applicable to the natural intermediate host of the parasite in the wild. *T. gondii* infection may affect hippocampal function, as the parasite has been shown to invade hippocampal neurons and glial cells (Creuzet et al., 1998), and the septohippocampal pathway is involved in the innate fear of feline odour (Vyas et al., 2007a). In terms of the *T. gondii*–rat manipulation–schizophrenia model, there is evidence for the hippocampus playing an important role in the neuropathology of schizophrenia (Harrison, 2004). *T. gondii* may also modulate host anxiety levels (Kaushik et al., 2012), and anxiety levels may be disrupted in OCD (Miman et al., 2010b) as well as other human mood disorders (Arling et al., 2009; Jones-Brando et al., 2003; Ling et al., 2011; Yagmur et al., 2010).

Morris water maze

What it measures

Spatial learning and reference memory. It is sensitive to hippocampal synaptic plasticity and NMDAR function (Vorhees and Williams, 2006).

Relevance for *T. gondii* research

Schizophrenia patients show dysfunction in learning and memory (Aleman et al., 1999; Saykin et al., 1991) and therefore using a test

that assesses these deficits in *T. gondii*-infected rodents allows us to further understand the link between *T. gondii* infection and schizophrenia observed in humans (Torrey et al., 2007). There is also much evidence that NMDAR hypofunction is involved in schizophrenia (Coyle et al., 2003; Mohn et al., 1999; Olney et al., 1999) and therefore this test provides a relatively non-invasive means of testing this link in rodents.

Five-choice serial reaction time task (5-CSRTT)

What it measures

Impulsivity, attentional processes, accuracy of discrimination, response control (Bari et al., 2008). The test is sensitive to discrete brain lesions and neurotransmitter depletions. The test is particularly used to elucidate neuropsychological mechanisms that are disrupted in pathologies characterised by attentional dysfunction, such as schizophrenia, ADHD, Alzheimer's disease and Parkinson's disease (Bari et al., 2008).

Relevance for *T. gondii* research

Highly relevant for the *T. gondii*–rat manipulation–schizophrenia model, as attentional dysfunction is a pathology found in human sufferers of schizophrenia (Laurent et al., 1999) and other neurological disorders associated with *T. gondii* infection in humans (Kusbeci et al., 2011; Torrey et al., 2000). The test has also been shown to have good translational value, as it was originally adapted from a human task and has been successfully extended to mice and primates (Higgins and Breyse, 2008). This assay provides a useful, less invasive/aversive alternative behavioural assay relevant to the aetiology of schizophrenia compared with that of the more widely used PPI assay.

T-maze test for impulsivity

What it measures

Impulsivity, attention deficits (Bizot et al., 2007; Denk et al., 2005). This test is often used in studies looking at ADHD, as impulsive behaviour (i.e. the selection of small immediate gains in preference to larger delayed gains) is a symptom displayed in ADHD patients (Winstanley et al., 2006).

Relevance for *T. gondii* research

As for the 5-CSRTT assay above, highly relevant for the *T. gondii*–rat manipulation–schizophrenia model, as attentional dysfunction is a pathology found in human sufferers of schizophrenia (Laurent et al., 1999) and other neurological disorders associated with *T. gondii* infection in humans (Kusbeci et al., 2011; Torrey et al., 2000). The test has also been shown to differentially involve the dopaminergic and serotonergic systems in rats (Denk et al., 2005).

Attentional set shifting

What it measures

Impairments in executive processes. This is a characteristic symptom of certain neuropsychiatric conditions where pathophysiology of the prefrontal cortical dopaminergic system is thought to be an underlying cause (Floresco et al., 2006).

Relevance for *T. gondii* research

Impairments in attentional set shifting are observed in patients with schizophrenia and ADHD, due to alterations in dopaminergic functioning within (Floresco et al., 2006). This has been seen in the Wisconsin card sorting task in humans (Lysaker and Bell, 1994; Riccio et al., 1994a; Riccio et al., 1994b; Rybakowski et al., 2005; Tsuchiya et al., 2005). Rodent models of attentional set shifting

may be used to assess mechanisms in the brain involved in such cognitive deficits.

Novel odour tests

What it measures

Novelty-seeking and levels of neophilia/neophobia, as well as olfactory responses and discrimination.

Relevance for *T. gondii* research

Novelty-seeking is an innate characteristic of rodent behaviour, where, in general, mice are neophilic whereas rats are neophobic (Barnett, 1958; Barnett and Cowan, 1976). Infection with *T. gondii* has been shown to decrease neophily in mice and decrease neophobia in rats (Webster, 2001). As rodents rely heavily on their olfactory abilities both for food acquisition and for predator avoidance (Dielenberg et al., 2001), altered neophilia/neophobia in the intermediate host may alter transmission probabilities of the parasite, either by increasing their chance of ingesting the parasite *via* foraging or through increasing their risk of predation by the definitive host. Humans are innately neophilic, and studies have suggested a negative correlation between novelty-seeking and latent toxoplasmosis in humans similar to that observed in rodents (Flegr et al., 2003; Novotná et al., 2005; Skallová et al., 2005). Novel odour assays in rodents may thereby be valuable for evolutionary and mechanistic studies into parasite altered innate behaviour. In terms of the *T. gondii*-rat manipulation–schizophrenia model, *T. gondii*-induced neophobia/neophilia can be ameliorated using D2 antagonists or selective dopamine uptake inhibitors, indicating involvement of the dopaminergic neuromodulatory system on exploratory behaviours (Skallová et al., 2006; Webster et al., 2006). Olfaction dysfunction is also frequently observed in patients with schizophrenia, with the greatest impact on odour identification (Cohen et al., 2012), which may thereby present a parallel to that observed in *T. gondii*-infected rodents.

Gambling test

What it measures

Impulsivity, addiction and risk-taking behaviours (deVisser et al., 2011; Winstanley, 2011; Zeeb et al., 2009).

Relevance for *T. gondii* research

Increased impulsivity and risk-taking are symptoms displayed in several clinical conditions such as schizophrenia and ADHD (Winstanley et al., 2006). Certain drugs that target the dopaminergic (and/or serotonergic) system, in particular that of dopamine agonists, may result in, as a side-effect, increased impulsivity, risk-taking or addictive traits such as gambling in humans (Winstanley, 2011). *T. gondii* infection can increase risk-taking behaviours in rats (Berdoy et al., 1995; Webster, 2001) and in humans (Flegr, 2007), potentially through related alterations of the dopaminergic system (Prandovszky et al., 2011; Webster and McConkey, 2010).

Object recognition test

What it measures

The ability to discriminate between a novel and familiar object, and thereby intact recognition memory of the familiar object, in addition to novelty-seeking behaviour (Bevins and Besheer, 2006). This can test various forms of object recognition, such as the alteration of a particular dimension, recognition of object location or of the environment in which an object was previously encountered.

Relevance to *T. gondii* research

T. gondii alters novelty-seeking behaviours in mice, rats and humans (Webster, 2001), potentially due, in part, to alteration of novelty discrimination mechanisms (Hodkova et al., 2007). As this test is a standardized test used in the field of learning and memory, this may provide a further understanding as to the observed link between *T. gondii* infection and schizophrenia (Torrey et al., 2007; Torrey and Yolken, 2007), as schizophrenia patients display impairments in recognition memory (Danion et al., 1999; Pelletier et al., 2005). Dopamine receptors have been implicated as having a role in functioning of this task (Besheer et al., 1999), which ties in with the potential link between *T. gondii* infection and alterations in the dopaminergic pathways (Prandovszky et al., 2011; Webster and McConkey, 2010). This test does not require exposure to aversive stimuli, or food or water restriction, and has been replicated in many laboratories using both mice and rats (Bevins and Besheer, 2006).

The Holeboard test

What it measures

Anxiety levels (Takeda et al., 1998) and exploration of a novel environment (File and Wardill, 1975a; File and Wardill, 1975b).

Relevance to *T. gondii* research

Widely used behavioural assay for anxiety and exploratory behaviours in rodents and is therefore replicable and comparable with other related tests. Unconditioned response based on natural behaviours seen in wild rats (Barnett, 1958). Non-aversive and can detect subtle changes such as those expected to arise from *T. gondii* infection (Webster, 2001). *T. gondii*-infected mice have been shown to display increased exploratory behaviours in the holeboard test in previous studies (Skallová et al., 2006). This effect has been shown to be suppressed by a selective dopamine uptake inhibitor, suggesting that this test may provide further insights into the association between *T. gondii* infection and changes in the dopaminergic neuromodulatory system (Skallová et al., 2006) and thereby the potential link with schizophrenia in humans (Torrey et al., 2007; Torrey and Yolken, 2007).

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