Strathclyde Institute of Pharmacy and Biomedical Sciences

T. gondii chronic and congenital infection and host manipulation: behavioural, immunological and metabolomics consequences in the brain

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Abstract

Previous studies have demonstrated that *Toxoplasma gondii* infection changes behaviour in rodents. In humans, *T. gondii* is considered a possible environmental and neurodevelopment risk factor for the pathogenesis of psychiatric disorders. During chronic infection *T. gondii* can form cysts in the brain, an optimal site to mediate these effects. Herein, the ability of *T. gondii* infection to alter murine behaviour was assessed in both adult acquired chronically infected, congenitally infected mice and in mice exposed to the maternal immune response to *T. gondii* infection during gestation. The immune-neuro environment of these mice was investigated by measuring the levels of cytokine and tryptophan metabolism transcripts by quantitative RT-PCR and the levels of identified metabolites by mass spectrometry. Hyperactivity and lower anxiety levels were observed in chronically infected mice. Higher anxiety levels were observed in congenitally infected mice and hyperactivity was measured in the mice exposed to maternal immune activation during *T. gondii* infection. Changes were also observed in the neuro-immune environment; in particular upregulation of inflammatory cytokines and higher levels of KYNA metabolites, which are known to be neurotoxic, were detected in the chronically adult acquired and congenitally infected mice. Instead, a down regulation of the immune mediators was measured in the brain samples of mice exposed to maternal immune activation. Hyperactivity of amino acid metabolism was observed in the metabolomic profiling of the brain samples from all groups of mice infected with *T. gondii*. Although it cannot at present be determined if the changes in the brain chemistry caused by congenitally acquired *T. gondii* infection are induced via the immune response or are directly mediated by the parasite. However, the studies described here provide a better understanding of how the parasite can influence behaviours and provide possible insights into new therapeutically approaches for treatment of mental health and degenerative disorders.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
AIDS	Acquired Immune Deficiency Syndrome
AMP	Adenosine Monophosphate
APC	Antigen Presenting Cells
ATP	Adenosine-5'-Triphosphate
BAG	Bradyzoite Antigen
BH4	Tetrahydrobiopterin
BLAST	Basic Local Alignment Search Tool
CCR5	Chemokine Receptor Type 5
CD	Cluster of Differentiation
cDNA	complementary DNA
CENI	Congenital Exposed Not Infected
CI	Congenital Infected
CMV	Cytomegalovirus
CNS	Central Nervous System
COX	Cyclooxygenase
CRP	C reactive protein
CSF	Cerebrospinal Fluid
CST1	Cyst Wall Antigen
CTL	Cytotoxic T cell
CXCR	Chemokine Receptor
Cy-18	Cyclophilin-18
DCs	Dendritic Cells
DDT	DL-Dithiothreitol
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide Triphosphate
EcoRI	Restriction enzyme I
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum

ESI	Electrospray Ionization
FcRn	neonatal Fc Receptor
FWHM	Full Width at Half Maximum
GABA	γ-Aminobutyric Acid
GPI	Glycosylphosphatidylinositol
GRA	Dense Granules proteins
GTP	Guanosine-5'-Triphosphate
HCV	Hepatitis C Virus
HD	3 Hydroxyanthranilate 3,4 Dioxygenase
HFFs	Human foreskin fibroblast cells
HIV	Human Immunodeficiency Virus
HPA	Hypothalamus Pituitary Axis
HPLC	High-Performance Liquid Chromatography
HS	Herpes Simplex
HSP70	70 kilodalton Heat Shock Proteins
IDO	Indoleamine 2,3-dioxygenase
IF	Intermediates Filaments
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IRF-1	Interferon Regulatory Factor 1
JAK	Janus Kinase (Just Another Kinase)
KA	Kynurenic acid
KO	Knock Out
KYN	Kynurenine
KYNA	Kynureninase
KYNA AT1	Kynurenine Aminotransferase I
KYNA AT2	Kynurenine Aminotransferase II
KYNA AT3	Kynurenine Aminotransferase III
KYNAMO	Kynurenine 3 Monooxygenase
L-DOPA	L-3,4-dihydroxyphenylalanine

lacZ	bacterial enzyme β-galactosidase
LB	Lysogeny broth
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharides
LTQ-Orbitrap	Linear Trap Quadrupole-Orbitrap
MAPK	Mitogen-Activated Protein Kinases
MCP	Monocyte Chemotactic Protein
MEApd	Postural Dorsal Medial Amygdala
MHC	Major Histocompatibility Complex
MIC	Microneme proteins
MIF	Migratory Inhibition Factor
MJ	Moving Junction
MPEG	Moving Picture Experts Group
MTs	Microtubules
MyD88	Myeloid Differentiation primary response gene (88)
NF-kB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
p value	Probability value
PAMPs	Pathogens Associated Molecules Patterns
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pen/strep	Penicillin Streptomycin
PF	Personality Factors
PIBF	Progesterone Induced Blocking Factor
poly: IC	Polyinosinic: polycytidylic acid
PPI	Pre Pulse Inhabitation
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuole Membrane
qRT-PCR	Quantative Real Time PCR

QUIN	Quinolinic acid
RNA	Ribonucleic Acid
ROI	Reactive Oxygen Intermediates
ROP	Rhoptries release proteins
RT	Reverse Transcriptase
SAG	Surface Antigen
STAT	Signal Transducer and Activator of Transcription
TBE	Tris/Borate/EDTA
TBP	Tata-binding Box Protein
TCI	Temperament and Character Inventory
TDO	Tryptophan 2,3 Dioxygenase
TGF	Transforming Growth Factor
Th	T Helper cells
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor
TPH	Tryptophan hydroxylase
Tregs	Regulatory T cells
UV	Ultraviolet
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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1 Introduction

1.1 Introduction to parasite manipulation hypothesis

Since the early 20th century observations have been made about the ability of parasites to modify the behaviour of the hosts they infect to facilitate their survival. The most popular example and that reported in many textbooks is the ability of *Dicocoelium dendriticumi,* a trematode "brain worm", to manipulate the behaviour of the ants, which they infect as intermediate host. The infected ants climb grass blades thus increasing the chances of transmission of the parasite being eating by sheep, the definitive host of the parasite (Gandon, 2005). Numerous other observations on the ability of parasites to manipulate host behaviour have been postulated over the years since 1952 until the present day (van Dobben, 1952; C. J Barnard and J. M Behnke, 1990; Combes, 1991, n.d.; Poulin, 1998; Poulin and Thomas, 1999; Moore, 2002).

There are a number of mechanisms postulated that could account for host behavioural changes. A possible direct action of parasite on host physiology has been described, for example releasing neuroactive molecules that have a direct effect on the host brain chemical environment (Adamo, 2002). This method would be biologically costly to the parasite, as it would require a lot of energy to release a lot of molecules in order to have a global effect on the host. An alternative mechanism is through indirect action via small physiological changes to the host, for example via the immune response. This has been proposed as a more plausible hypothesis although a combination of direct and indirect action of the parasite on the host is perhaps more realistic (Moore and Gotelli, 1990).

In this study we investigate the ability of the intracellular protozoan parasite *Toxoplasma gondii* to modify behaviour in the infected murine host and the interactions of infection, immune response and central nervous system are analysed. *T. gondii* infection of mice is a good model to utilise in order to fully appreciate the interaction between the different physiological systems. First of all, the immunological response to *T. gondii* infection is very highly regulated and complex (and it is specific to the parasite's life cycle). During chronic infection *T. gondii* resides in tissue cysts that can be found in many tissues, but are mainly located in the brain, an anatomically favorable position to interact with the nervous system (Dubey, 1997). In addition, studies have already described the ability of the parasite to modify behaviour in the infected host, in particular in rodents. In humans, antibodies to *T. gondii* are more prevalent in schizophrenic patients compared with the population as a whole establishing an association that is not yet fully understood (Buka et al., 2001; Conejero-Goldberg et al., 2003; Alvarado-Esquivel et al., 2006, 2011; Torrey et al., 2007; Zhu et al., 2007; Hinze-Selch et al., 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007; Niebuhr et al., 2008; Ahmad et al., 2010; Hamidinejat et al., 2010; Okusaga et al., 2011; Pedersen et al., 2011; Tedla et al., 2011; Horacek et al., 2011; Ling et al., 2011). Herein, the parasite's life cycle and biology, modes of transmission and the immune response induced by infection will be described in detail. Furthermore, the impact of infection on host behaviour will be discussed. The possible mechanisms that infections mediate behavioural changes though the immune response and and resultant tryptophan degradation will be analysed.

1.2 *Toxoplasma gondii* life cycle

Toxoplasma gondii was discovered by Nicolle and Manceaux (1908) in the spleen of the north African rodent the gondi (*Ctenodactylus gundi*) and later that year by Splendore in a rabbit (*Oryctolagus cuniculus*) (Splendore, 1908; Nicolle and Manceaux, 1909). It took 60 years after its discovery to fully understand the life cycle and transmission routes of *T. gondii* (Figure 1.1). In 1954 it had been suggested that undercooked meat could be a source of infection (Weinman and Chandler, 1954) and this hypothesis was supported by the identification of the tissue cyst stage of the life cycle (Jacobs et al., 1960). The ability of cat faeces to transmit disease, the description of the sexual stages and the resultant oocyst stage (and thus the coccidian nature of the parasite) were described in 1969 and provided the explanation of how herbivores became infected (Hutchison et al., 1969; Frenkel, 1970). *T. gondii* was first recognised as causing disease in humans in 1923 when congenital infection was identified as the cause of ocular disease in a Czechoslovakian infant (Janků, 1923). Congenital transmission and ocular disease were quickly confirmed in independent studies (Wolf and Cowen, 1937; Wolf et al., 1939). Irrespective of the mode of infection, *T. gondii* initially multiplies in almost every tissue of the body as the fast dividing tachyzoite stage, before transforming into the slowly dividing bradyzoite stage that forms long-lived cysts in skeletal muscle and the central nervous system (Lyons et al., 2002).

T. gondii has at least five clonage lineages (Types I-V), that differ slightly genetically and by the disease symptoms they can cause (Wendte et al., 2011). It is widely known that Type I strains (RH) cause fulminant disease in both humans and

rodent models, whereas Type II (Me49 and Beverly) cause chronic toxoplasmosis. Type III strains (VEG) are usually found in the animal reservoir and types IV and V are defined as atypical as they are likely to be the result of sexual recombination. These are largely found in geographical pockets in South America (eg Brasil)(Vaudaux et al., 2010). Disease severity is usually linked with strain type as recent studies have also demonstrated that different molecules from each strain may manipulate the host cell. This will be discussed throughout the chapter.

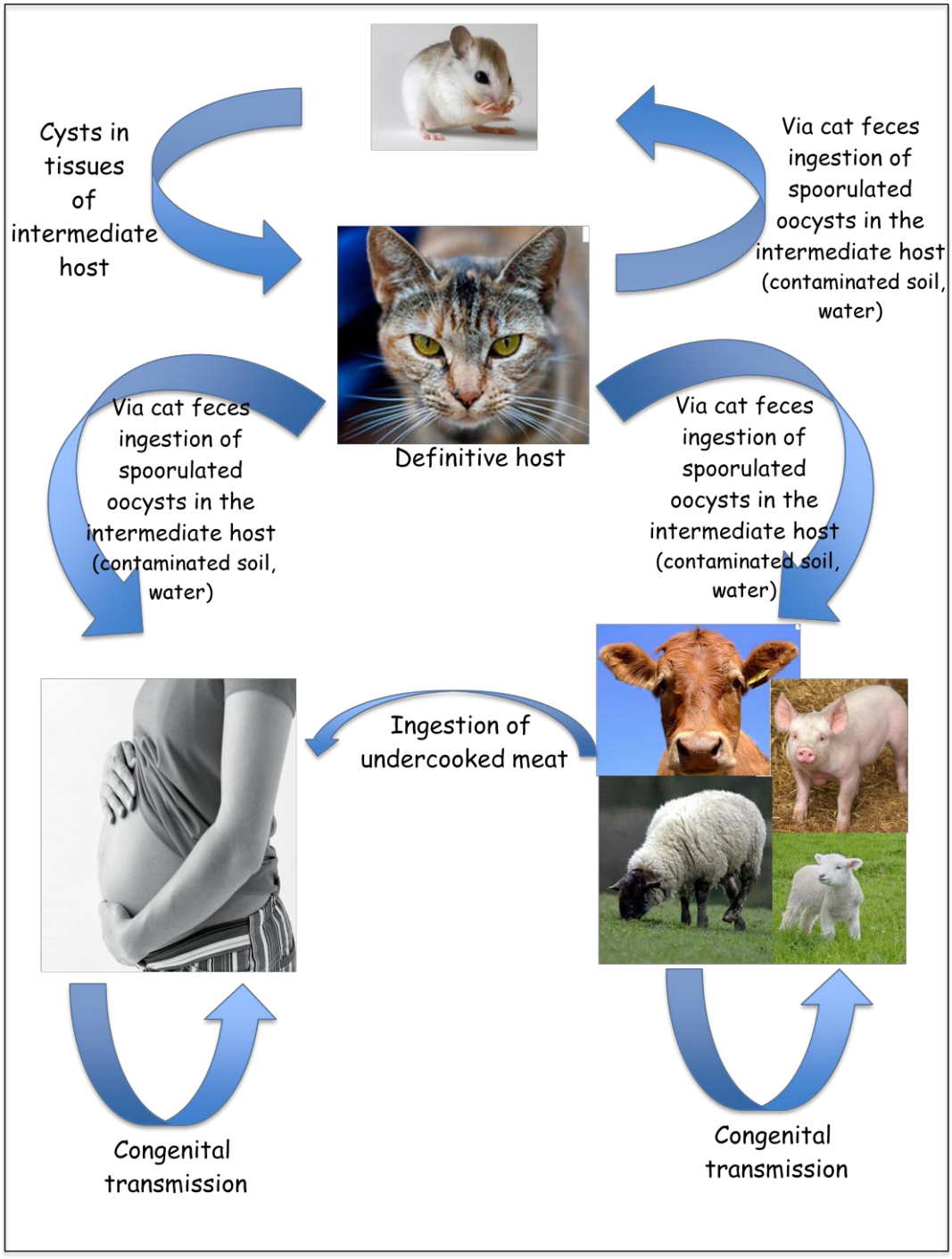


Figure 1.1: The representation of *T. gondii* asexual life cycle and mode of transmissions. The sexual life cycle occurs in the gut of the cat. The asexual life cycle begins with the ingestion of sporulated cysts released in cat faeces, which can contaminate soil, food and waters. Tachyzoites undergo a fast multiplication in the intermediate host and begin converting into the bradyzoites form, which reside in tissue cysts that are mainly located in the brain. Vertical transmission can occur during pregnancy if the mother has been exposed to *T. gondii* infection.

1.3 *T. gondii* morphology and cell biology

T. gondii undergoes a complicated life cycle characterised by different life cycle stages that have different mechanisms of interaction with the infected host cells. For this reason, *T. gondii* possesses different proteins for each stage of its life cycle in order to interact properly with its host during infection. Some of these molecules are known to modulate the host immune response. This will be discussed in detail in section 1.4. Other molecules released by *T. gondii* could interact and have an effect on the neuro functions of the host, which will be discussed in further detail in paragraph 1.7.2. *T. gondii* is a very successful parasite as it has established infection across the world and numerous species. This can only be explained by the ability of *T. gondii* to create an intimate relationship with the infected host through specific molecular interactions.

1.3.1 The Tachyzoite

The tachyzoite stage has a crescent shaped pointed anterior, a conoidal end and a rounded posterior. It is 5μm long and 2μm wide. The nucleus is centrally located and the parasite possesses a rough and smooth endoplasmic reticulum, a Golgi organelle and several inclusion bodies such as amylopectin granules. The apical organelles, named so for their location in the apical tip of the parasite include rhoptries, micronemes and the dense granules (Soldati et al., 2001; Boothroyd and Dubremetz, 2008; Nishi et al., 2008). They are very important in invasion and mechanisms of the parasite survival during its different life stages. In particular, microneme proteins (MIC) are involved in the motility of the parasite, in the host cell recognition and in the binding of the host cell membrane (Sheiner et al., 2010). MICs proteins are

involved in the attachment to the host cell wall by binding to specific receptors. Rhoptries release proteins (ROP) during the internalisation process within the host cell and have a likely role in the actual formation of the parasitophorous vacuole (PV). ROP proteins together with the MIC proteins are involved in the formation of the moving junction (MJ), essential to form the PV membrane from the host cell membrane. This strategy is used in order to avoid fusion with host cell lysosomes and to maintain a stable environment for multiplication of the parasite (Laliberté and Carruthers, 2008). GRA proteins, released from the dense granules approximately ten minutes after invasion, have been given a putative role in the development, maintenance, organisation and structure of the PV and its location in the host cell by reorganising the intermediates filaments (IF) and microtubules (MTs) of the host cell. *T. gondii* is an auxotroph for many essential nutrients such as tryptophan, arginine, polyamine, purines, cholesterol, iron and therefore it utilises the host cell provisions for its own metabolism. Therefore the PV membrane (PVM) possesses pores in order to facilitate a bidirectional exchange by diffusion of small molecules with the cytoplasm of the host cell. However, some essential nutrients are insoluble or attached to larger molecules and other routes of acquisition are used. With immunocytochemistry and electron microscopy techniques it has been possible to identify the interaction of GRA proteins with the organelles of the host cell. Some organelles such as the endoplasmic reticulum were bound to the PV through these GRA proteins and giving them a putative role in the metabolic activity of the parasite. In addition, many *T. gondii* proteins are characterised by phosphatase and kinase activity, which enable them to modulate the host cell signaling pathways and gene

expression to provide nutrients from different routes, to control infection and to ablate host cell apoptosis mechanisms to increase the parasite's survival.

Motility is essential for the parasite survival during the tachyzoite stage and it is characterised by a gliding motion (Heintzelman, 2003). Movement is regulated by an actin myosin complex and also through the interaction of the MIC proteins constitutively secreted. This secretion is increased once the parasite attachment to the host cell membrane occurs (Laliberté and Carruthers, 2008). *T. gondii* enters in the host cell by an active penetration and it egresses the cell by an active mechanism very similar to the invagination process (Roiko and Carruthers, 2009), when the intracellular environment becomes adverse for parasite survival. During invasion, *T. gondii* re-orientates itself on the host cell membrane exposing its apical complex first and releases the contents from this complex via a mechanism described as "kiss and spit" by Boothroyd and Dubremetz (2008). Membrane proteins appear on the parasite's surface such as proton pumps, which have a role in preventing the vacuole acidification (McIntosh and Vaidya, 2002). During the formation of the parasitophorous vacuole in which the parasite resides the rhoptries and GRA proteins are released. Their function still remains to be elucidated but it has been suggested that they may be important in the communication between the internalised parasite the host cell organelles (Laliberté and Carruthers, 2008).

1.3.2 The Bradyzoite

The morphology of the bradyzoite stage is very similar to the tachyzoite stage. It has a similar shape 7μm long and 1.5μm wide. However, it is characterised by a higher

number of micronemes and a lower number of dense granules (Lemgruber et al., 2011). It also contains amylopectin granules (Dubey et al., 1998). Differentiation between the tachyzoite and the bradyzoite can be achieved through morphological analysis and through the identification of different surface molecules. For example, SAG1 antigen is specific for the tachyzoite stage and BAG1 or CST1 for the bradyzoite stage and tissue cyst, respectively (Skariah et al., 2010). When *T. gondii* infects the intermediate host it develops firstly into the fast multiplication phase of the tachyzoites. With the onset of host immunity, the parasite develops into bradyzoites leading to the chronic infection phase. Nevertheless, it is also true that bradyzoite formation is an important stage of the parasite's life cycle and therefore independent from the host immune regulation (Dubey, 2008). Bradyzoites reside in tissue cysts preferentially positioned in the CNS, eye, lung, lymphoid, skeletal and smooth muscle tissues. Tissue cysts can reactivate with the conversion of bradyzoites into tachyzoites. Generally the reverse transformation of bradyzoites into tachyzoites happens in immune suppressed individuals and tissue cysts remain dormant in immune competent individuals and any possible tissue cyst rapture is immediately controlled by the host immune response (Dubey et al., 1998).

1.4 Immune Response to *T. gondii*

The immune response against *T. gondii* is a highly regulated process. The parasite participates actively in the host immune responses in order to increase its own chances of survival by creating equilibrium between activating and evading the host defence. The knowledge so far acquired of the immune response against *T. gondii* has been mainly through the analysis of infection using the murine model. While

many aspects of murine and human toxoplasmosis differ, the murine model provides a good starting point to elucidate many aspects of the immune response against *T. gondii* infection and to understand the delicate balance between parasite control and host pathology. The importance of the innate immune response during infection is well established. *T. gondii* activates the immune response through immunological activating molecules, PAMPs (pathogens associated molecules patterns), which bind with receptors present on the host cell, in particular TLRs (toll-like receptors) and CCR5 (Egan et al., 2009). The first cells to be involved in this process are neutrophils and dendritic cells (DCs). These cells are stimulated to produce IL-12, which promotes the further stimulation and activation of other immune cells, such as NK cells, involved in the release of IFN-γ, the most important cytokine in the protective response against *T. gondii* infection (Miller et al., 2009). Herein, the close interaction between the parasite infection and the host immune response will be described in detail.

1.4.1 Intimate relationship of *T. gondii* and its host and the effect on the immune system

Several immunomodulatory molecules are released by *T. gondii* in order to interact with specific host cell receptors and regulate the outcome of infection. A number of *T. gondii* proteins bind to the TLRs specifically. For example, profilin couples to the TLR-11 and HSP70 binds both TLR-2 and -4. In particular, TLR-11 when coupled with profilin activates the cell-signaling molecule MyD88 as does TLR-2 and -4 and it is essential to control the acute phase of infection in mice.

In humans TLR-11 is present only as a pseudogene and the equivalent mechanisms still have to be found (Pifer and Yarovinsky, 2011). Nevertheless, the signaling molecule MyD88, essential for the activation of the innate immune responses against *T.gondii* infection seems to play a similar role in humans as in mice (LaRosa et al., 2008). Other parasites molecules also bind to TLRs. GPI anchors are present on a number of *T. gondii* surface proteins and interact with TLR-4. *T. gondii* cyclophilin-18 (Cy-18) mimics the properties of a chemokine and it is capable of binding CCR5 (Dobbin et al., 2002; Bell et al., 2006; Yarovinsky and Sher, 2006). Many proteins released by *T. gondii* during the different stages of infection, tachyzoites as well as bradyzoites, have phosphatase and kinase activity that could interfere with the host cell pathways and gene expression. Moreover, some proteins are found to be located directly in the nucleus of the host cell and studies have been shown that they play an essential role during infection. In particular, ROP 16 and 18 have been found abundantly in the host nucleus and it seems that they interfere with the JAK/STAT pathway that is responsible for the production of IL-4 (Saeij et al., 2006). This has recently been determined to be strain type specific as ROP16 from a type II strain activates STAT6 and is responsible for alternative macrophage activation, whereas GRA15 activates NF-κB in the host cell, thus promoting a classical macrophage activation (Jensen et al., 2011). However, the essential role of these proteins has not yet been defined. GRA6 and GRA10 interact with the STAT6 signaling pathway of the host cell, which would then lead to the final production of anti-inflammatory cytokines (Ahn et al., 2006). ROP2 and GRA3 are co-localised to the host cell mitochondria and ER respectively (Henriquez et al., 2005) probably to access more nutritional metabolites and in the case of ROP2 also to interfere with apoptosis

mechanisms (Park et al., 2004). It is known that *T. gondii* disrupts apoptosis in the host cell, which is initiated by a number of inducers, which can be mechanical, chemical or physical. It is also known that both tachyzoites and bradyzoites prevent apoptosis in the host cell in order to increase its survival (Laliberté and Carruthers, 2008).

1.4.2 Cell mediated response

As previously mentioned, *T. gondii* usually enters the intermediate host by the oral route, for example by the ingestion of undercooked meat and the first cells to actively be infected are enterocytes. Therefore, these cells are also the first to react and to begin the defense mechanisms by releasing chemokines and recruit the immune cells to the site of infection (Mennechet et al., 2002). Of note, there has been a recent increase in attention given also to the interaction and the balance created with the commensal gut bacteria following *T. gondii* oral infection (Pifer and Yarovinsky, 2011). Commensal gut bacteria may also contribute to early *T. gondii* infection by contributing to the release of inflammatory cytokines by inducing expansion and activation of TLR-4 receptors (Heimesaat et al., 2007). The cell mediated response consists of two types: an early nonspecific innate T cell independent response and a specific T cell response (Lieberman and Hunter, 2002).

1.4.2.1 Innate immune response

The role of the innate immune response is dual, first of all it limits the replication of tachyzoites during an acute infection and then it directs the development of the adaptive response by producing IL-12 and IFN-γ and the development of a type 1

response (Miller et al., 2009). Macrophages, dendritic cell and neutrophils are the first cells to respond to *T. gondii* infection by producing IL-12, IL-18 and TNFα, which stimulate NK cells to produce IFNγ (Bliss et al., 1999).

Neutrophils are critical for the control of the early stages of *T. gondii* infection not only for the antimicrobial activity and production of ROI (reactive oxygen intermediates) and NO (nitric oxide) but also by releasing cytokines, in particular IL-12, and chemokines and recruiting more immune cells. Studies have shown a negative outcome of the infection in neutrophil- deficient mice (van Gisbergen et al., 2005). In humans the chemokine IL-8 is recruits neutrophils during *T. gondii* infection (Del Rio et al., 2001). Neutrophils abundantly express the CXCR2 receptor and mice deficient in this receptor, have increased susceptibility to infection (Del Rio et al., 2001).

The primary function of dendritic cells (DCs) is to present the parasite antigens and activate the T and B cell response. In addition, DCs act as Trojan horses during *T. gondii* infection (Tait et al., 2010). DCs transport the parasite through the blood vessels and infection is spread throughout the host organism. In this manner, *T. gondii* is also able to enter and invade the brain where the immunological environment favors its survival (Channon et al., 2002). DCs also have antimicrobial activity and they produce IL-12, which again directs Th1 expansion (Scott and Hunter, 2002). DCs are, in fact, probably the main producers of IL-12 and extremely important for a positive outcome of infection (Scott and Hunter, 2002). The study by Liu et al. (2006) demonstrates how the depletion of DCs during acute infection

results in lower production of IL-12 and increased mortality and that the reconstitution of DCs from wild type mice during the acute phase of infection results in the rapid production of IL-12 and IFN-γ and a protective immune response.

Macrophages in a manner similar to DCs facilitate the spread of the parasite infection throughout the host. It has been shown that classically activated macrophages are characterised by a strong antimicrobial activity and that are able to restrict *T. gondii* multiplication via (iNOS) inducibile nitric oxide synthase expression (Sibley et al., 1993). However, *T. gondii* is able to interfere with macrophage and DC antimicrobial activities. For example, the parasite promotes the activation of STAT3, which results in the down regulation of classical macrophage activation (Saeij et al., 2006). Nevertheless, despite STAT3 activation macrophages and DCs produce IL-12 and IL-15 and activate NK cells, that play a crucial role in the control of the early stages of *T. gondii* infection (Fehniger and Caligiuri, 2001). NK cells produce large amounts of IFN-γ and therefore they promote classical macrophages activation activities.

As previously mentioned earlier, IFN-γ is the most important cytokine during *T. gondii* infection and it activates and regulates many activities. It has been observed that IFN-γ deficient mice die rapidly during acute infection even though IL-12 production is normal (Scharton-Kersten et al., 1996). Furthermore, anti-IFN-γ antibody therapy during chronic infection has been shown to result in the conversion of bradyzoites into tachyzoites and increased mortality (Yap and Sher, 1999). IFN-γ is clearly involved in directing the infection from the acute phase to the chronic

phase and in the suppression of the conversion from bradyzoites to tachyzoites (Jones et al., 1986) during chronic infection. IFN-γ regulates many antimicrobial activities. In combination with TNF-α it induces the production of nitric oxide, which also plays a role in controlling the chronic infection phase (Khan et al., 1997). IFN- γ also participates in the activation of the ROI species (Chang and Pechère, 1989) and iron depravation, especially in enterocytes in order to limit parasite growth (Dimier and Bout, 1998). IFN-γ also promotes tryptophan starvation in all the cells of the infected host, including brain cells, by up regulating the activity of the enzyme indoleamine 2,3-dioxygenase (IDO), the first step of the tryptophan degradation pathway. Not only does this serve to limit parasite growth but also to limit the immune system response and avoid inflammatory pathologies (Däubener and MacKenzie, 1999). IFN-γ also up regulates the antimicrobial activity of the p47 GTPases proteins, mainly involved in regulating the infection in mice (Butcher et al., 2005).

1.4.2.2 Specific T cell response

CD4+ Th1 cells are activated by antigen presenting cells (APC) through the MHC II (Bliss et al., 1999). They produce IFN-γ, and therefore they participate in promoting the activation of macrophages and the production of IL-12, and they also produce IL-2, responsible for the activation and development of CD8+ T cells.

CD4+ Th2 cells are considered anti–inflammatory as by producing IL-4 and IL-10 they inhibit the effects and induction of Th1 cells. For this reason, for a long time they were thought to be detrimental during *T. gondii* infections (Dawson et al., 2005).

However, it is now known that CD4+ Th2 cells have an important role in regulating the Th1 inflammatory response during infection and reduce the detrimental effects of pathology associated with the over production of pro-inflammatory cytokines (Dawson et al., 2005). Furthermore, IL-4 and IL-13, both produced by the CD4+ Th2 cells, alternatively activated macrophages, leading to a decrease in the antimicrobial activities and an increase in wound healing, such as increase of arginase, polyamines and collagen production (Varin and Gordon, 2009).

CD8+ T cells, also known as CTL (cytotoxic T lymphocyte), also play an important role in the outline of *T. gondii* infection. They are the main mediators of resistance as they detect infection by derived parasite peptides or MHC I (Bliss et al., 1999). They act in two ways. First of all, they directly lyse the infected cells and secondly they also participate in the production of IFN-γ and consequently, the anti parasitic effects of this cytokine.

1.4.2.3 Tregs and Th-17

Other populations of T cells such as Tregs and Th-17 have been demonstrated to play significant roles in the outcome of *T. gondii* infections. Treg cells expansion depends on TGF-β in the absence of IL-6 and they are generally considered to be anti-inflammatory because of their production of IL-10 and TGF-β (Bettelli et al., 2006). In contrast, Th17 cells are stimulated to differentiate by TGF-β and IL-6, their amplification is mediated by IL-21 and later stabilised by IL-23 (Bettelli et al., 2007). They are involved in autoimmune disease and also in the mobilisation and generation of neutrophils during responses to the pathogens through the production of IL-17

(Bettelli et al., 2007)(Sakaguchi, 2000). Treg cells have recently been demonstrated to be the major source of IL-10 production during *T. gondii* infection and are responsible for the protection of the host against excessive inflammation that was previously attributed to Th2 cells (Jankovic et al., 2007). Gazzinelli et al. (1996) demonstrated that previously IL-10 was essential to control the pro-inflammatory response induced following *T. gondii* infection and in absence of IL-10 mouse death is extremely rapid.

1.4.3 Humoral response

There is specific production of antibodies against *T. gondii* in the infected host, such as IgM, IgG and IgA (Correa et al., 2007). Antibodies are thought to not have a predominant role against parasite infection, since *T. gondii,* for the majority of its life cycle and host infection, is intracellular and therefore not susceptible to the action of antibodies (Correa et al., 2007). However, studies have demonstrated the importance of IgA in mucosal immunity in the event of an oral infection with *T. gondii* (Correa et al., 2007) and in the avoidance of re-infection (Correa et al., 2007). IgM has been shown to play a role in the acute phase of infection by limiting parasite dissemination and its invasion in host cells (Couper et al., 2005). In addition, the strategic role of B cells in the development of resistance to the parasite has been described (Correa et al., 2007). It is known that the humoral response also promotes the production and release of the IFN-γ cytokine during *T. gondii* infection (Menard et al., 2007) and that B cell deficient mice die during *T. gondii* infection. However, the mechanism and the prime role of the humoral response has not yet been clarified and fully understood.

1.4.4 Immune response in the brain

T. gondii invades the brain by infecting macrophages and dendritic cells that migrate through the blood vessels (Carruthers and Suzuki, 2007). Here, the tachyzoites are free to penetrate and multiply in other cells such as microglia, astrocytes and neurons where they form cysts (Wilson and Hunter, 2004). It is not yet clear if *T. gondii* preferentially infects a particular cell or if there is a preferential brain region of infection. However, it is known that the immunological consequences of tachyzoite invasion in the brain are several.

As well as systemic effects, *T. gondii* induces the production in the brain of a number of cytokines including IL-1β, IL-6, TNF-α, GM-CSF, IL-2, IL-4 and IFN-γ (Hunter, Roberts, and Alexander, 1992; Hunter, Roberts, Murray, et al., 1992; Burke et al., 1993; Schlüter et al., 1997, 2003), chemokines (GRO1, GRO2, LIF, and MCP1) and different transcription factors (Hunter et al., 1991; Burke et al., 1993; Carruthers and Suzuki, 2007). Microglial cells and astrocytes are associated with IL-1, IL-6, IL-10, IL-12, and TNF-α expression, while infiltrating $CD4^+$ and $CD8^+$ T cells are the presumed sources of IL-2, IFN-γ, IL-4, IL-10 and TNF-α. Expression of cytokines, specifically those associated with T cell expansion (IL-2 and IL-4), may be temporally or sequentially regulated. IL-10 is expressed during chronic infection and would appear necessary to regulate inflammation (Burke et al., 1993; Schlüter et al., 1997, 2003; Wilson et al., 2005).

It is now known that microglial cells can also produce IL-17 and IL-23, which may also contribute to inflammation, although the role of these cytokines in the brain of *T. gondii* infected mice remains to be established (Dunn and Wang, 1995; Flegr et al., 2000, 2003; Novotná et al., 2005; Skallová et al., 2005; Yereli et al., 2006; Flegr, 2007; Hodková et al., 2007; Müller and Schwarz, 2007; Kawanokuchi et al., 2008; Li et al., 2008). These cytokines may influence mood and behaviour in the host through their ability to modulate neurotransmission including monoamine and glutamate dependent mechanisms (Dunn and Wang, 1995; Müller and Schwarz, 2007) in the brain.

The close regulation of the host immune response by the parasite itself could be explained as an evolutionary advantage and it is beneficial to the parasite. In fact, it is necessary for *T. gondii* that the host survives at least 14 days after infection in order, for the parasite, to develop into bradyzoites, the infecting stage of the parasite, to increase the chances of transmission (Dubey, 1998). Most probably this is the reason why *T. gondii* possesses immunostimulatory molecules that induce a protective immune response and facilitate host survival for a longer period after infection (Bell et al., 2006). Moreover, tachyzoites can manipulate different routes of apoptosis, antimicrobial effectors mechanism and immune cell maturation (Carruthers and Suzuki, 2007).

1.5 Disease

Approximately 30% of the world's population has *T. gondii* infection and harbour cysts in the brain, and overt disease symptoms such as encephalitis and ocular

toxoplasmosis are only evident during immune suppression (Lyons et al., 2002) or in children infected by congenital transmission (Gagne, 2001). In fact, the occurrence of disease symptoms during *T. gondii* infection in healthy individuals is considered rare.

However, in recent years it has been described, mainly in South America, that atypical or recombinant strains of *T. gondii* are able to induce severe ocular disease or even lung disease and splenomegaly in immune competent individuals (Jones et al., 2006). In addition, an increasing number of studies are now providing evidence that disease is associated with subtle changes in behaviour in animals and humans (Buka et al., 2001; Conejero-Goldberg et al., 2003; Alvarado-Esquivel et al., 2006, 2011; Torrey et al., 2007; Zhu et al., 2007; Hinze-Selch et al., 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007; Niebuhr et al., 2008; Ahmad et al., 2010; Hamidinejat et al., 2010; Okusaga et al., 2011; Pedersen et al., 2011; Tedla et al., 2011; Horacek et al., 2011; Ling et al., 2011) and in some reports it has been noted that the incidence of mental diseases such as schizophrenia is greater in *T. gondii* infected individuals (Wang et al., 2006).

The level of the relationship is not clear and many hypotheses are being postulated and studied. Under these new circumstances, therefore, the assumption of chronic infection being described as asymptomatic is now being challenged (Henriquez et al., 2009).

Herein, the evidence that *T. gondii* infection can cause these changes and potential mechanisms that may account for these observations are discussed.

1.6 *T. gondii* and host behaviour manipulation

1.6.1 Animal behaviour

T. gondii infection is relatively common among both wild and domestic animals (Hill et al., 2005). As *T. gondii* has a complex life cycle with predator-prey interactions, manipulation of the behaviour of the prey can facilitate parasite transmission (Berdoy et al., 1995a, 2000). During the chronic phase of infection the parasite resides in tissue cysts that are in various anatomical sites including smooth muscle, heart, lung and eye. However it is the presence of the parasite within the brain that provides the greatest opportunity for manipulation of the host's behaviour (Webster et al., 1994; Hill et al., 2005).

A number of studies have demonstrated the ability of the parasite to manipulate the behaviour of rodents in relation to predator-prey interactions (Hutchison et al., 1980; Hay et al., 1983; Hay, Aitken, and Graham, 1984; Hay, Aitken, Hair, et al., 1984; Webster, 1994; Webster et al., 1994; Berdoy et al., 1995a, 2000; Vyas et al., 2007). Early studies examined the ability of *T. gondii* infection to affect the behaviour of laboratory mice (Hutchison et al., 1980; Hay et al., 1983; Hay, Aitken, and Graham, 1984; Hay, Aitken, Hair, et al., 1984). These studies found that infected mice were more active, explored novel areas of the apparatus and showed reduced grooming activity compared to uninfected animals. It was noted that some infected mice walked in circles while bending their head, leading to the suggestion that this may facilitate their capture by the cat host and transmission of the parasite (Webster, 2001). Similar observations were reported in studies of rats although these were not so pronounced, perhaps reflecting the generally reduced parasite loads in the brains

of these rodents (Webster, 2001). More sophisticated studies carried out recently on naturally infected and uninfected wild and wild/laboratory hybrid rats housed in semi-natural outdoor conditions, demonstrated that *T. gondii* infection reduces the natural aversion of rats and mice to cat odour, and even to attract the rats to the odour (Berdoy et al., 2000). Furthermore, this alteration was highly specific and not due to destruction of the olfactory regions of the brain, as neophobia towards food of novel scent remained unaltered (Webster et al., 1994). The innate fear normally exhibited towards cat urine was ablated or in some cases reversed so that *T. gondii*-infected rodents were attracted to the feline odour. This would serve to allow an increased exposure to feline attack and therefore an increased opportunity for *T. gondii* sexual reproduction in the definitive host (Berdoy et al., 2000). No disturbance to the innate preference of the rodents to their own odour or that of rabbits was observed implying that the difference in odour perception may relate to impaired processing of emotionally relevant stimuli rather than a general olfactory impairment (Berdoy et al., 2000). Recently, it has been demonstrated that the mechanisms to create this ablation of the rodent's natural fear towards the odour of the cat it is regulated by interfering with the neuronal network controlling sexual behaviour in the rodents (House et al., 2011).

Further evidence supporting the ability of *T. gondii* to modulate behaviour is shown by the ability of the antipsychotic drug, haloperidol, to inhibit growth of the tachyzoite form of *T. gondii* in vitro (Jones-Brando et al., 2003) and to reduce the altered neophobic behavioural response of rodents to cat odour (Webster et al., 2006).

1.6.2 Human behaviour

The ability of *T. gondii* to alter human behaviours and personality has been investigated in recent years. Examination of 3 separate cohorts of subjects from the Czech Republic by Cloninger's TCI (Temperament and Character Inventory) revealed that irrespective of gender, people infected with *T. gondii* had reduced novelty seeking (Flegr et al., 2000, 2003; Novotná et al., 2005; Skallová et al., 2005; Flegr, 2007; Hodková et al., 2007). Nine out of 11 tests using various subject groups found gender differences between some of the factors assessed by Raymond Catell's 16 Personality Factors (16PF) questionnaire. Men infected with *T. gondii* were generally found to have lower rule consciousness, but higher vigilance than uninfected males, whereas infected women had higher warmth and rule consciousness compared with uninfected women. Apprehension was increased in all infected subjects independent of gender. It is hard to determine if the observed correlations are due to infection or if certain personality types are more likely to become infected with *T. gondii* (Flegr et al., 2000, 2003; Novotná et al., 2005; Skallová et al., 2005; Flegr, 2007; Hodková et al., 2007).

T. gondii infection has also been associated with a modest negative effect on reaction times, which may account for the observation from 2 independent studies that *T. gondii* infected individuals are more likely to be involved in road traffic accidents (RTAs) (Flegr et al., 2000; Yereli et al., 2006). However, causation criteria in the clinical field are not easy to achieve and these associations may be due to other factors including the possibility that some personality types are more likely to be involved in RTAs and to be infected with *T. gondii.*

1.6.2.1 Schizophrenia

Several studies have demonstrated that schizophrenic patients have an increased incidence of *T. gondii* infection compared with control volunteers (Brown et al., 2005; Wang et al., 2006; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007). Importantly, a recent meta-analysis of 23 studies found an increased prevalence of *T. gondii* antibodies in patients with schizophrenia. Whilst the odds ratio of 2.73 is modest it exceeds that for other environmental and genetic factors measured to date. This suggests that *T. gondii* infection is associated with a large numbers of cases of schizophrenia (Hinze-Selch et al., 2007).

The reasons why only a proportion of individuals that have been infected with *T. gondii* develop schizophrenia are unclear. Possibilities include differences in genetic susceptibility, mode of infection (tissue or oocysts), and/or timing of infection (*in utero*, childhood or adulthood). Alternatively, it has been suggested that behavioural traits associated with schizophrenia could result in increased infection with the parasite. For example, the transmission of the parasite could be related to a lack of personal hygiene, which is a characteristic of schizophrenic patients (Wang et al., 2006). However, this is unlikely to explain all cases and since *T. gondii* encysts in the brain there is a clear potential to affect neuronal function directly. Moreover, evidence is accumulating for immune-mediated monoaminergic and glutamatergic interactions that could contribute to psychiatric disorders including schizophrenia (Dunn, 2006; Müller and Schwarz, 2007). Dysregulation of these neurotransmitter systems have long been recognised to be important in behavioural deficits associated with schizophrenia. In addition, Hinze-Selch et al. (2007) report an abnormal

immune response to *T. gondii* in patients with schizophrenia, which could contribute to the increased vulnerability of this patient group (Hinze-Selch et al., 2007).

1.6.2.1.1 Depression and other neuropsychological illnesses

There is emerging evidence that *T. gondii* infection can cause depression in certain individuals. For example, the incidence of *T. gondii* infection as determined by serology was greater in depressed patients compared with a control group (Delgado García and Rodríguez Perdomo, 1980). Furthermore, in a reported case, a patient was unresponsive to conventional antidepressants, but after treatment for an underlying *T. gondii* infection the depression was ameliorated (Kar and Misra, 2004). Clearly more studies are required in this area to determine if there is a causal link between *T. gondii* infection and depressive illnesses. The general possibility that parasitic infections may contribute to depression also merits study.

More recently, *T. gondii* infection has also been linked through epidemiological studies to neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Celik et al., 2010; Miman et al., 2010; Kusbeci et al., 2011). In particular, it is hypothesised that chronic infection in the brain caused by *T. gondii* promotes neuronal death and therefore contributes to the development of neurodegenerative diseases.

1.7 Potential mechanisms whereby *T. gondii* might interfere with neural function

Three possible avenues are under investigation to understand the mechanisms of how *T. gondii* modifies behaviour in the infected host. Firstly, a direct mechanism of

action where the parasite alters behaviour in the infected host, through the release of proteins and molecules in the brain is under consideration (Prandovszky et al., 2011). Secondly, it is possible that *T. gondii* acts indirectly through the immune response. As previously described, *T. gondii* infection develops a unique immune response that can participate in altering the physiological interactions throughout the host. There is also now evidence that certain cytokines can influence mood and that some drugs used to treat mood disorders also affect immunological parameters (Jones-Brando et al., 2003; Webster et al., 2006). However, it is not yet clear whether the role of the immune system is part of the pathology of the behavioural disorder or a cause. Lastly, congenital transmission of *T. gondii* might mediate effects as part of the 'neurodevelopment hypothesis' that has been proposed for the development of schizophrenia.

1.7.1 The direct action of *T. gondii* in the brain

In mice, *T. gondii* has been demonstrated to predominantly infect neurons (Jones-Brando et al., 2003). Consequently, infection could directly affect neuronal function and thus explain neuropsychological deficits. Cysts are assumed to be present in the brain for the life of infected humans, although rarely reported at routine post-mortem. As encephalitis is the normal disease manifestation during reactivation of *T. gondii* infection in immunocompromised individuals, significant numbers of cysts are likely to be present in this tissue. Studies in certain strains of mice indicate that cyst number can decline with time suggesting that individual cysts have a limited life-span (Brown and McLeod, 1990; Hunter, Roberts, and Alexander, 1992; Blackwell et al., 1993; Villard et al., 1995; Gazzinelli, Amichay, et al., 1996; Gazzinelli,

Wysocka, et al., 1996; Roberts et al., 1996; Suzuki et al., 1996; Alexander and Hunter, 1998; Nickdel et al., 2004). This would suggest that cysts are perpetually turning over in the brain and causing neuronal cell death that could ultimately result in neurological impairment.

Neurochemical changes have also been demonstrated in mice with *T. gondii* infection. During acute infection a 40% rise in homovanillic acid levels and a reduction in noradrenaline levels as compared with controls has been reported. Dopamine levels were unchanged during acute infection but were increased in the mice with chronic *T. gondii* infections and serotonin and 5-HIAA levels were not altered (Stibbs, 1985). Whether these changes were directly attributed to *T. gondii* infection in the cells of the brain or as a consequence of complex neuroimmunoendocrinological interactions is not known. However, the *T. gondii* genome is known to contain two aromatic amino acid hydroxylases (GenBank ACB99413 and ACB99414), specifically for phenylalanine and tyrosine, which if found to be released into the infected host could directly affect dopamine and/or serotonin biosynthesis (Gaskell et al., 2009). Furthermore, the *T. gondii* genome contains a protein homologous to human 14-3-3 (Assossou et al., 2004), which has phosphorylation activity when combined with other molecules and appears to be involved in the regulation of tyrosine hydroxylase activity. Finally, the macrophage migratory inhibition factor (MIF) has also been identified in *T. gondii* (Sommerville *et al.*, in prep). This protein has been shown to have different enzymatic activities. In particular, its tautomerase activity could deprive dopamine substrate, L-DOPA,

towards the production of melanin and therefore decreasing the neurotransmitter formation and have a further effect on the neurochemical environment (Figure 1.2).

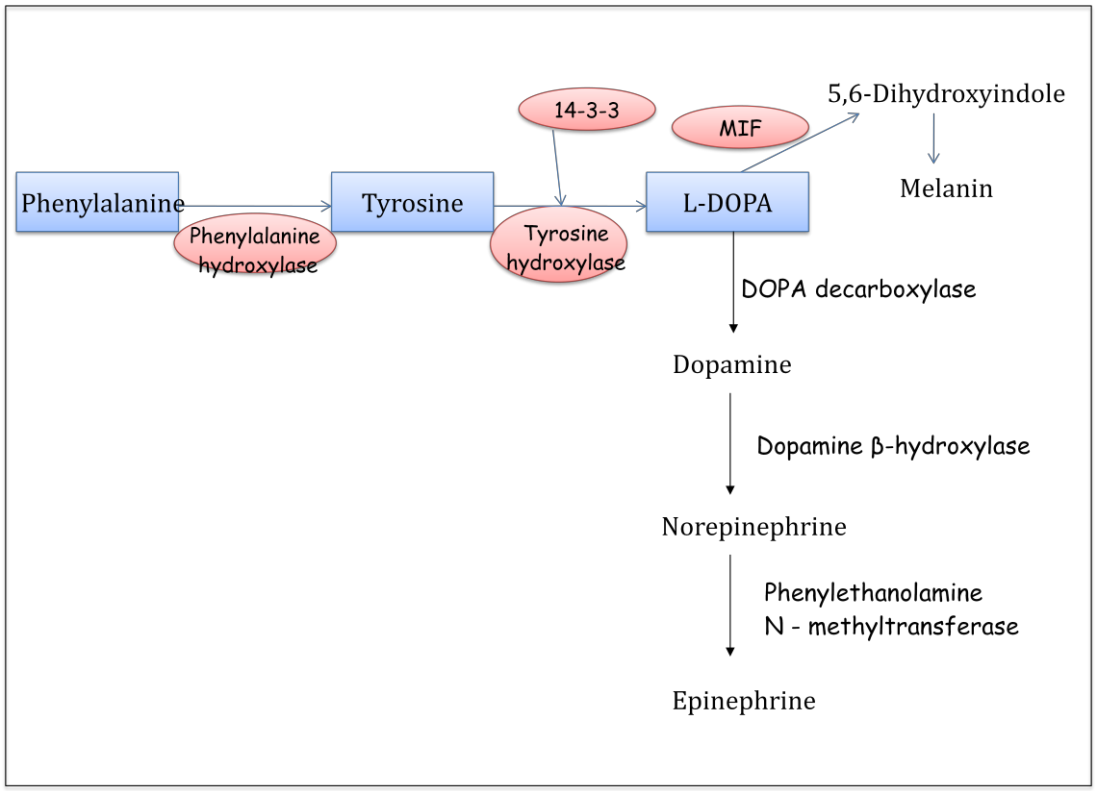


Figure 1.2: Illustration of the potential involvement of *T. gondii* secretory molecules in the dopamine synthesis pathway. In red are illustrated the enzymes that are also present in the *T. gondii* genome and at what stage they could interfere with the dopamine pathway.

1.7.2 The Indirect modulation of *T. gondii* of the host brain physiology

1.7.2.1 The immune-nervous system interaction

The close relationship and interaction between the immune system and the nervous system has been described in many settings such as through the investigation of several neurodegenerative diseases such as, Alzheimer's disease and Parkinson's disease (Celik et al., 2010; Miman et al., 2010; Kusbeci et al., 2011). In addition, through the study of processes such as 'sickness behaviour'(Damm et al., 2012), there is a significant correlation between psychiatric symptoms (in particular major depression) and inflammatory disorders, such as cancer, rheumatoid arthritis, infectious diseases, autoimmune diseases and cardiovascular (Evans et al., 2005). It is now known that the immune system and nervous system share several mechanisms and communication systems. A bidirectional communication from the early stage of development till further on in life has been described (McAllister and van de Water, 2009), hypothesising a common ancestral development for the two systems and cooperation between several functions (Ottaviani et al., 1991).

Cytokines can be produced and released in the brain by astrocytes, microglia and neurons often as a result of stimulation by peripheral cytokines, which enter the brain through the blood brain barrier by activated transport or in response to nervous processes such as stress (Müller and Ackenheil, 1998). During stress, the neurotransmitter noradrenalin is upregulated and it stimulates the release of IL-6 in the brain to implement the action of the hypothalamus pituitary axis (HPA) during this homeostatic mechanism (Norris and Benveniste, 1993). Other neural mechanisms have been demonstrated to function through the vagus nerve from the

abdominal organs directly to the brain (Wieczorek et al., 2005). Specifically, macrophages and dendritic cells in the perineural sheath of the vagus nerve have been demonstrated to respond to LPS through production of IL-1 that stimulates sensory activity on IL-1 receptors expressed by the nerve (Konsman et al., 2002). Surgical procedures that serve the vagus nerve are sufficient to prevent brainstem, hypothalamus and limbic activation following peripheral LPS or IL-1 administration (Wieczorek et al., 2005).

Soluble mediators produced in peripheral tissues can also convey immunological signals to the brain. IL-1 produced in the periphery stimulates the endothelial cells that make up the cerebral blood vessels to induce PGE2 that acts on the hypthothalamic area of the brain, stimulating the HPA axis (Tilders et al., 1994). Inhibitors of cyclo-oxygenase 2, (part of the PGE2 synthesis pathway) such as Celecoxib have been demonstrated to have beneficial effects for the treatment of major depression (Müller et al., 2006). A number of cytokines are known to act directly on the HPA axis and these include IL-1β, IL-6 and TNF-α (Turnbull and Rivier, 1995) all of which are produced in abundance during *T. gondii* infection (Hunter, Roberts, Murray, et al., 1992; Müller et al., 2006) (Figure 1.3).

Cytokines in the brain have an effect on the dopaminergic, noradrenergic and serotonergic pathways (Müller and Ackenheil, 1998). Neurons have been characterised with cytokines receptors and several in vitro studies have shown that at physiological levels IL-1, IL-2 and IL-6 increase the release of dopamine in a dose dependent manner (Zalcman et al., 1994).

Clinically, patients affected with viral infections undergoing therapy with IFN-α have lower levels of serotonin, dopamine and tetrahydrobiopterin (BH4), a co-factor of tyrosine hydroxylase, the limiting step towards dopamine production, and of nitric oxide synthase (Kitagami et al., 2003).

Conversely, neurotransmitters have an effect on the cytokines production as mentioned during the stress response (Norris and Benveniste, 1993). Furthermore, high levels of group of cytokines, in particular IL-1β and IL-6, have been measured in CSF samples taken from psychiatric patients before treatment and also the same pattern was found in post mortem brain tissue of psychiatric patients (Smith et al., 2007). In particular, in the blood and CSF patients with Major depression during sickness and healthy stages of the illness, increased levels of TNF-α, IL-6 and C reactive protein (CRP) were found (Zorrilla et al., 2001; Dowlati et al., 2010).

1.7.2.2 *T. gondii* and immune imbalance in the brain

As described above, *T. gondii* infection in peripheral tissues may contribute to immunologically mediated events in the brain during *T. gondii* infection. However, as *T. gondii* is also present within the brain it can also directly induce the production of immunological mediators from within this site during the reactivation of the tissue cysts during chronic infection (Hermes et al., 2008). This exposure to the immune challenge in the brain could lead to a chronic condition of an immune-neurochemical imbalance in the brain environment.

Early studies demonstrated that a plethora of immunological transcripts are induced in the brains of infected mice (Hunter, Roberts, and Alexander, 1992; Hunter, Roberts, Murray, et al., 1992; Burke et al., 1993). Notably, a number of these mediators including IL-1β, IL-6 and TNF-α are able to stimulate PGE2 or act directly on the hypothalamus. Although stimulation of the HPA axis has been linked to behavioural changes including depression, no mechanism has been definitively demonstrated (Konsman et al., 2002). It is indeed possible that the activation of the HPA axis may be indicative of immunological activity affecting the brain, but not be the cause of behavioural changes. However end products of the HPA axis are glucocorticoids. Glucocorticoids not only feed back to limit HPA stimulation, but also affect a number of other systems (Konsman et al., 2002). Notably, glucocorticoids have profound effects on the immune system including macrophages, dendritic cells and T cells (Konsman et al., 2002; Belvisi, 2004; Martinez et al., 2008). Glucocorticoid antagonists such as RU486 and ketoconazole have been demonstrated to function as antidepressants in certain individuals (Gallagher et al., 2008). In addition glucocorticoids affect tryptophan metabolism through increasing the levels of the tryptophan 2, 3, dioxgenase (TDO) in the liver and IFN-γ induced indoleamine 2,3-dioxygenase (IDO) in immune cells (Comings et al., 1995; Türck et al., 2005). Therefore tryptophan metabolism, the HPA axis and the immune system are inextricably linked (Figure 1.3).

T. gondii induces a very strong type 1 response, with the release of pro inflammatory cytokines such as IFNγ, which not only actively controls the infection (Konsman et al., 2002), but also promotes the development of tachyzoites into bradyzoites (Yano

et al., 2002). IFN-γ has also direct consequences on the chemical brain environment by inducing the tryptophan degradation pathway. IFN-γ, via inflammatory signals STAT1a, IRF-1, NF-κβ, p38 MAPK (Fujigaki et al., 2006), upregulates the activity of Indolamine 2, 3 Dioxygenase (IDO), the enzyme involved in the first step in the tryptophan degradation pathway (Schwarcz and Pellicciari, 2002; Pfaff et al., 2008) (Figure 1.3). This leads to several consequences: the down regulation of the inflammatory response during infection by limiting the production of T cells, the regulation of intracellular parasite multiplication by limiting the availability of host tryptophan, the consequential depravation of the substrate for serotonin (5-HT) formation, an important neurotransmitter, and the production of kynurenine metabolites, which are neurotoxic. The importance of tryptophan and its degradation pathway in the brain and behavioural aspects has also been described clinically. In fact, a vulnerable population treated with acute dietary depletion of tryptophan have been characterised with depressive symptoms (Delgado et al., 1994). Administration of LPS and in which the IDO enzyme activity was inhibited did not exhibit the depressive-like symptoms that other studies of LPS administration have shown (O'Connor, Lawson, André, Briley, et al., 2009; O'Connor, Lawson, André, Moreau, et al., 2009).

Therefore, *T. gondii* infection can modulate the interactions between the immune system and nervous system via several routes resulting in a most probable immune-neurochemical imbalance in the brain. The molecules involved in this interaction between *T. gondii*, the immune system and nervous system are going to be described in detail below.

Serotonin

Serotonin or 5-HT (5-hydroxytryptamine) is a neurotransmitter synthesized by the serotonergic neurons in the central nervous system (Mohammad-Zadeh et al., 2008). It has been suggested that it is an important regulatory factor for various behavioural and physiological functions such as aggression, mood, sexuality, vomiting, appetite, body temperature and sleep. Serotonin is synthesized mainly in the gastrointestinal tract (Robert S. Feldman (Robert Simion), 1997) and tryptophan hydroxylase (TPH) is central to its production (Figure 1.3). This enzyme has two forms: TPH is present in different tissues of the body, in particular in the intestines, and TPH2 is the specific isoform present in the brain (Zhang et al., 2004). TPH is regulated by the concentration of the substrate, tryptophan (Maes et al., 1993). Therefore, the level of tryptophan in the brain is the major component responsible for serotonin production. TPH activity has also been associated with the interaction of ovarian hormones, explaining the possible role of serotonin in the mechanism of post partum depression and pre menstrual stress syndrome (Mohammad-Zadeh et al., 2008). Serotonin is also up regulated by electrical stimulation and down regulated by high levels of serotonin receptors (Mohammad-Zadeh et al., 2008). In vivo studies have reported that acute exposure to cytokines and cytokines inducers, such as LPS also have an effect on the turnover of serotonin activity in different brain regions (Dunn and Wang, 1995; Dunn et al., 1999; Anisman et al., 2005). Patients treated with interferon in order to eradicate viral infections often show symptoms associated with depression. These symptoms are diminished when these patients are also treated with antidepressants that block the serotonin reuptake, such as paroxetine (Musselman et al., 2001; Raison et al., 2007). These studies clearly show the close relationship

between cytokines and levels of serotonin in the brain. Serotonin levels have also been associated with other disorders, in particular low levels of serotonin are found in relation with aggression, depression, obsessive-compulsive disorder, anxiety bipolar disorder, migraine and irritable bowel syndrome (Mohammad-Zadeh et al., 2008).

Quinolinic acid and kynurenic acid

The products of the kynurenine (KYN) pathway can influence neural activity. Also KYN itself has an effect on behaviour (Wichers et al., 2005). In fact, the administration of KYN has been shown to produce symptoms-like schizophrenic behaviour in rodents (O'Connor, Lawson, André, Moreau, et al., 2009). The products of KYN are quinolinic acid (QUIN) and kynurenic acid (KA). QUIN is mainly produced in microglia cells. It activates the NMDA (N-methyl-D-aspartic acid) receptor and upregulates the consequential release of glutamate, promoting the increase of the CNS excitotoxicity effect (Schwarcz and Pellicciari, 2002).

Kynurenic acid (KA) is mainly produced in astrocytes (Kiss et al., 2003). It inhibits the release of glutamate and therefore it participates in the regulation of the release of dopamine (Borland and Michael, 2004). In fact, a study by Wu et al. (2007) demonstrates that the administration of KA reduces the levels of dopamine in the striatum of the rat brain, indicating a clear involvement of KA in the regulation of the dopamine neurotransmitter. In addition, high levels of KA have been measured in schizophrenic patients (Schwarcz et al., 2001) and rodents treated with KA have

impaired sensory gating mechanism (Erhardt et al., 2004), a condition also present in schizophrenic patients (Mansbach and Geyer, 1991).

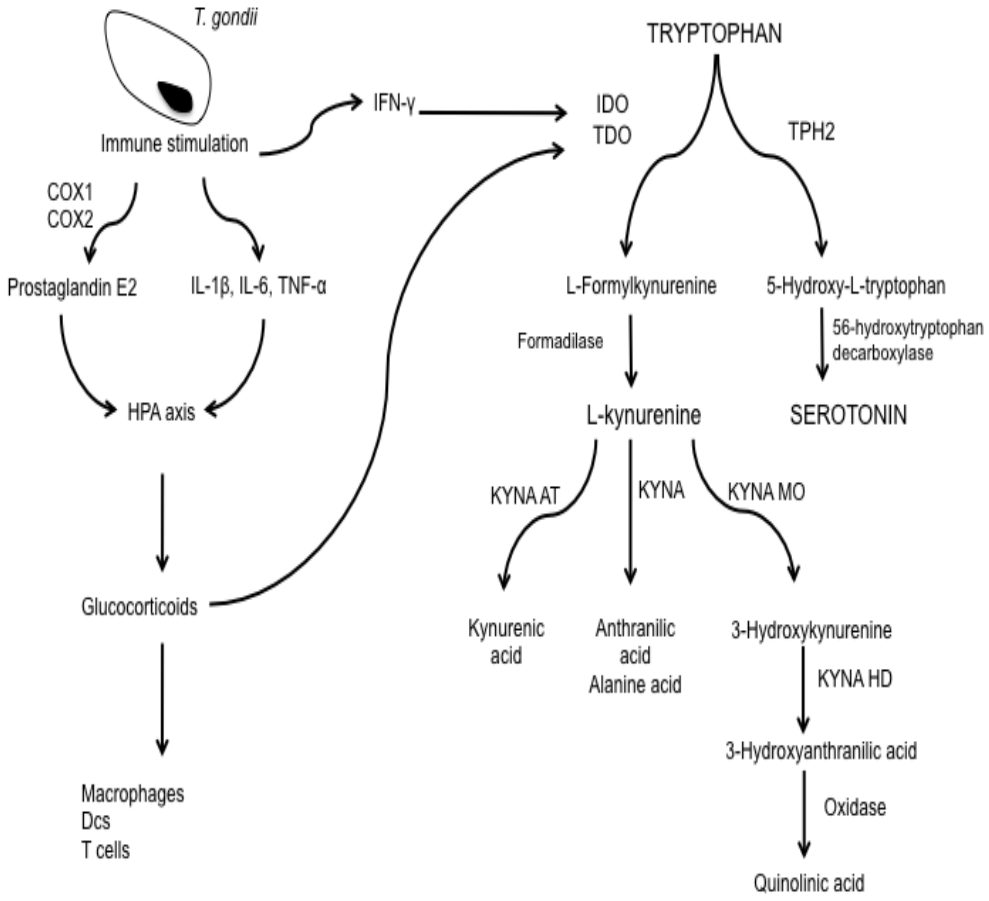


Figure 1.3: Release of cytokines induced by *T. gondii* and their involvement in the tryptophan degradation pathway, which results in the production of kynurenine metabolites. These metabolites are found to have neurotoxic effects and are present at high levels in blood and cerebral spinal fluid samples of psychiatric patients.

1.7.3 The influence of *T. gondii* on Neurodevelopment

1.7.3.1 Vertical transmission

Transmission of infection can also occur congenitally. Tachyzoites are able to infect the foetus by crossing the placenta (Abbasi et al., 2003). In humans, this happens usually only when the mother is infected by *T. gondii* for the first time during pregnancy although exceptions occur where chronically infected women have transmitted infection vertically (Elbez-Rubinstein et al., 2009). Congenital transmission results in the manifestation of congenital toxoplasmosis and a high risk of foetal abortion (Rorman et al., 2006).

The immune response has a major role during pregnancy. A healthy pregnancy is characterised by the inhibition of nitric oxide (NO), TNF-α, IL-6 and the up regulation of IL-10 in macrophages in proximity to the foetus. During pregnancy a down regulation of the inflammatory response occurs, which is regulated by steroid hormones (Mor and Cardenas, 2010). Progesterone also promotes the activation of NK cells by stimulating the release of PIBF (progesterone induced blocking factor) from lymphocytes and the production of IL-4 from Th1 cells (Mor and Cardenas, 2010). The presence of a type 2 immune response contributes to a positive ending of pregnancy. Exposure to *T. gondii* infection for the first time during pregnancy could disrupt any of these regulatory mechanisms of the immune response and result in foetus abortion (Ortiz-Alegría et al., 2010). A second possible consequence of infection during pregnancy is the transmission of the parasite from the mother to the foetus resulting in foetal abnormalities such as hydrocephalus, intracranial calcifications and retinochoroiditis (Ortiz-Alegría et al., 2010). Approximately 20-

33% of acute infection in pregnant mothers results in parasite transmission to the foetus. Different factors influence the outcome of congenital infection, including the immune status of the mother, the number of parasites and the virulence of the parasite strain and the timing of infection during gestation. In fact, during the first trimester there is a risk rate lower than 6% for the parasite to be transmitted to the foetus, during the second trimester there is a chance between 20-40% for the parasite transmission to the foetus an at the third trimester of pregnancy there is a higher risk, between 58-72%, of transmission of the parasite from mother to foetus (Ortiz-Alegría et al., 2010). However, the risk of abortion is increased when infection occurs during the first trimester. Disease is more severe in the foetus when the parasite is transmitted during the early stages of gestation (Ortiz-Alegría et al., 2010). There are several possible routes of transmission from mother to foetus. The most probable mode is for *T. gondii* to infect the foetus via the haematogenous transplacental path (Carlier et al., 2012). First of all, the parasites traverse the trophoblastic barrier, the first line of protection for the foetus, to infect the mesenchymal tissues and finally invade the foetal vessels. In vitro studies show how *T. gondii* develops into the bradyzoite form to cross the trophoblast layer to then transform into tachyzoites to subsequently invade the following layers of the placenta and the foetus (Abbasi et al., 2003; Jack S. Remington, 2006; Kurt Benirschke, 2006). Another possible route for parasite congenital transmission is via breaches and tears that appear on the placenta as a natural progress towards the end of the pregnancy or as a result of the mother's inflammatory response towards infection (Kurt Benirschke, 2006). It has also been reported that infection can occur via the amniotic fluid by a placental infection derived from the amniotic membranes

(Carlier et al., 2012). However, the mechanisms of this route of transmission are still unclear. Once the parasite invasion occurs, the placenta reacts with an innate immune response (Abrahams and Mor, 2005; Koga and Mor, 2010). Microbes can be detected by the TLRs expressed on trophoblasts, syncytiotrophoblasts, fibroblasts, hofbauer phagocytic cells and dendritic cells in the placenta. TLR-2 and TLR-4 recognise *T. gondii* infection resulting in overexpression of TLRs and stimulation of systemic maternal inflammatory responses, with the release of cytokines, chemokines, reactive oxygen and nitrogen species and upregulation of IDO. Trophoblastic FcRn (neonatal Fc receptor) is stimulated to produce maternal antibodies to be released in the foetus for further protection against pathogens (Egan et al., 2009). The outcome of this immune response is not usually successful in protecting transmission of *T.gondii* infection. In contrast, in other congenitally transmitted diseases, such as *T. cruzi* congenital infection, the immunological responses involved as described above are usually beneficial to overcome foetal infection (Altemani et al., 2000).

Generally in *T. gondii* infection, placenta inflammation has negative consequences on foetal damage and it increases the chances of parasite infection by the development of abrasions on the placenta wall from where the parasites have easier access to the foetus (Garcia-Lloret et al., 2000). Placentas are also known to decrease the actual antibody exchange from mother to foetus (Cumberland et al., 2007). As previously mentioned, the genotype of the parasite influences the outcome of congenital transmission. *T. gondii* Type II genotype is the most frequent cause in Europe and North America of adult acquired and congenital infection, while I and III

genotypes and new forms of recombinant genotypes, I/II and I/III are more frequent in Africa and South America (Fuentes et al., 2001; Ajzenberg et al., 2002; Nowakowska et al., 2006; Peyron et al., 2006; Boughattas et al., 2010; Kieffer et al., 2011). Occurrence of congenital transmission in subsequent pregnancies is rare. However, reinfection with other parasitic genotypes during pregnancy could explain the occurrence of *T. gondii* congenital transmission in chronically infected mothers (Elbez-Rubinstein et al., 2009). Also, the parasite burden during the acute phase of infection can favour transmission to the foetus.

Although not yet fully developed, the foetus' immune response is also an important variable on the outcome of infection (Carlier et al., 2012). Between 85-90% of congenital transmitted disease is asymptomatic at birth and symptoms usually can develop a few days after birth. Symptoms include low birth weight, premature birth, fever, hepatosplenomegaly, jaundice, pneumonitis (Freeman et al., 2005; Remington, 2006). Growth retardation is both the result of the multisystemic diffusion of the parasite in the foetus and of placenta inflammation during gestation (Carlier et al., 2012). Later effects of *T. gondii* on the development of the neonatal infant include meningoencephalitis, convulsions, brain calcifications, hydrocephaly that could lead to psychomotor retardation and or the development of choroidorentinitis (Remington, 2006). A long-term consequence of *T. gondii* congenital transmission is the imprinting of the foetal immune system that could lead to the development of hypersensitivity reactions to other infectious agents or vaccines in later life (Carlier et al., 2012). In addition, the effect of infectious agents on the developing foetus is considered an environmental risk factor and a neurodevelopmental model in the pathogenesis of psychiatric diseases in adulthood (Pearce, 2001).

1.7.3.2 Neurodevelopmental hypothesis

Epidemiological and experimental studies have described an association between general infection during pregnancy and the development of schizophrenia in adulthood (Buka et al., 2001; Wen et al., 2003; Brown et al., 2005; Wang et al., 2006; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, Torrey, et al., 2007; Xiao et al., 2009; Pedersen et al., 2011). Many *in vivo* studies carried out in different laboratories are aimed at elucidating the mechanisms involved in brain development, the damage caused by congenital infection and the possible behavioural, morphological and chemical consequences in the offspring (Fatemi et al., 1999; Shi et al., 2003). Despite, following different protocols in different laboratories similar observations and conclusions have been made. These are going to be described below. However, the understanding of the possible mechanisms that are involved during neurodevelopment and the consequences of an infection during pregnancy are still not clear.

Bacterial and viral congenital infections have been mimicked through the administration of LPS and poly: IC to pregnant dams via different modes of delivery such as intraperitoneal, subcutaneous, intravenous or intranasal at different time points of gestation. Immunologically, an increase of the production of IL-1, IL-6 and TNF-α cytokines in the mother has been observed through TLR-4 or TLR-3 ligation according to the type of infection (Patterson, 2009). These cytokines could lead directly or indirectly to brain dysfunction of the foetus through an increase of

glucocorticoid, production fever, anorexia and decreased levels of zinc (Brown and Derkits, 2010). Additionally, TNF-α can directly cause placenta dysfunction increasing the possible damage to foetal brain development (Figure 1.4). Morphological characteristics have also been examined and a decrease through cellular death in the white matter and a decrease in the myelin basic protein have been observed in the brains of offspring affected by congenital transmission (Brown and Derkits, 2010). Neurochemical measurements have shown an alteration in neurotransmitter levels in the brain of the congenitally infected offspring such as, dopamine levels, a decrease in serotonin, increase of oxidative stress, increase of cytokine levels and an increase of glial fibrillary acidic protein (Krystal et al., 1999; Coyle et al., 2003). Behavioural studies have also been carried out in the congenital infected offspring at different time points after birth. A deficit in the pre pulse inhibition, a behavioural aspect also observed in schizophrenic patients and deficits in the spacial learning and the novel object recognition test have been observed (Brown and Derkits, 2010). Additionally, an amphetamine-induced locomotor-like behaviour has been observed in offspring treated by Poly IC during pregnancy (Shi et al., 2003).

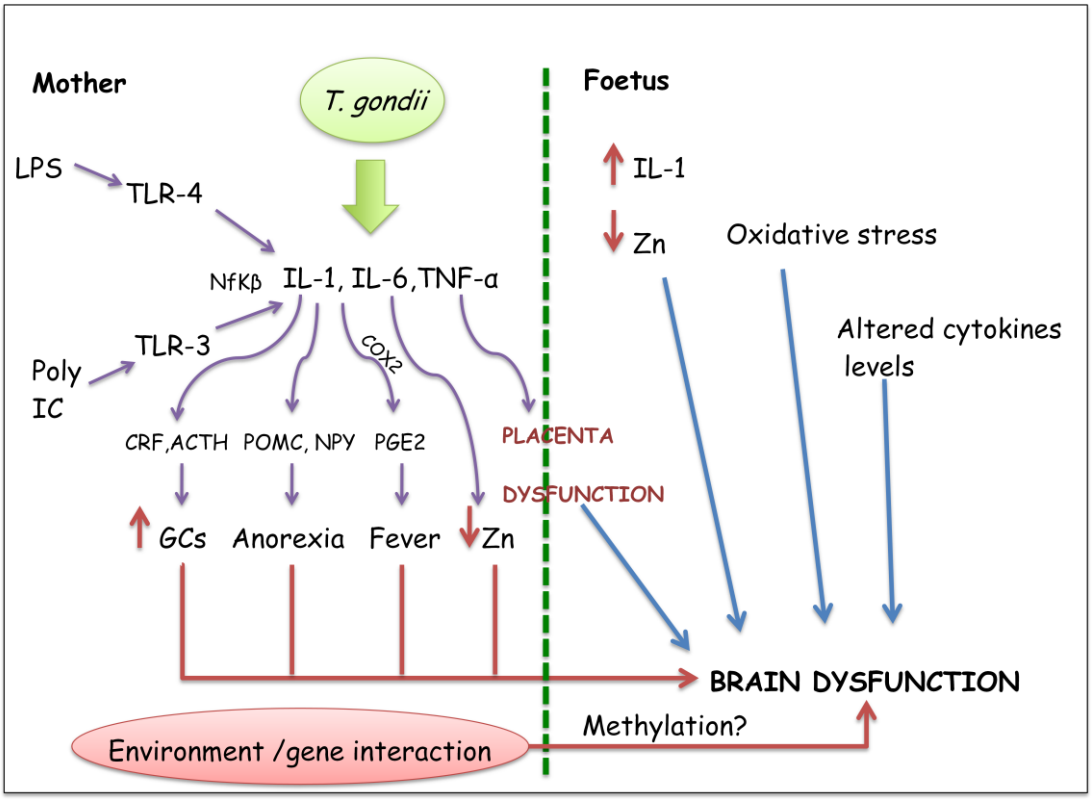


Figure 1.4: Diagram of cytokines released during congenital infection and their impact on the brain. Cytokines have an impact on the body's physiology. In particular, during pregnancy the immune system is involved to maintain and guarantee a successful pregnancy. An imbalance created by, for example infection, can alter this process. This could lead to several consequences, from abortion to creating a negative environment for the development of the foetus and incrementing the risks of brain pathology in adult life.

As previously mentioned, one of the outcomes of *T. gondii* congenital transmission is chronically infected offspring. Therefore, not only the interaction between the maternal immune response during infection and the central nervous system in the neurodevelopment processes is involved as a potential consequence for the development of psychiatric disorders. Moreover, all the mechanisms described

previously during acquired chronic infection in adulthood might be involved and should be taken into consideration in this model.

1.8 Treatment

Treatment of toxoplasmosis most frequently involves the use of a combination of sulphonamides and pyrimethamine, which inhibits the multiplication of tachyzoites, but has no effect on bradyzoites (Fung and Kirschenbaum, 1996). These drugs act synergistically interfering with the biosynthesis of folic acid in the parasite (Fung and Kirschenbaum, 1996). Atovaquone can also be used in combination with pyrimethamine (Pearson et al., 1999). This drug also inhibits bradyzoite viability and reduces the number of cysts in the brain as it acts on mitochondrial respiration with high affinity for the parasite's organelles (Pearson et al., 1999). Spiramycin is normally used to treat women with acute toxoplasmosis during pregnancy (Montoya and Remington, 2008). There are currently still no vaccines available against *T. gondii* infection in humans, although there is one developed for veterinary use called Toxovax (made by Intervet). Hygiene and cooking habits remain the best form of prevention. Some countries, for example Italy, have compulsory screening for *T. gondii* seropositivity as a routine test in pregnant women in order to limit the consequences of a possible congenital transmission occurring. In addition, in spite of gaining evidence that *T. gondii* induces behaviour changes in the infected host there is still no treatment to eradicate *T.gondii* infection. Clinical evidence has demonstrated that depression therapies are only successful on patients once the *T. gondii* infection was treated (Kar and Misra, 2004) but still no common practice has been yet outlined.

1.9 Aims and objectives

Understanding the interactions between the immune system and nervous system of the host is likely to be important in defining the mechanisms responsible for parasite manipulation of the host behaviour. Many behavioural consequences could be also explained as a different aspect of the hosts' sickness behaviour. Recently, mental illness has been associated with infection and it is also known that different types of immune responses can promote different behavioural outcomes. Therefore increasing the understanding of the mechanisms involved will contribute to the development of more specific treatments. In addition, the study of the behavioural outcome of the interaction between parasite and host could be beneficial for a psychiatrist to understand the evolution and the interconnection of the brain. Moreover, social behaviour such as aggression, reproductive and parental behaviours, modified by parasite infection through affecting the limbic region of the brain is present in many classes of vertebrates (Klein, 2003) thus demonstrating one of the phylogenetically primitive structures of the vertebrate brain.

Promoting the study and the discussion of the interaction between parasite and its host between parasitologists, immunologists and neuroscientists with a critical and analytical approach could be beneficial in understanding the mechanisms of regulation for each system and how the global interaction of each system contribute to the biology of the living organisms.

The overview of the literature regarding *T. gondii* infection and the possible behavioural consequences in the infected host has raised many questions that have to be addressed.

The aim of the present study is to evaluate the ability of *T. gondii* to modify behaviour in the infected host during chronic infection in BALB/c mice. General locomotor activity, anxiety levels and pre-pulse inhibition of the startle response in infected animals was measured and compared to uninfected controls. The effects of different timing of infection were also assessed. The first part of this study evaluated the possible behavioural changes in adult mice infected during adulthood. This way the parasite was examined as a sole risk factor involved in the possible behavioural changes in the host without the additional age susceptibility to the infection. The second part of this study assessed behaviour of adult mice affected by *T. gondii* congenital transmission and adult mice challenged by the mother immune response during gestation by *T. gondii* infection. This allowed assessing both, the possible role of *T. gondii* congenital infection and the role of the maternal immune response activated by *T. gondii* infection, as possible environmental risk factors in the neurodevelopmental hypothesis of the pathogenesis of psychiatric disorders.

In studies of adult and congenitally acquired *T. gondii* infection described in this thesis, changes in the expression of immunological and metabolic transcripts as well as metabolites are examined. Specifically, the expression levels of the transcripts for enzymes involved in the tryptophan degradation pathway responsible for the formation of kynurenine metabolites as described in figure 1.3 were assessed by real

time PCR. In addition, gene expression of inflammatory cytokines were measured in the infected brain. Finally, mass spectrometry was employed to measure changes to metabolites and neurochemicals in *T. gondii* infected mice relative to healthy control mice.

2 *T. gondii* infection: behavioural, immunological and metabolomics consequences in the brain

2.1 Introduction

In the past 20-30 years there has been an increase of interest in the study of the relationship between infection and the nervous system. In particular, it has been hypothesised that infection could be an environmental risk factor in the development of psychiatric disorders (Patterson, 2009). There are various epidemiological data that demonstrate increased incidences of infectious agents in psychiatric patients compared with the population as whole (Arias et al., 2012). In addition to *T. gondii*, viruses such as *cytomegalovirus* or *Herpes simplex* have also been investigated and have been implicated as risk factor for psychiatric disorders (Shirts et al., 2008; Carter, 2009). As neuropsychiatric disease can be associated with behaviours that increase the risk of some infectious disease, it is difficult to demonstrate a causative relation ship. However, causative relationship between *T. gondii* and neuropsychiatric disease is supported by numerous studies that demonstrate its ability to manipulate behaviour in the experimental hosts under laboratory conditions (Hay et al., 1983; Hay, Aitken, and Graham, 1984; Hay, Aitken, Hair, et al., 1984; Webster, 1994; Webster et al., 1994; Berdoy et al., 1995b; Skallová et al., 2005; Gonzalez et al., 2007; Papini et al., 2009; Gulinello et al., 2010). Furthermore, mechanistic insights are now being gained. For example, it has been shown that the natural aversion towards the odour of cat urine is inhibited in infected rodents (Berdoy et al., 2000; Webster et al., 2006; Vyas et al., 2007; Lamberton et al., 2008). More recently it has been reported that the infected rodents are attracted to the cat's odour that stimulates an increase in the neural activity of the MEApd (postural dorsal medial amygdala), which crosses the regions of the rat brain associated with sexual excitement (amygdala and hypothalamus). In addition this area of the brain is

anatomically positioned next to the rat 'defence' mechanisms pathway mediated by the VMH (ventromedial hypothalamus) brain region (House et al., 2011). This ability to modify host behaviour exemplifies the 'parasite manipulation hypothesis', which states that parasites manipulate the host behaviour for their own survival (da Silva and Langoni, 2009; Lagrue and Poulin, 2010). The life cycle of *T. gondii* is completed only in the gut of the cat, in which the sexual cycle occurs, so reasonably to the hypothesis that *T. gondii* has evolved a mechanism to facilitate transmission. A possible link between *T. gondii* infection and behaviour in humans has also been investigated. For example, there have been psychological studies to identify similar traits in humans infected by the parasite. Generally, it has been observed reduce novelty seeking and a lower rule of consciousnesses and slower reaction times in infected individuals resulting in an increased involvement in car accidents compared to the uninfected population. Also gender differences have been observed in the infected populations studied (Lindová et al., 2006; Hodková et al., 2007). However these studies do not take into consideration the time of infection or the strain of the parasite with which they were infected (Flegr et al., 2000; Macpherson, 2005; Flegr, 2007; Arling et al., 2009; Yagmur et al., 2010; Groër et al., 2011) or other parameters such as age or intelligent quotient (IQ). Determining the mechanism(s) responsible especially in humans is a challenge. In addition to the parasite-mediated mechanisms already described whereby *T. gondii* releases aromatic amino acid hydroxylase to affect dopamine levels (Gaskell et al., 2009), other mechanisms including immunologically-mediated changes could play a role. The involvement of the immune system and its contribution to changes in neuronal mediators and function has been of great interest as this could lead to behaviour dysfunction and/or

neuropsychiatric disease (Blume et al., 2011; Capuron and Miller, 2011). This has been particularly studied in the context of the development of new drug therapies for the treatment of psychiatric disorders. For example, it has been observed that patients that undergo IFN-α treatment in hepatitis C infection develop depression-like symptoms and if treatment is administered for a long length of time, patients also manifest deficits in cognitive functions (Stasi et al., 2011).

When *T. gondii* infects a host it promotes a very strong Th1 immune response, eliciting IFN-γ (Suzuki et al., 2011). The immunological pressure also influences and triggers chronic infection, which is characterized by stage switching from the tachyzoite to the bradyzoite stage and the formation of tissue cysts (Lyons et al., 2001). Tissue cysts are long-lived and their effects on the brain are poorly understood. The production of IFN-γ has several different outcomes during the immune response as it upregulates indoleamine 2,3-dioxygenase (IDO), which controls the first step of the tryptophan degradation pathway leading to a potential array of consequences (Yasui et al., 1986). Firstly, as *T. gondii* is a tryptophan auxotroph a reduction in available tryptophan limits *T. gondii* growth (Schmitz et al., 1989). Secondly, reduced tryptophan availability limits the extent of the host inflammatory response that could otherwise have adverse consequences for the host (Logan et al., 2002). Thirdly, tryptophan degradation decreases substrate availability for the production of serotonin (5-HT), an important neurotransmitter for mood, emotional behaviour and sleep (Myint and Kim, 2003; Müller and Schwarz, 2007). Finally, tryptophan degradation results in the production of kynurenine metabolites that in high levels are known to be neurotoxic and have been linked to numerous

pathologies in the brain (Müller et al., 2011). Other metabolic pathways might also be influenced in a similar manner either due to direct parasite-mediated or indirect immunologically-mediated effects of infection. For this reason, I have utilised a variety of techniques, to assess the levels of enzyme and immunological mediator transcripts by qRT-PCR and metabolomic analyses as a tools to acquire an overview of the effects of *T. gondii* chronic infection in the brains of mice. Prior, to molecular and biochemical analysis, behavioural analysis of the infected mice versus the non-infected was conducted to determine the impact of infection upon behaviours related to anxiety and psychosis.

2.2 Material and Methods

2.2.1 Experimental plan

Behavioural baseline measurements (open field, elevated plus maze and pre-pulse inhibition (PPI)) were taken from BALB/c male mice at 6 weeks old. The mice were then divided in two groups. Both groups displayed the same baseline pattern of behaviour. One group was orally infected by gavage with 10 cysts of *T. gondii Beverley* strain 200µl of 1X PBS (Invitrogen, UK). The second group is the non-infected control group. Progress of infection was monitored daily and the weights of the mice were measured. The control animals were also weighed in order to maintain parity of stress exposure to the mice in each group and to rule out possible differences in behaviour being attributable to handling. Behavioural testing was then performed at 1 month and 2 month after infection to monitor behaviour from the beginning of the chronic phase of infection, once the mice are fully recovered from the acute phase of infection and the parasites are beginning to form tissue cysts in the brain. At each time point 5 mice per group were killed and the brain of each mouse was extracted for molecular gene analysis and metabolomics. This experimental plan was repeated three times. The first time 45 BALB/c mice were used; 24 infected and 21 controls. The second time 40 mice were used; 25 infected and 15 controls. In the third run 36 mice were used; 20 infected and 16 control. For run 3, 7 mice were sacrificed each time point in order to increase the number of samples per group for the metabolomics analyses and behaviour was performed only to measure pre-pulse inhibition in these animals. The data analysis was carried out on all the runs pulled together.

2.2.2 Animals

BALB/c mice were chosen as a model to study behaviour during *T. gondii* chronic infection due to the relatively low number of cysts that they develop following infection compared to other mouse strains. Chronic infection in the BALB/c mouse is therefore somewhat comparable to human chronic infection where the number of cysts formed in the human brain is low (Wen et al., 2010).

2.2.3 Caging

Each cage contained 5 mice with bedding, nesting material, food and water ad libitum. The room was maintained at a constant temperature and it was held on a 12 hours light and dark cycle.

2.2.4 Infection

Infection, 10 cysts of *T. gondii Beverley* strain (type II) in 200µl of PBS (Invitrogen, UK), was orally delivered by gavage. Humans are commonly infected by the parasite by the ingestion of undercooked meat contaminated by *T. gondii*.

2.2.5 Behavioural Studies

The behavioural tests were carried out on each individual mouse in a random sequence between and within the groups for baseline measurements and then at 1 and 2 months post infection. At each time point, the order of the experiments was performed from least to most stressful for the animal. Therefore the first experiment consisted of the observations of the general locomotor activity with the open field test, followed by the measure of anxiety levels by the elevated plus maze method and

last the measure of pre-pulse inhibition. Experimental testing was carried out during the 12 hour light cycle.

2.2.5.1 Open field

Open field behaviour was carried out following the protocol previously described by Holmes et al. (2002). Testing was carried out in a control room with continuous monitoring of the temperature, the light and the noise between each test to eliminate variation. The mice were placed one at a time into a 40cm x 40cm box with black persepex walls 36cm in height (Figure 2.1). Locomotor behaviour was monitored and tracked by the Ethovision 3.0 program (Noldus Information Technology, Netherlands) for 10 minutes. With this program the tracking was acquired from a digital video file (MPEG). The boxes were washed between each trial. For each animal tested a number of parameters were studied: the total distance moved in the open field, the number of the entries in the centre of the box and the duration of time spent in the centre of the box. In the open field animals tend to explore the whole arena, but typically spend less time in the more aversive central zone.

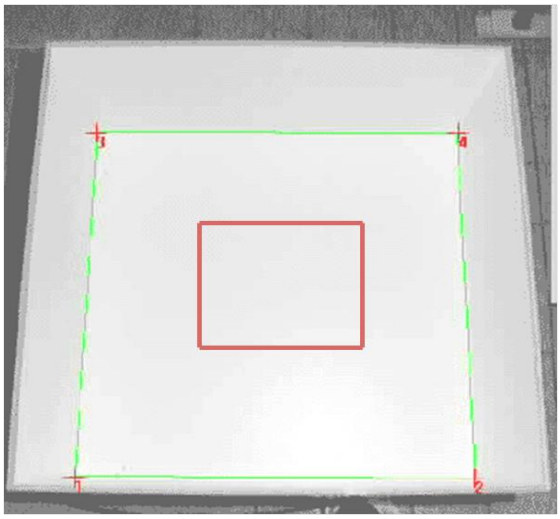


Figure 2.1. The figure illustrates the open field box (40x40x36cm). Green line is the outline perimeter, next to the wall. Red line is the perimeter of the central zone.

2.2.5.2 Elevated plus maze

The elevated plus maze is shaped as a cross, characterised by two open arms and two enclosed arms. Each arm is 30 cm long and 6 cm wide. The height of the closed arms is 16cm (Figure 2.2). Each mouse is placed in the centre of the maze for a period of 10 minutes and monitored and tracked by the Ethovision 3.0 program (Noldus Information Technology, Netherlands) as previously described by Hogg, (1996). Testing was carried out in a temperature-controlled room with continuous monitoring of light and noise to eliminate variation between each test. In particular, the intensity of the light was 20/22 lux for each open arm of the plus maze. Between each trial the plus maze was washed before testing a new animal. The anxiety level of the animals is measured by counting the number of entries and the time spent into the open arms compared to the entries into the closed arms. Anxious animals venture more often into the closed arms compared to animals that are less anxious. The elevated plus

maze is considered to be a more refined measure of anxiety compared to the open field test.

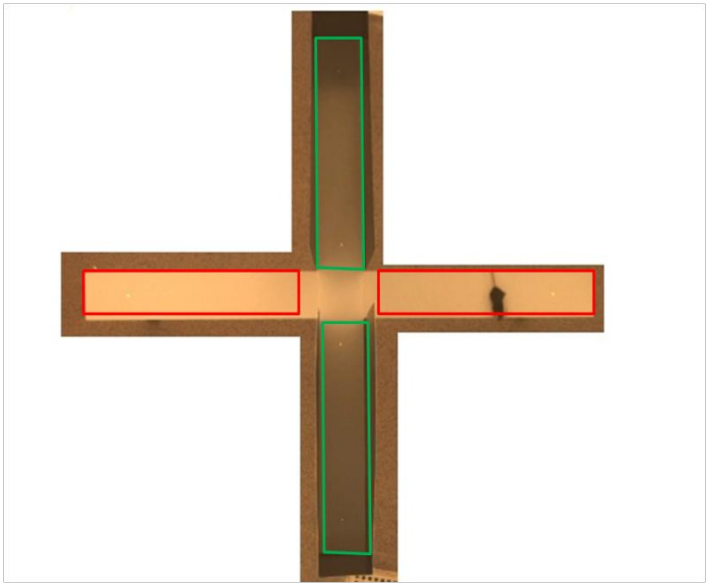


Figure 2.2. The figure illustrates the elevated plus maze (30x6cm). The red lines are the open arm perimeters; the green lines are the closed arm perimeters with walls of 16cm in height.

2.2.5.3 Pre-Pulse inhibition of the startle response.

The PPI test is considered to measure underlying sensorimotor gating deficits which can be observed in schizophrenic patients (Swerdlow et al., 1999; Geyer et al., 2002). This process is present in several species, as well as rodents. It is an inhibited reaction of the animal to a strong external impulse, such as noise, if previous to the strong impulse a weaker stimulus has been experienced. The PPI experiment tests the ability to suppress a normal response to a starting stimulus after being preceded by a weak pre-stimulus (Perry and Braff, 1994). Therefore in this study, the levels of the

inhibition of the response by the pre-pulse were taken and the two groups (infected and control) were compared.

The equipment used in these studies for the PPI acoustic startle response was that of San Diego Instruments (California, USA). In brief, the animals are placed in restrainers on movement sensitive platforms inside chambers next to a speaker. The startling stimulus of 120 db is presented 100ms prior to the presentation of the pre-pulse stimuli of 4, 8 and 16 db. The program used for these experiments is characterised by the first 5 minutes of acclimation and then followed by 3 block of trials. The first block consists of 6 trials where the animals are accustomised to the startle and the pre pulses. The second block is where the animal is challenged by a pseudo randomly present pre pulse followed by the startle. The final block takes the measurement only of the reaction to the 120db startle, as a control to make sure that the animals are not habituated to the noise throughout the test. This program lasts for 30 minutes. Between each trial the boxes are washed and dried before testing a new animal. The PPI measures were taken over two days. Since responses were less variable on the second day, these measures were selected for data analysis.

2.2.5.4 Statistics for behavioural tests

The ANOVA test was used to assess differences in means between infected and control mice using Minitab V. 15. Statistical significance was set at $p<0.05$. To identify any significant differences between individual groups the post hoc value Bonferroni-Dunn test was performed.

2.2.6 Molecular analysis

5 mice per group, from the infected and non-infected control groups from experiment run 1 and run 2 were sacrificed at each studied time, at 1 month and 2 month after infection, in order to carry out the molecular and the metabolomics analyses on the mouse brains. The animals were sacrificed using CO_2. The left hemisphere of the brain was homogenized and stored in Trizol at -80°C, which followed RNA extraction, cDNA synthesis and Real time PCR on the samples. The quantitative gene expression analysis was achieved via real time PCR through the use of standards curves constructed for each considered gene (from 30 to $3x10^6$ gene copy number) with the exception of COX1, the gene copies of which were calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

Standards curves preparation

Standards curves necessary for the gene expression analysis were made for the house-keeping gene TBP (Tata-Binding box protein) IDO (Indoleamine 2,3-dioxygenase), TDO (Tryptophan 2,3 Dioxygenase), TPH2 (Tryptophan hydroxylase 2) (GenBank^{TM} accession numbers respectively NM_008324, NM_019911 and NM_173391), IFN- γ (Interferon γ), TNF-α, IL-1β (Interleukin 1β) and IL-6 (Interleukin 6) in previous studies (Henriquez, 2009 MRes thesis, for the exception of TNF-α, IL-1β and IL-6 prepared by Roberts CW laboratory). COX-2 (Cyclooxygenase 2), HD (3 Hydroxyanthranilate 3,4 Dioxygenase), KYNA (Kynureninase), KYNAMO (Kynurenine 3 Monooxygenase), KYNA AT1

(Kynurenine Aminotransferase I), KYNA AT2 (Kynurenine Aminotransferase II) and KYNA AT3 (Kynurenine Aminotransferase III) were prepared in this study.

Oligonucleotide primer design

Oligonucleotide primers for COX1, COX-2, HD, KYNA, KYNAMO, KYNA AT1, KYNA AT2 and KYNA AT3 were designed using MacVector™ 7.0 (Oxford Biomolecular). The primers were chosen on the basis of their predictive annealing temperature to be the highest as possible (Table 2.1).

Table 2.1: Oligonucleotide Primers for COX1, COX-2, HD, KYNA, KYNAMO, KYNA AT1, KYNA AT2 and KYNA AT3. Annealing temperature for each pairs is predicted to be 60˚C.

Primer	Forward (5' to 3')	Reverse (3' to 5')	Predicted size (bp)	NCBI number
COX1	AAGGAGTCTCTC GCTCTGGTTTCC	TTCTGGCACGG ATAGTAACAACA GG	126bp	NM_008969
COX2	CCCATCTGTTCT CCTCAATACTGG	TGGCTGTTTTGG TAGGCTGTGG	163bp	NM_011198
HD	ATGGGGCTGGT GATTGAGAGAA GG	TGGATGATGGGT GCTAACTGCGT G	140bp	NM_025325
KYNA	TCAGCAAGCAA CTATGACTGCG	TTGGGAATGGAA AAGGTGAGTG	186bp	NM_027552
KYNAMO	GGAGTCCTATGC CAATGCGAAG	TGCTGAGTGTAA TCAAAGCGGG	198bp	NM_133809
KYNA AT1	CAAGTTCACACC TCGCACCAAG	CCTCATCAGAGA AGCACAGGACA TC	137bp	AK042391
KYNA AT2	AGCCATCTCCCA TCAGAACTACAG	GGTGCTTCCGTT TTCCACAGTG	137bp	AF072376
KYNA AT3	CAAAAACGCCAA ACGAATCG	AAGTGCTGGAT GACCAAAGCCC	202bp	AF363737

PCR

Each PCR reaction contained 6.25µl 2X ReddyMix (Thermofisher, UK), 25pmol of forward and Reverse Primers (Invitrogen, UK) each, 5.25µl Molecular H_2O and 1µl cDNA from brain infected murine samples. In the negative control DNAse and RNase free H_2O replaced the template. Thermocycling conditions were as follows: Initial denaturation at 94˚C for 3 minutes, followed by 35 cycles of 94˚C for 30 seconds, annealing 60-64˚C for 45 seconds and extension at 72˚C for 1 minute. A final extension step was carried out at 72˚C for 10 minutes. The PCR products were electrophoresed on a 2% Agarose, containing 0.00003% ethidium bromide in 1X TBE at 120 volts for approximately an hour. DNA bands chelated with the ethidium bromide were visualised on a UV transilluminator.

DNA gel extraction

DNA bands were excised from the agarose gel using a clean scalpel. DNA was extracted using the QIAgen gel extraction kit (Qiagen, UK) according to the manufacturer's instruction.

Ligation

Purified PCR products were ligated into the Strataclone™ vector mix (Stratagene, UK) as follows: 0.5µl of Strataclone™ vector mix was added to 1µl PCR product and 1.5 µl of buffer and incubate for 5 minutes at room temperature and then place on ice.

Transformation of plasmid into Strataclone™ SoloPack® competent cells

Transformation of the ligated plasmid was carried out following a modify version of the protocol first described by Cohen et al. (1972). In brief, 2µl of the ligation reaction were added to a tube of Strataclone™ SoloPack® competent cells, slowly unfrozen and incubated for 20 minutes on ice. This is followed by a heat-shock for 2 minutes at 42˚C and by incubation 2 minutes on ice. 250µl of pre warmed LB broth was added and followed incubation at 37˚C in shaker at 225 rpm for 40 minutes to 1 hour in order to stimulate the recovery of the competent cells after the temperature shock. The transformed cells were then spread onto an agar plate treated with 100µg/ml of Ampicillin (USB Corporation) and 50 µg/ml of X-gal (Apollo Scientific, UK) and 100µM IPTG (Apollo Scientific, UK) and incubated at 37 ˚C overnight.

Plate screening and colony selection

The Strataclone™ vector mix contains the lacZ gene that is disrupted if ligation of a DNA fragment with the plasmid is successful. If the plasmid does not successfully ligate the fragment transformant colonies will appear blue in the presence of X-gal as the lacZ gene is switched on. However, if ligation is successful the lacZ is not switch on and the colony will appear white. Therefore only white colonies will possess the DNA fragment of interest. In this transformation, a single white colony was picked by aseptic technique and incubated at 37 °C in a LB broth with 100µg/ml of ampicillin and shaking at 225rpm overnight.

Plasmid isolation and digestion

The plasmid was then isolated and extracted from the bacterial culture using the QIAgen miniprep plasmid isolation kit according to the manufacturer's protocol (QIAGEN). The restriction enzyme digest was then carried out in order to insure the insertion of the DNA fragment into the plasmid. In a 10µl reaction 3µl plasmid was added to 1µl EcoRI (Promega, UK), 1µl 10X EcoRI buffer and 5µl molecular H_2O and incubated at 37 °C for 1 hour. The visualisation of the digestion product was through a 2% agarose gel as previously described.

Standards preparation

Standards curves were made by calculating the mass of the plasmid with the insert containing the copy numbers of interest (Giulietti et al., 2001). The copies of number chosen were $3x10^6$, $3x10^5$, $3x10^4$, $3x10^3$, $3x10^2$, $3x10^1$ and they were prepared with a series of dilutions. The standards were then stored at -20°C until use.

RNA extraction and isolation from the brain

Following behavioural assessments after the final time point, 10 mice from the control group and all 30 mice born from infected mothers during pregnancy were sacrificed according to the Home Office UK law by exposing the animals to an increasing level of CO_2. When the animal was pronounced dead the extraction of the brain was carried out. The right half of the brain was stored in 0.1% of formic acid for metabolomics analyses. The other half of the brain was homogenised with a 21 gauge needle in 1 ml of TRIzol (Invitrogen) and stored at -70°C for the RNA extraction.

RNA extraction

500µl of the brain sample in TRIzol™ was taken and an additional 500µlof Trizol was added. The remaining of the brain sample in TRIzol™ was then stored at – 80°C. The RNA extraction was then completed on the rest of the sample. The method used is a single step extraction based on the acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987). Each sample was further homogenised through a 21gauge needle. The sample was further homogenised through a 25gauge needle in order to shear the genomic DNA. 500µl of Chloroform was added and mixed to the solution and incubated at room temperature for 2 minutes. After a centrifugation at 4°C for 15 minutes at 10000*g* the top aqueous layer was retained and added to 600µl of ice-cold isopropanol. The samples were then mixed vigorously and incubated at room temperature for 10 minutes. Centrifugation was followed at 4°C for 10 minutes at 10000*g*. The isopropanol was gently discarded in order not to disturb the pellet formed at the bottom of the tube. A second extraction was immediately performed by adding 500 ml of TRIzol to the samples and the protocol was repeated. Finally, the pellets were washed in 1 ml of 70% (v/v) of ice-cold Ethanol and centrifuged at 4°C for 15 minutes at 7000*g*. The ethanol was then discard and the pellets dried in a sterile environment. 50µl of molecular H_2O (Sigma, Poole, UK) was added to each pellet and the RNA was solubilised at 60°C for 5 minutes. The RNA was then stored at -80°C until further use.

DNase treatment

Prior to cDNA synthesis, the RNA was tested by PCR in order to ensure of the absence of genomic DNA contamination. The house-keeping gene TBP was used as a positive control for the reaction. If bands appeared in the agarose gel where the RNA samples were loaded then 2µl of DNAse (Deoxyribonuclease I, Invitrogen, UK) treatment on contaminated samples was performed at 37°C for 2 hours. RNA samples were PCR tested and if contamination still appeared a second DNase treatment or TRIzol™ extraction were carried out.

RNA concentration

Once the RNA samples were free from genomic contamination the concentration of the samples were measured using the Nanodrop spectrophotometer (ThermoScientific, USA). All samples met the condition of purity with the ratio of the absorbance 260/280 approximately 2.

cDNA synthesis

Complementary DNA (cDNA) was synthesised from the murine brain RNA using AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene, UK). The initial step of the reaction required 2µg of RNA, 1µl of Random Primers (Promega, UK) and molecular H_2O was added to a final volume of 14.2µl, followed by an incubation at 65°C for 5 minutes. Samples were then left to cool down at room temperature for 10 minutes in order to permit the correct annealing of the random primers. Finally, 2µl of 10X Affinity Script RT buffer, 2µl of 100mM DDT, 0.8µl of 100mM of deoxynucleotide triphosphate (dNTPs) mix (10mM each of dATP, dGTP,

dCTP, dTTP at neutral pH) and 1µl of RT enzyme were added to the samples to a final volume of 20µl. The samples were then incubated at 25°C for 10 minutes, 55°C for 1 hour and 70°C for 15 minutes in order to inactivate the reaction. cDNA samples were then stored at -20°C.

Quantative Real Time PCR (qRT-PCR)

The Stratagene Mx3000p Realtime PCR thermocycler was used for all the qRT-PCR reactions performed. Each sample was analysed in duplicate and the final volume of the reaction was 12.5µl containing 6.25µl of SYBR green (Sigma, UK), 25pmol of forward and reverse oligonucleotide primers, 1µl of cDNA template or negative control and molecular H_2O to arrive to volume. The reactions were then carried out using the following programme: 1 cycle of denaturing at 95°C for 10 minutes, 40 cycles of denaturing at 95°C for 30 seconds, annealing at 64°C for 45 seconds and extension at 72°C for 1 min followed by 1 cycle of 95°C for 1 min and 55°C for 30 seconds. The genes of interest analysed were IDO, TDO, IFN-γ, TPH2, TNF-α, IL-1β, IL-6, COX1, COX2, HD, KYNA, KYNAMO, KYNA AT1, KYNA AT2 and KYNA AT3 and their expression levels were calculated as a percentage of the house-keeping gene TBP. For each gene of interest standards curves were previously produced as described. For the exemption of COX1 gene that was added to the analysis of this study with a limited time available to construct the standard curve. However, the gene expression was calculated by using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001), as previously mentioned.

Molecular Statistics

Mann-Whitney U test was performed for the comparison of the expression levels of IDO, TDO, IFN-γ, TPH2, TNF-α, IL-1β, IL-6, COX1, COX2, HD, KYNA, KYNAMO, KYNA AT1, KYNA AT2 and KYNA AT3 between the control and the infected brain samples. Minitab V. 15 was used in order to carry out these calculations.

2.2.7 Metabolomic analysis

5 control mice and 5 infected mice from experiment run 1, 2 and 7 control mice and 7 infected mice from experiment run 3 were sacrificed at each time point, 1 month and 2 month after infection and the right hemisphere of the brain was extracted in order to perform metabolomics analysis. At each sacrificed time point the samples were analysed by mass sepctometery using a LTQ-Orbitrap (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) the following day after sacrifice and sample preparation for the exemption of the last experiment, run 3, which samples obtained from 1 and 2 month after infection were run at the same time after the final time point.

Sample preparation

The right hemisphere of the brain was extracted and homogenized in 0.1% of formic acid through a 21-gauge needle. The sample preparation was then centrifuged for 10 minutes at 4000 rpm. In a 4:1 ratio acetonitrile was added to the sample and left incubating at room temperature for 10 minutes. The sample was then vacuum filtered

and stored at -20°C overnight and used the following day from sacrifice and preparation.

Orbitrap LTQ

LTQ-Orbitrap (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) was used in order to collect high-resolution, accurate mass data, allowing the acquisition of a single molecular formula for each m/z measured within the described range (Makarov, 2000). The Orbitrap was connected to a Surveyor HPLC pump (ThermoElectron, Hemel Hempstead) running the a gradient of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The gradient started at 80% of solution A and 20% of solution B, decreased to 60% of solution A and 40% of solution B over 6 minutes, held at 60% of solution A and 40% solution B for 12 minutes, decreased to 10% of solution A and 90% of solution B over 4 minutes, held at 10% of solution A for 2 minutes, increased to 80% of solution A over 2 minutes and then held at this initial composition for 14 minutes to re-equilibrate. All samples were injected (10 μL) into a ZIC-HILIC column (Merck Sequant, 150 x 4.6 mm, 3.5 μm) (t' Kindt et al., 2010). The settings for the Electrospray ionisation (ESI) source settings were optimised and set as follows: Source Voltage – 4.50 kV, Capillary voltage - 25 V Capillary temperature - 250 ^{o}C, Sheath gas flow – 60 AU, Aux gas flow – 25 AU, Sweep gas flow - 0 AU. All spectra were recorded in profile mode across the range 50-1250 m/z at 30,000 resolutions FWHM.

Data Processing

The data was processed using Proteowizard (Smith et al., 2006; Kessner et al., 2008) and MZMatchR (Scheltema et al., 2011) within the R (Dean and Nielsen, 2007) environment. The MSConvert tool from Proteowizard was used to convert the .RAW files produced by the mass spectrometer to .mzXML files. These were then processed using centWave (Tautenhahn et al., 2008) from XCMS to extract the features and then the following pipeline from the tools available through MZMatchR.

1. Conversion from xset to .peakml files. Xsets are produced by XCMS and need to be converted so MZMatchR can read process the file. A single file is produced for each mass spectrometry file.
2. Combination of biological replicates. This tool combines all of the files that represent the same biological conditions into a single file. All of the detected features from each file are included and any that a found across one or more files are grouped together to create a set.
3. Biological replicates gap-filled. If a peak from a biological replicate is absent in one or more of the other replicates this tool will manually integrate the .mzXML file over the specific mass range and retention time to see if the peak has been missed by the peak picking stage. This is important as missing peaks can cause the RSD filter to remove a peak set that should not have been removed.
4. RSD filter applied. The RSD filter will remove any peak set that shows a deviation in one or more of the peaks of more than the set fraction of the RSD. This is user defined and in this work 0.5 was used.

5. Combination of all different conditions. This combines all of the different biological replicates into a single file. As with the previous combine step, it will combine and peak sets that are the same in two or more biological replicates into a single peak set.
6. Noise filter applied. A noise filter is applied to remove any peaks that do not exceed the pre-determined value.
7. Intensity filter applied. An intensity filter is used to remove and peaks that are deemed to be below the pre-determined threshold where validity of a feature becomes questionable.
8. Filtered file gap-filled. The final filtered file is gap-filled to ensure that not peaks that should be present have been removed by any of the filters.
9. Related peaks matched. Related peaks, such as isotopes, adducts etc. are labelled for ease of browsing the data after analysis.
10. Converted to text. The final file is converted to a .txt file so it can be opened in Excel and processed using an in-house macro.

The data was then processed using an in-house macro to identify potential metabolites. The identification was done by comparing the list of masses to a database of known metabolite masses contained in an Excel worksheet. If the experimental mass matched the database mass to within 3 ppm of the known metabolite mass the feature was labeled as that metabolite. Where multiple isomers were known for a single mass all of them were listed.

The data of the experiment Run 1 and Run 2 was further analysed by the expertise of the Metabolomic group of the Univeristy of Strathclyde, led by professor David Watson using the software program SIEVE 2.0 (Thermo Scientific, USA).

Metabolomics Statistics

The student t test was performed to establish the presence of possible significance differences between the metabolites detected in control and infected mouse brains.

2.3 Results

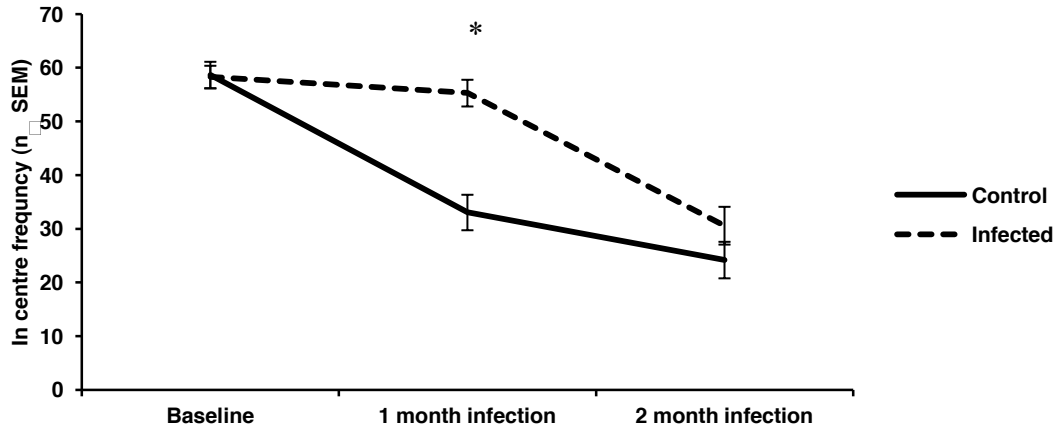
2.3.1 *T. gondii* infection alters mouse behaviour as determined by the Open Field test

At 1 month after infection the frequency of entries (n) into the central zone of the open field was significantly higher in the infected mice compared with controls ($p<0.001$). This difference was not observed at 2 Month after infection (Figure 2.3 A).

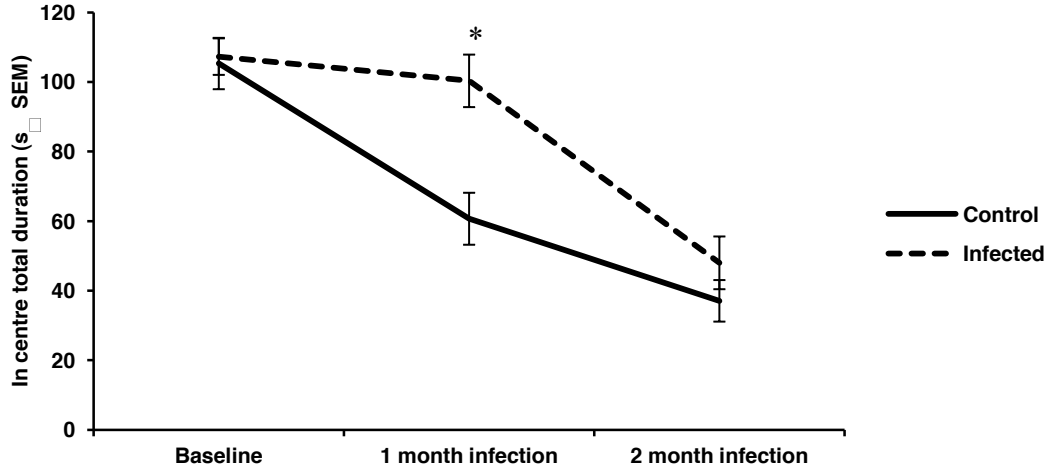
Similarly, at 1 month after infection infected mice were significantly spending more time (s) in the central zone compared to controls ($p<0.001$). This difference between the two groups of the time spent at the center of the box was not observed at 2 month after infection (Figure 2.3 B).

At 1 month after infection the total distance moved (cm) of the infected mice was significantly higher compared to controls ($p<0.01$). At 2 month after infection no difference was observed between the two groups of the distance moved around the field (Figure 2.3 C).

A


70
60
50
40
30
20
10
0
In centre frequncy (n SEM)
*
Baseline
1 month infection
2 month infection
Control
Infected

B


120
100
80
60
40
20
0
In centre total duration (s SEM)
*
Baseline
1 month infection
2 month infection
Control
Infected

C

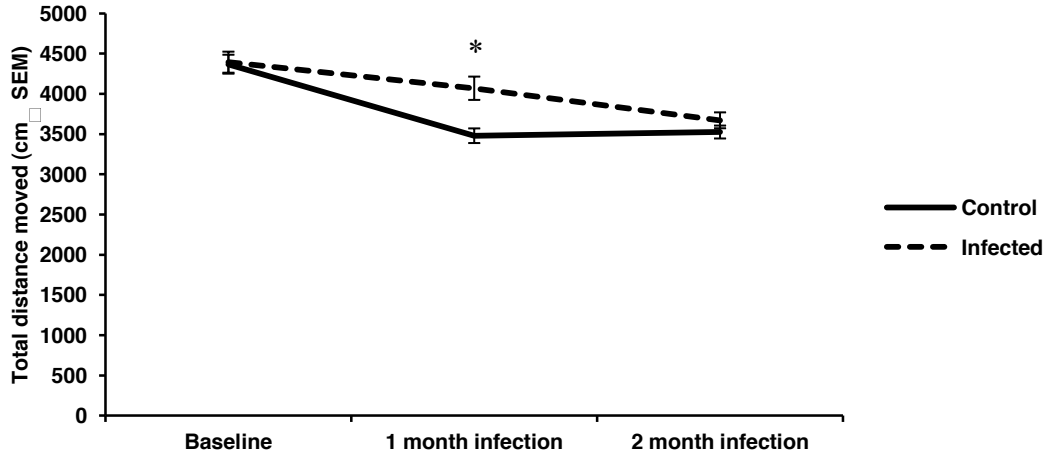

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Total distance moved (cm SEM)
*
Baseline
1 month infection
2 month infection
Control
Infected

Figure 2.3: Performance of BALB/c male mice in the open field test task. The measurements were taken before infection with *T. gondii Beverley*, the baseline measurements, and at 1 month and 2 month after infection. The n number per group is: at 1 month after infection controls (continuous line) = 36 and the infected mice (discontinuous line) = 41, at 2 months after infection the controls=24 and infected mice= 30. Graph A represents the average number of entries (n) at the centre of the field for each group. At 1 after infection infected mice significantly entered into the centre of the field more frequently compared to the uninfected controls ($p<0.001$). This significance is represented by *. Graph B represents the average of the time spent (s) at the centre of the field for each group. The infected group significantly spends more time in the centre of the field compared to the uninfected controls at 1 month after infection ($p<0.001$). This significance is represented by *. Graph C represent the average of the total distance moved (cm) in the field during the 10 minute task. At 1 month after infection the infected group significantly travelled more distance compared to the uninfected controls ($p<0.01$). This significance is represented by *.

2.3.2 *T. gondii* infection does not alter anxiety levels as determined by performance in the elevated plus maze

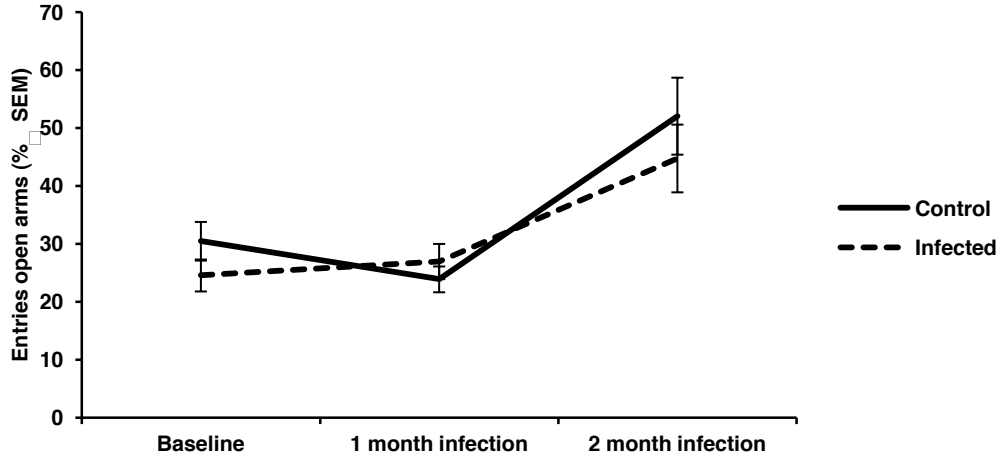
For each mouse, the numbers of entries into the open and enclosed arms were calculated as a percentage of the total number of entries. Similarly, the percentage time spent in the open and enclosed arms was calculated as a percentage of the total time spent in all arms.

The number of entries (%) in the open arms was not significantly different between the infected and controls mice at both the time points studied of the chronic infection (Figure 2.4 A).

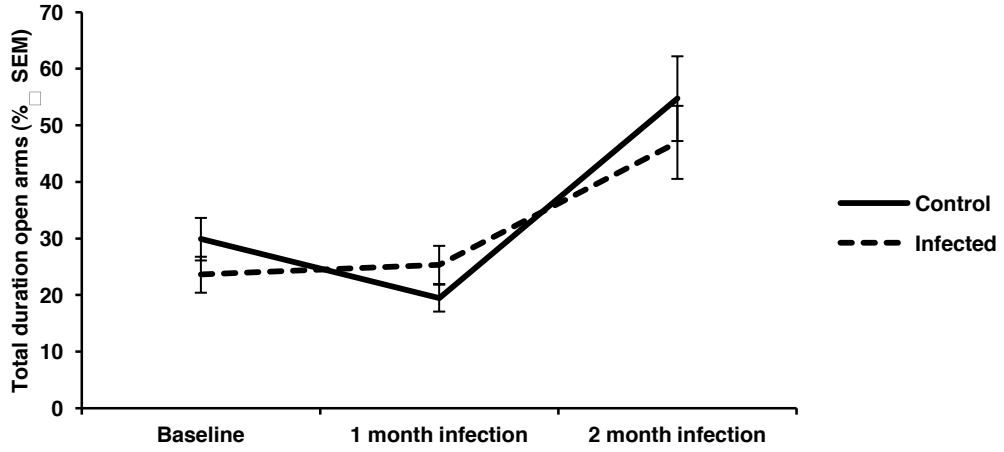
Similarly, the time spent (%) in the open arms was not different between infected and control mice at 1 month and 2 month after infection (Figure 2.4 B).

No difference was observed in the total distance travelled (cm) in the maze between the two groups at 1 and 2 month after infection, indicating the equal level of activity between the groups inside the maze (Figure 2.4 C).

A


Entries open arms (% SEM)
70
60
50
40
30
20
10
0
Baseline
1 month infection
2 month infection
Control
Infected

B


Total duration open arms (% SEM)
70
60
50
40
30
20
10
0
Baseline
1 month infection
2 month infection
Control
Infected

C

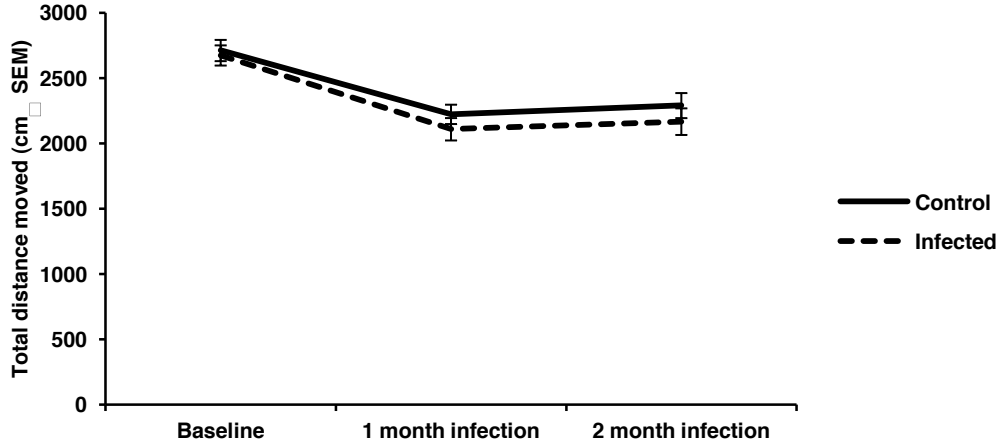

Total distance moved (cm SEM)
3000
2500
2000
1500
1000
500
0
Baseline
1 month infection
2 month infection
Control
Infected

Figure 2.4: Performance of the BALB/c male mice in the elevated plus maze task. The measurements were taken before infection with *T. gondii Beverley*, baseline and at 1 month and 2 month after infection. The n number per group is: at 1 month after infection controls (continuous line) = 36 and the infected mice (discontinuous line) = 42, at 2 months after infection the controls=26 and infected mice= 32. Graph A represents the average of percentage of entries in the open arms for each group. No differences were observed between the controls and infected group at any time point. Graph B represents the average of the percentage of the time spent in the open arm for each group. No differences were observed between the controls and infected group at any time point. Graph C represent the average of the total distance moved (cm) in the maze during the 10 minute task. No differences were observed between the controls and infected group at any time point.

2.3.3 *T. gondii* infection does not alter PPI levels in mice

The pre pulse inhibition was calculated using the following equation:

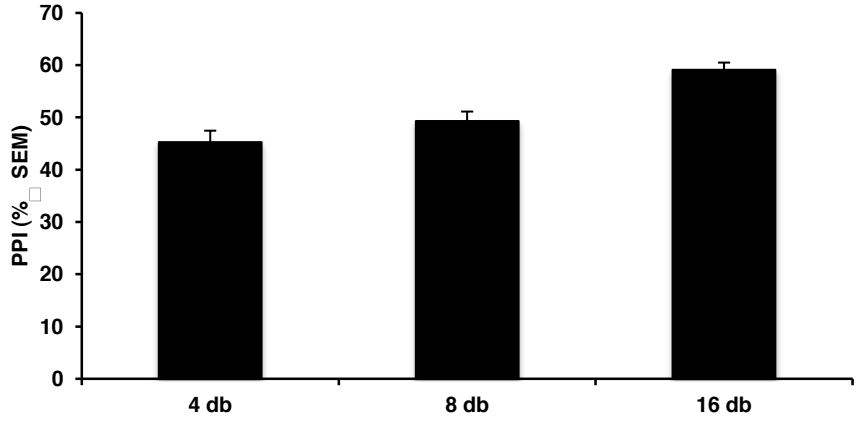
%PPI= [1-(startle response to pre-pulse + pulse)/ (startle response to pulse alone) X100].

The measure of the reaction to startling stimulus alone (120 db) was taken in the infected and control mice at each time point in order to assess whether there were no hearing deficits over time and/or habituation to the startle. No differences were observed in the startle reaction between the control and infected group (Figure 2.5 B-C).

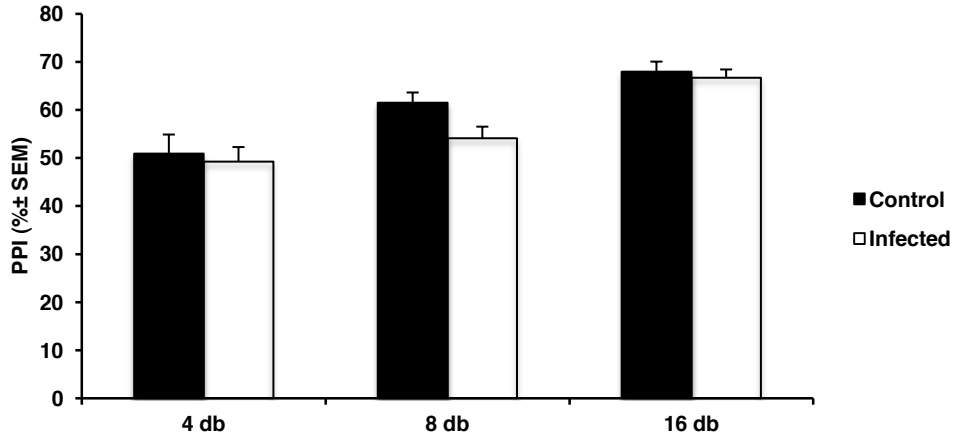
As predicted there was a pre-pulse dependent increase in PPI in control mice (Figure 2.5 A).

At 1 and 2 month after infection no difference was observed in the pre pulse inhibition (%) between the control and infected group.

A - Baseline



B - 1 month after infection



C - 2 month after infection

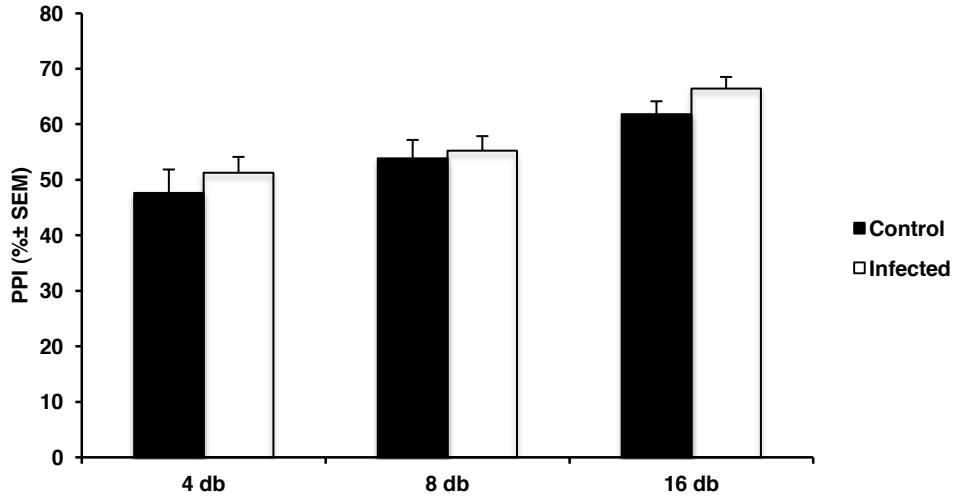


Figure 2.5: PPI levels measured and compared between the controls (black column) and the infected (white column) groups with *T. gondii Beverley*. Graph A represents the baseline measurements taken from all 85 BALB/c mice of the PPI levels challenged at different pre pulse intensities 4, 8 and 16 db. Graph B represents the measurements of the PPI challenge at 4, 8 and 16 db pre pulse intensities taken at 1 month after infection between the controls (n=36) and infected (n=41). No differences were observed between the controls and infected. Graph C represents the measurements of the PPI challenge at 4, 8 and 16 db pre pulse intensities taken at 1 month after infection between the controls (n=26) and infected (n=32). No differences were observed between the controls and infected.

2.3.4 Alteration of the immuno-neuro environment in the chronic infected mice compared to controls

Real time data was calculated as a percentage of the quantity of gene copies in relation to the TATA-binding protein (TBP) house keeping gene, with exception for COX1. COX1 expression was calculated using the ΔΔct method in relation to the ct of the house keeping gene TBP. The expression levels of the several genes were found by real time PCR to be significant different between control and infected cDNA brain samples.

Herein, we report the data obtained on the gene expression measured for the first and second run of experiments pulled together. At each time point 10 samples were analysed from individual controls brains and 10 samples from individual infected brains. The order of the figures is as follow: firstly, the effect of IFN-γ on the tryptophan pathway is examined, secondly all other immunological mediators in the brain during chronic infection compared to controls are observed.

At 1 month after infection the expression of the immunological mediators IFN-γ, IDO were found to be significantly higher in the infected brain samples compared to the controls with p values of 0.012 and 0.0012 (Figure 2.6, figure 2.7), respectively.

The gene expression of the enzyme involved in the tryptophan degradation pathway HD was found to be significantly higher in the infected brain samples compared to controls with p values of 0.013 (figure 2.10).

Instead, KYNA AT1 expression in the infected brain samples was significantly lower compared to the controls samples at 1 month after infection, p value of 0.0076 (figure 2.13).

Other immunological mediator found to be significantly increased in the infected brain samples compared to controls at 1 month after infection were IL-β (p=0.010) and TNF-α (p=0.038) (Figure 2.16, 2.17, respectively).

The levels of the expression for the rest of the genes taken into consideration were not different between the control and infected samples at 1 month after infection.

No differences were observed at 2 months after infection in the expression of the immunological mediators IFN-γ and IDO (figure 2.6, figure 2.7).

The level of gene expression of the enzyme involved in the tryptophan degradation pathway HD was significantly higher in the infected brain samples compared to controls brain samples with a p value of 0.045 (figure 2.10).

The levels of the expression for the rest of the genes taken into consideration were not different between the control and infected samples at 2 month after infection.

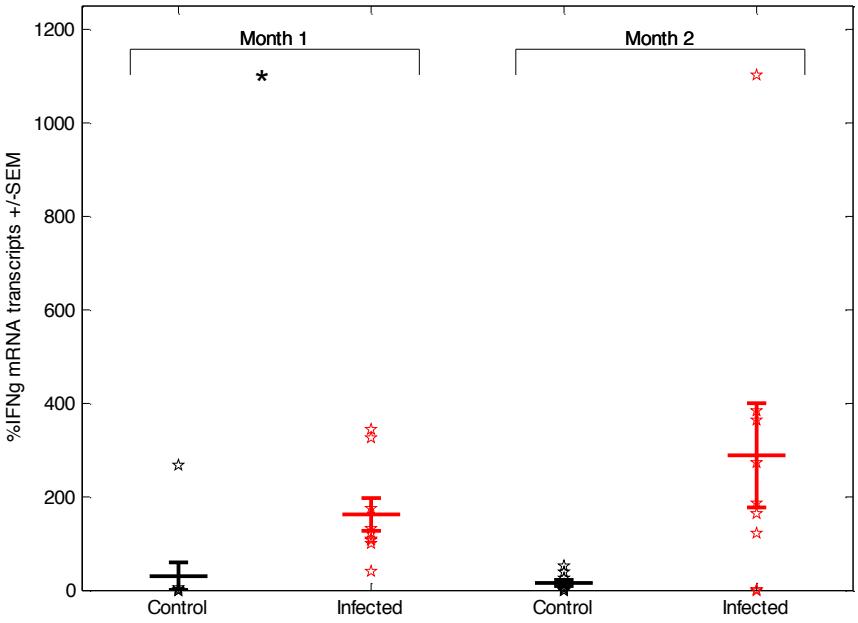


Figure 2.6: IFN-γ mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. IFN-γ expression was found significantly up regulated in the infected brain samples compared to controls at 1 month after infection (p=0.014). This is represented by an*.

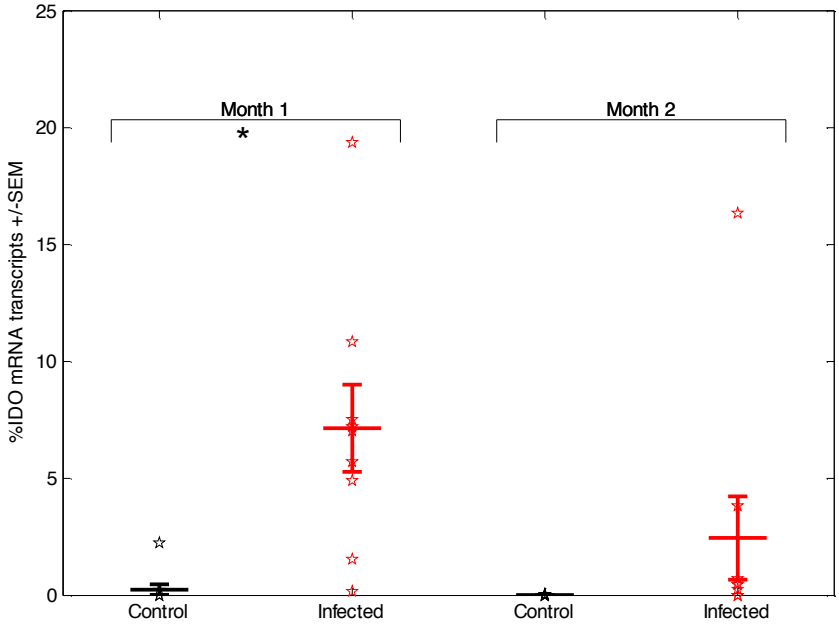


Figure 2.7: IDO mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. IDO expression was found significantly up regulated in the infected brain samples compared to controls at 1 month after infection (p= 0.0012). This is represented by an *.

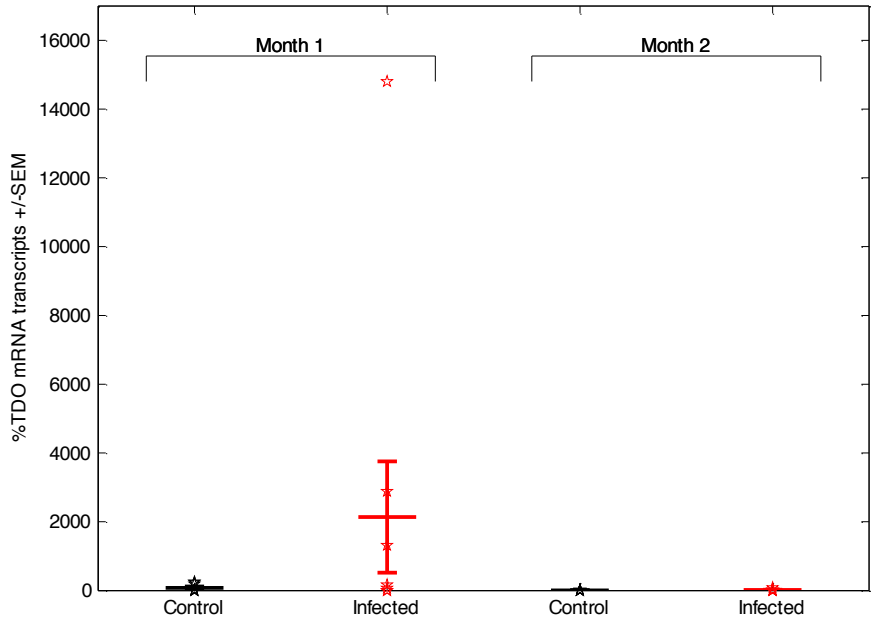


Figure 2.8: TDO mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

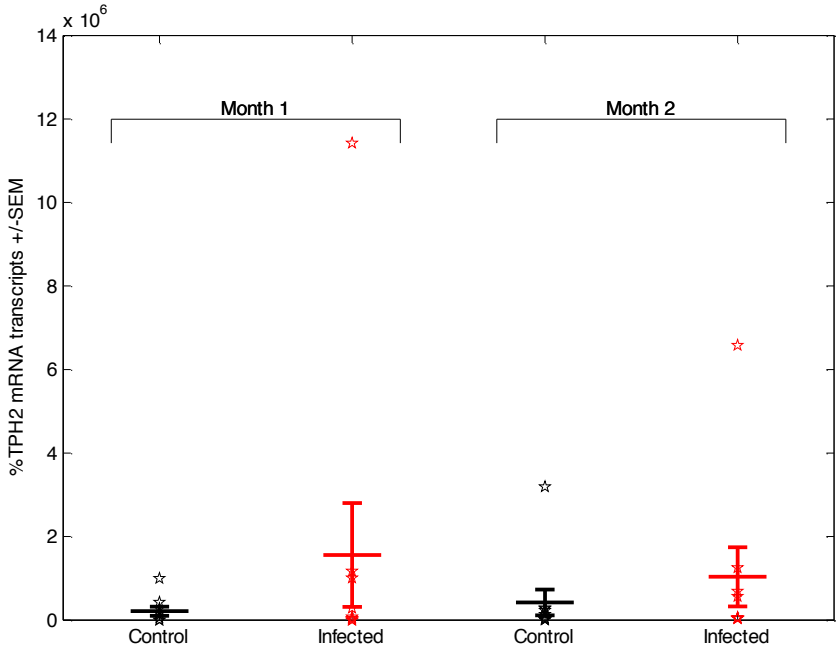


Figure 2.9: TPH2 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

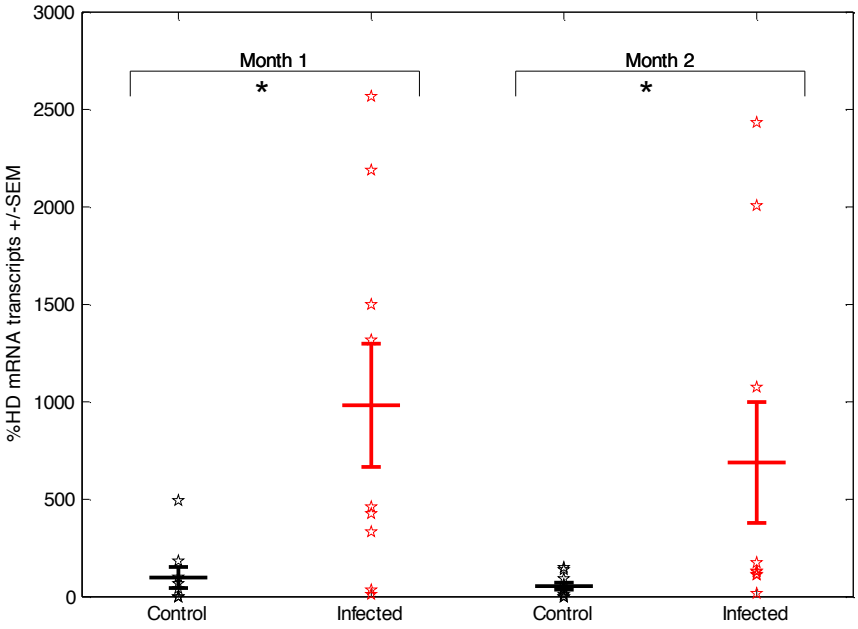


Figure 2.10: HD mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. HD expression was found significantly up regulated in the infected brain samples compared to controls at both time points (p=0.013 and 0.045). This is represented by an *.

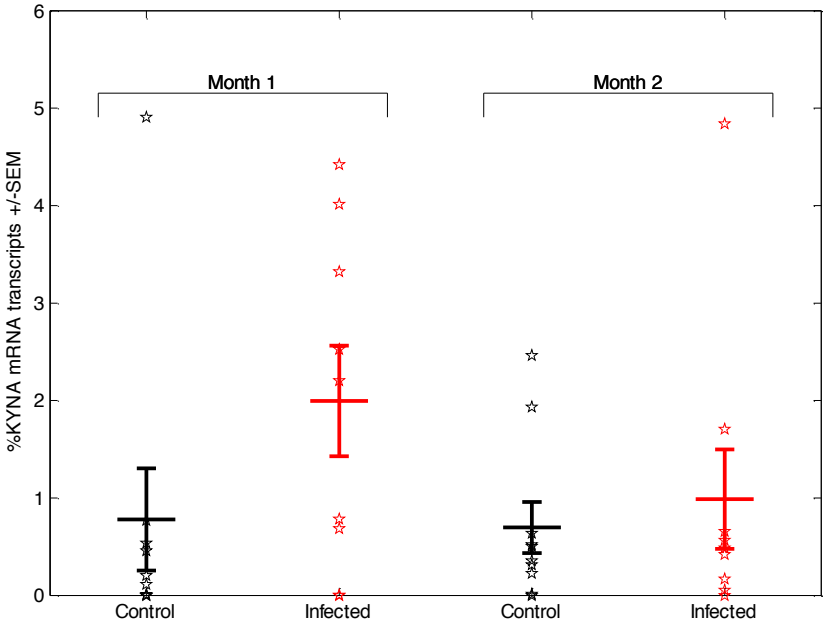


Figure 2.11: KYNA mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

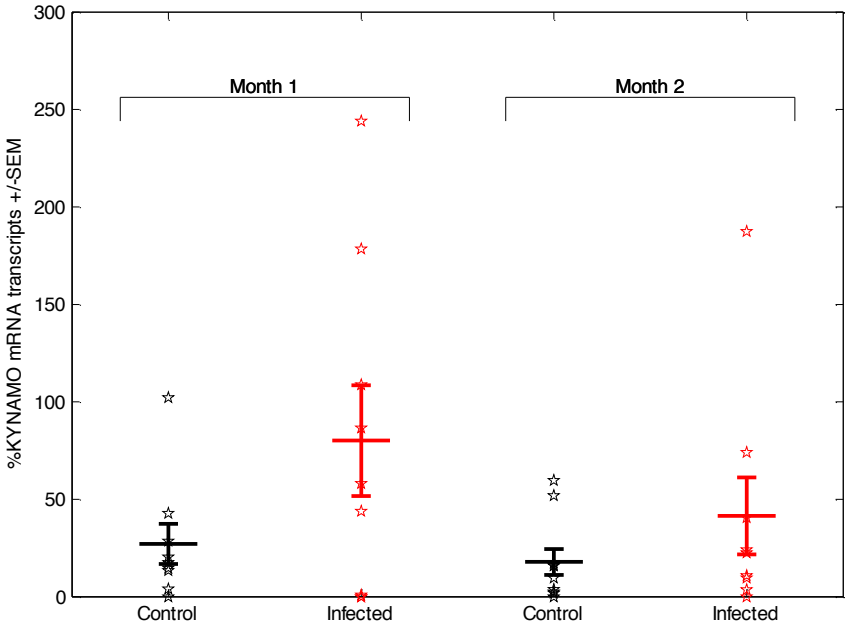


Figure 2.12: KYNA MO mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

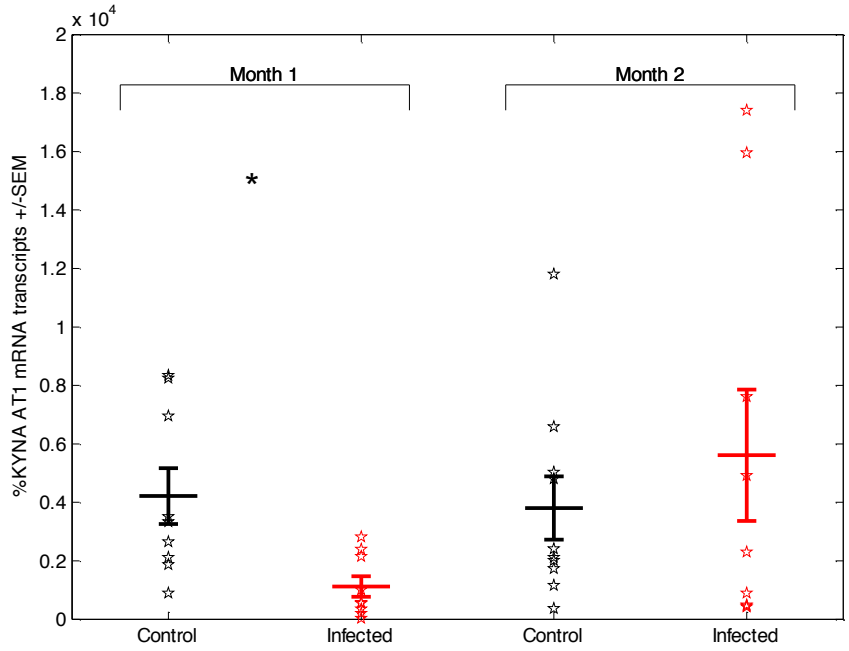


Figure 2.13: KYNA AT1 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. KYNA AT1 expression was found significantly down regulated in the infected brain samples compared to controls at 1 month after infection (p=0.0076). This is represented by an *.

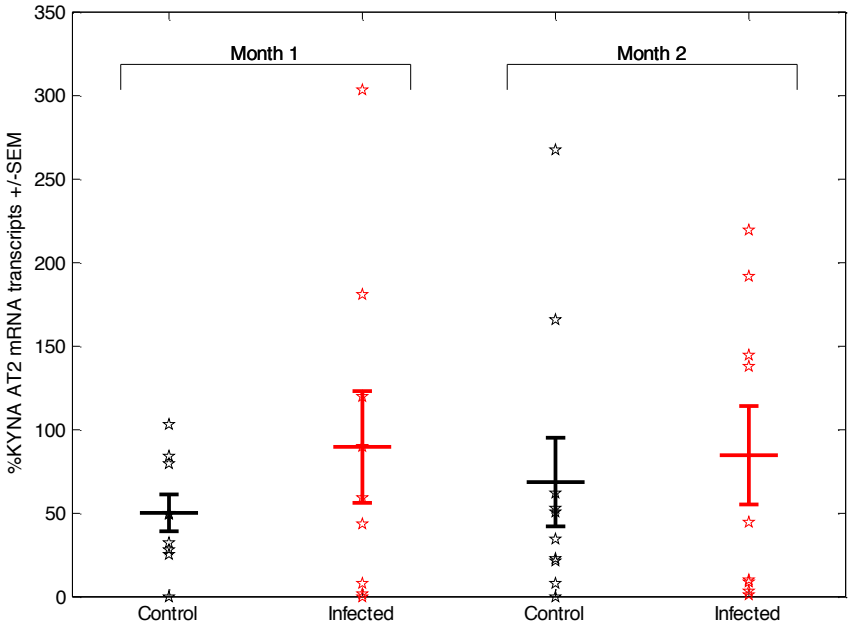


Figure 2.14: KYNA AT2 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

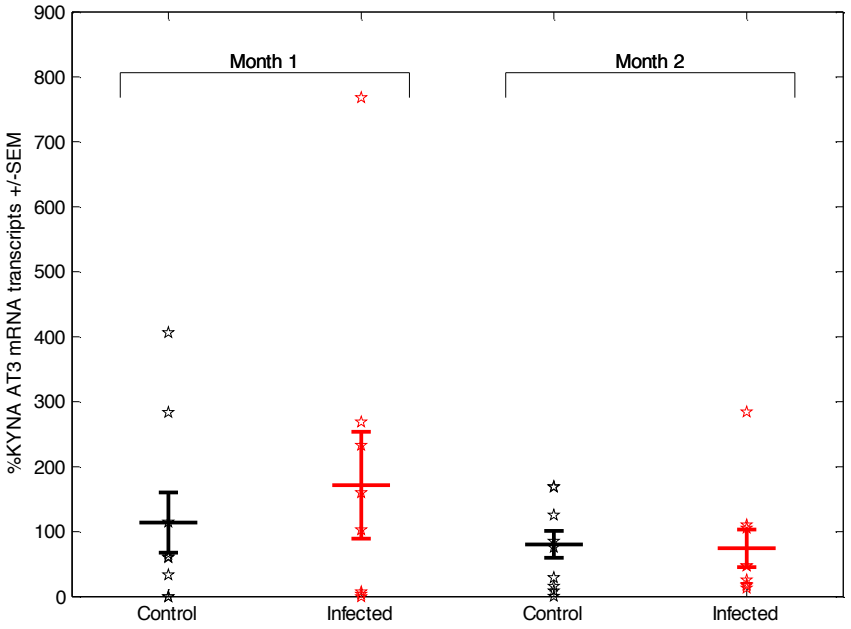


Figure 2.15: KYNA AT3 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

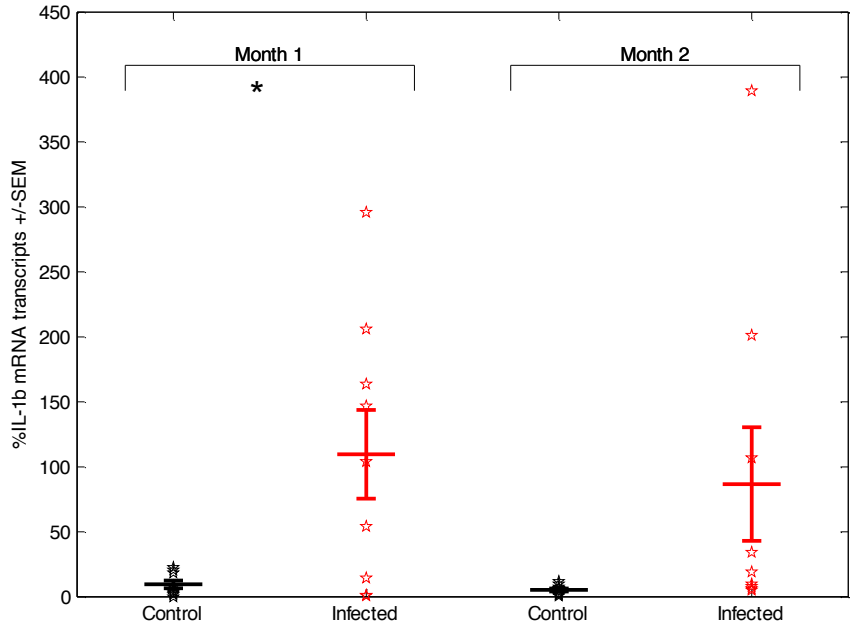


Figure 2.16: IL-1β mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. IL-1β expression was found significantly up regulated in the infected brain samples compared to controls at 1 month after infection (p=0.010). This is represented by an *.

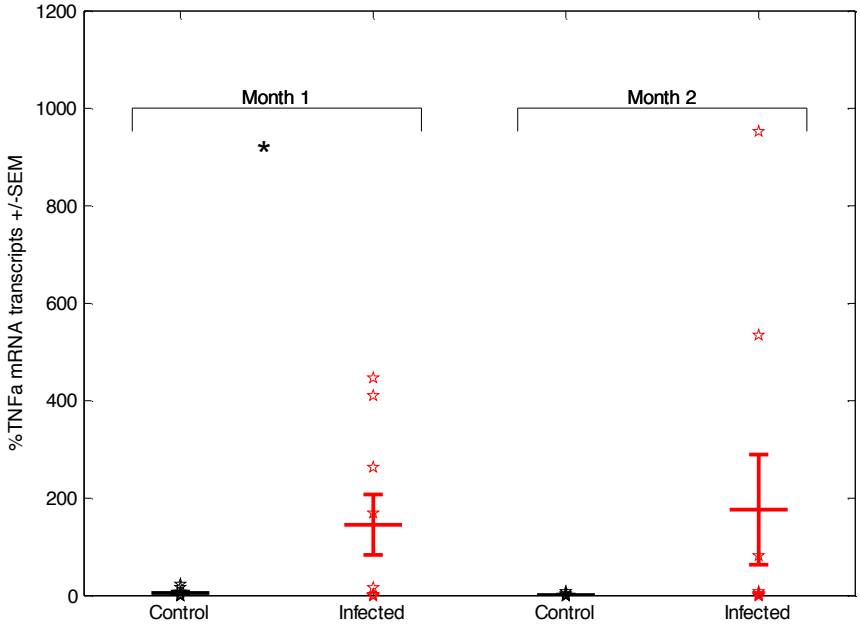


Figure 2.17: TNF-α mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. TNF-α expression was found significantly up regulated in the infected brain samples compared to controls at 1 month after infection (p=0.038). This is represented by an *.

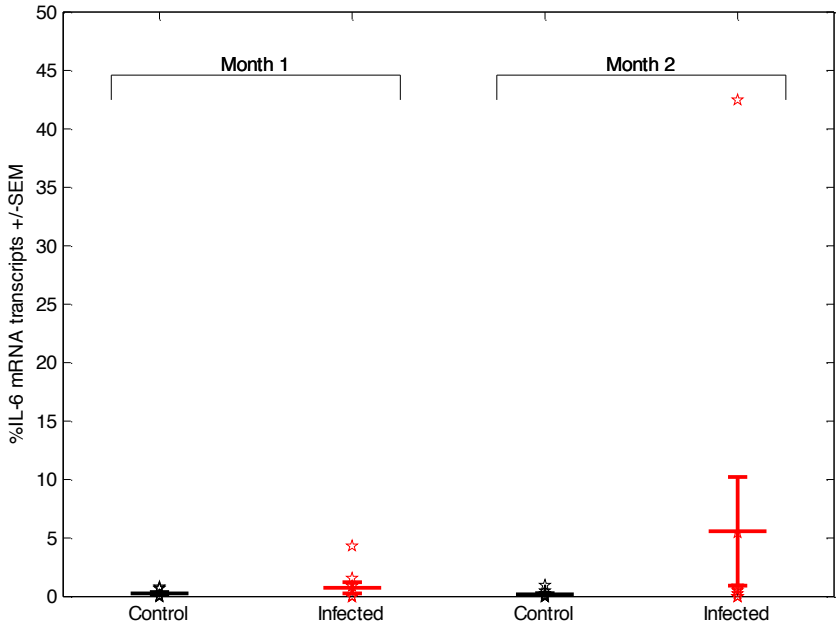


Figure 2.18: IL-6 mRNA mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

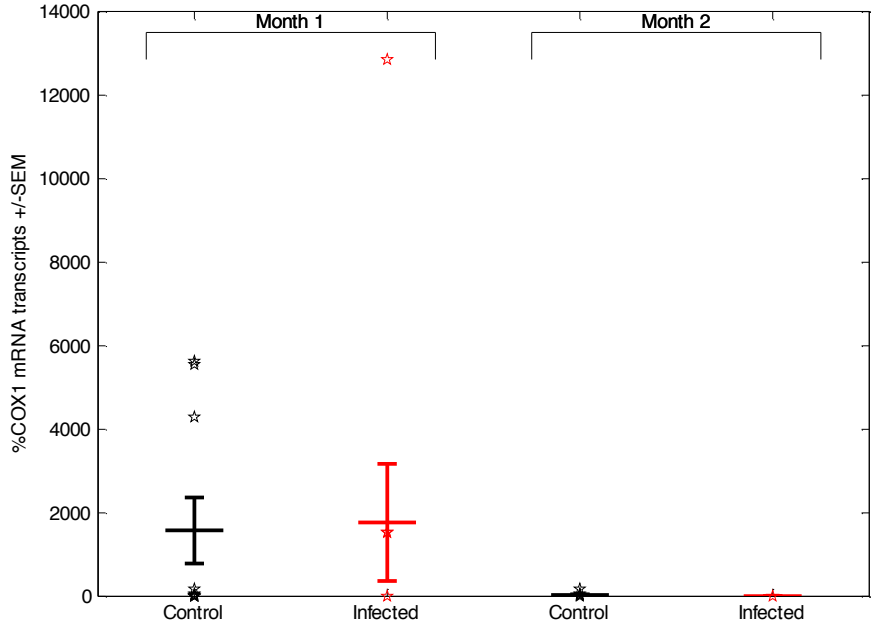


Figure 2.19: COX-1 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

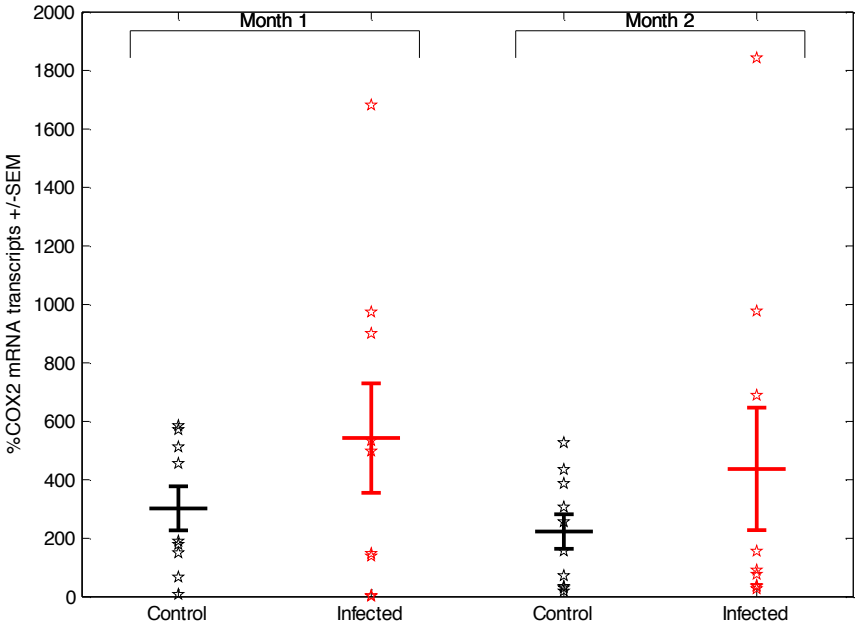


Figure 2.20: COX-2 mRNA transcripts expression in the percentage of the house keeping gene (TBP) in the controls (black stars) (n=10) and infected (red stars) (n=10) samples at 1 month and 2 month after infection. No differences were observed.

Figure 2.6-2.20: It is the analysis of the molecular expression by quantative real-time PCR of the enzymes involved in the tryptophan degradation pathway and the cytokines present in the brain during *T. gnodii* chronic infection (red stars) compared to the molecular expression of the molecules in the brain of uninfected controls (black stars) at 1 and 2 month after infection. The number of copies for each gene was expressed in the percentage of the relation of the number of copies of the house keeping gene. This measurement was done for all genes for the exception of COX-1, which was analysed using the $2^{\Delta\Delta C}{}_{T}$ method in relation of the house keeping gene TBP. At each time point the analysis was performed on 10 samples per group.

2.3.5 Metabolomics analysis

Figure 2.21 describes the analysis process of the metabolomic data. It was decided that the lipid profile in the mouse brain would not be taken into consideration for the final analysis in each experiment. This is because the lipid population is subject to frequent changes in the mouse brain and therefore it is more difficult to detect variables and to maintain consistency in the control samples (Rappley et al., 2009). Therefore, from the joint analysis of run 1 and run 2 experiments, 82 common unique masses were detected in positive ionization mode at 1 month and 2 month after infection and from the analysis of run 3, 672 unique masses were detected in positive ionization mode. In addition, a further step was taken to remove the compounds not detected in all the experiments from the analysis. The analysis was then carried out on the 52 unique common compounds identified in all the experiments (see appendix table 5.1). This was done to maintain consistency throughout the analysis of the data and to compare patterns between all the experiments. The analysis of the samples with the Proteowizard XCMS and MZMatchR programs also eliminated samples, in which the mass spectrum could not be aligned with the rest of the samples in the experiment. The final sample number analysed for each experiment at each time point is described in Table 2.2. The detection and identification of the compounds through the Proteowizard XCMS and MZMatchR analysis was further confirmed by the SIEVE software analysis by Prof David Watson and more compounds were identified (see appendix table 5.2), these compounds were included in the overall analysis of the metabolomic data (Table 2.4).

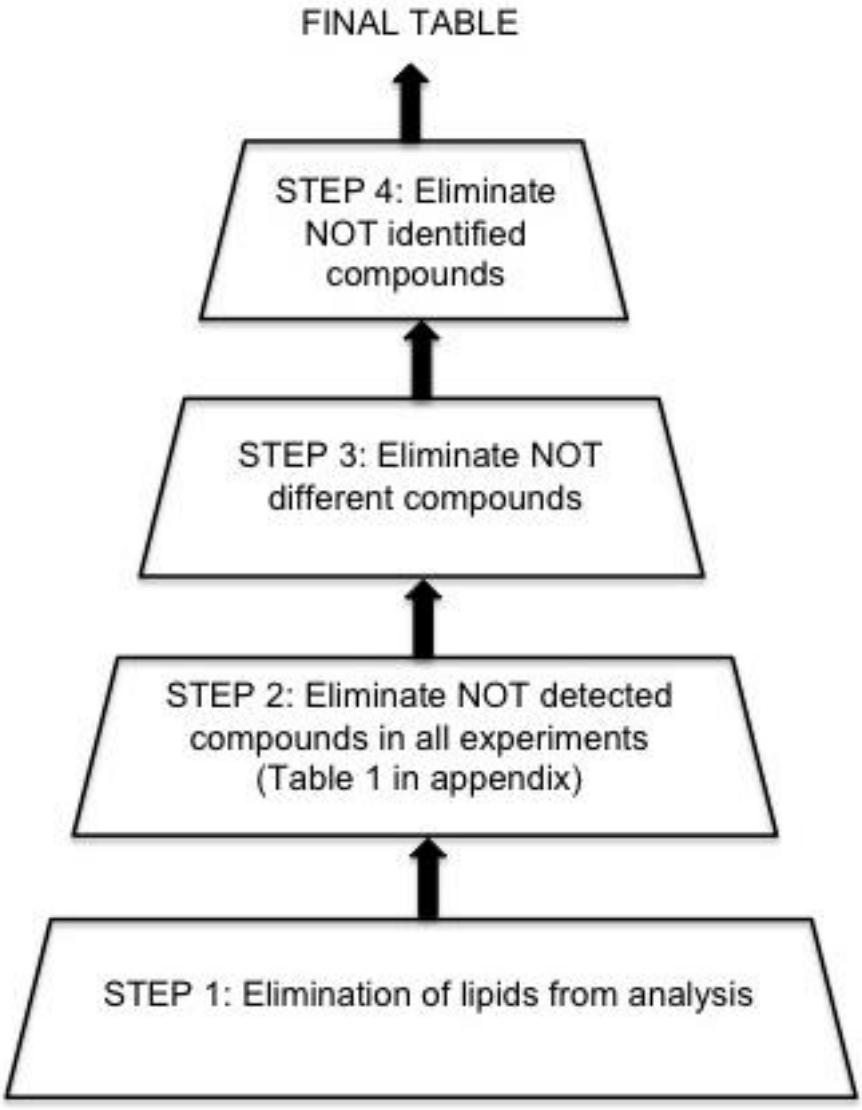


Figure 2.21: Diagram of the process of the analysis of the metabolomic data.

Table 2.2: The final number of control and infected samples analysed for each experiment at each time point.

	RUN 1		RUN 2		RUN 3	
Time point	1 month	2 month	1 month	2 month	1 month	2 month
CONTROL	4	4	4	5	4	6
INFECTED	5	2	4	5	4	6

The final analysis of the 17 compounds positively identified in all the 3 adult experiments, with significant differences in the brain samples between the controls and infected mice, observed at 1 and 2 month after infection, is summarised in Table 2.3. In table 2.4 are reported the extra 6 compounds detected by Dave Watson analysis. Although these compounds were not consistently detected in all the time points and experimental runs this data has also been included in the overall discussion and therefore the analysis has been reported together. In these Tables (2.3 and 2.4) we report the ratio of the peak intensity between infected/control of the brain samples at each time point for each experiment and it is colour coordinated to easily identify significant differences. If a difference in a compound between control and infected is consistent in more than one experiment, then there is the confidence that the difference observed is caused by *T. gondii* infection and not by other biological variable, such as age, the time of sacrifice or temperature.

The overall data is described on the appendix table (Table 5.1) were all the experimental runs were pulled together to analyse the overall differences. On this table statistical analysis such as standard error and p value are also included.

Table 2.3: Ratio Infected/Control of the compounds consistently detected analysed by the Proteowizard XCMS and MZMatchR softwares.

			RATIO RUN1		Ratio RUN 2		RATIO RUN 3	
Mass	FORMULA	Identification	1 month	2 month	1 month	2 month	1 month	2 month
85.089232	C5H11N	Piperidine	1.308	0.867	1.284	1.094	0.114	0.170
101.047753	C4H7NO2	1-Aminocyclopropane-1-carboxylate	0.875	0.468	0.880	0.633	0.464	1.071
103.063370	C4H9NO2	4-Aminobutanoate	0.877	0.551	0.962	0.795	0.734	0.002
105.042642	C3H7NO3	L-Serine	1.145	0.451	0.980	0.697	0.558	0.970
119.058318	C4H9NO3	L-Threonine	1.413	0.556	1.164	0.809	0.726	1.085
131.069450	C4H9N3O2	Creatine	0.923	0.621	0.926	0.658	0.629	0.919
131.094653	C6H13NO2	L-(iso)Leucine	1.298	0.895	1.358	1.069	1.167	1.657
133.037546	C4H7NO4	L-Aspartate	0.881	0.429	0.843	0.782	0.488	1.142
146.069144	C5H10N2O3	L-Glutamine	1.039	0.497	0.941	0.734	0.587	0.877
147.053128	C5H9NO4	L-Glutamate	0.898	0.480	0.864	0.716	0.518	0.890
151.049369	C5H5N5O	Guanine	0.787	0.780	1.426	0.931	1.172	1.255
165.079029	C9H11NO2	L-Phenylalanine	1.474	0.885	1.420	1.131	1.073	1.488
181.073924	C9H11NO3	L-Tyrosine	1.517	0.634	1.374	0.949	1.324	1.531
204.089948	C11H12N2O2	L-Tryptophan	1.374	0.855	1.241	1.066	1.052	1.366
221.090051	C8H15NO6	N-Acetyl-D-glucosamine	1.227	0.733	1.795	1.073	1.109	1.566
244.069682	C9H12N2O6	Uridine	0.900	0.709	0.844	0.820	1.373	0.848
283.091744	C10H13N5O5	Guanosine	0.786	0.794	1.485	0.932	1.128	1.252

TABLE LEGEND	
	>1 (p<0.05 Higher in Infected)
	<1 (p<0.05 Lower in Infected)
	No Difference

Table 2.4: Ratio Infected/Control of the compounds detected by the Sieve analysis.

			RATIO RUN1		Ratio RUN 2		RATIO RUN 3	
Mass	FORMULA	Identification	1 month	2 month	1 month	2 month	1 month	2 month
136.06187	C5H5N5	Adenine	0.419		0.447	0.541	0.426	0.751
153.04077	C5H4N4O2	Xanthine			1.763		2.202	1.585
154.08633	C6H3(OH)2-CH2-CH2-NH2	Dopamine	0.41	0.374	0.493		0.277	0.833
209.09218	C10H12N2O3	Kynurenine	6.252		3.525	4.332		
268.10385	C10H13N5O4	Adenosine	0.204	0.032	0.022	0.05		
348.07025	C10H14N5O7P	AMP		0.203	0.441	0.353	0.102	0.377

TABLE LEGEND	
	>1 (p<0.05 Higher in Infected)
	<1 (p<0.05 Lower in Infected)
	No Difference
	COMPOUND NOT DTECTED

From the analysis of this table it can observed that there are several compounds with consistent significant differences between the infected and control mice. However,

some differences in metabolite levels are not consistent in all experiments. Therefore, the analysis of all the metabolomic data from the 3 experiments performed is herein reported for the purpose of the deeper analysis and understanding of these results.

Metabolites involved in the tryptophan pathway

At 1 month after infection the levels of tryptophan (Table 2.3) and kynurenine (Table 2.3) were found to be higher in the infected brains compared to the controls consistently in the experiment run 1 and run 2 (respectively, p=0.000514, 0.008739 and p=0.017, 0.012).

At 2 months after infection the levels of tryptophan (Table 2.3) and kynurenine (Table 2.4) in the infected samples remained higher compared to controls only in one experiment, respectively experiment run 3 (p=0.036) for tryptophan and experiment run 2 for kynurenine (p=0.027).

Neurotransmitters, their precursors amino acids and metabolites

At 1 month after infection dopamine (Table 2.4) is lower in the infected brain samples in all three experiments compared to controls (p=0.0039, 0.018, 0.024). L-Glutamate (Table 2.3) was found lower in the infected brain samples compared to controls only in the last experiment, run 3 (p=0.008). However, the result has been reported because the measurements of L-glutamate at 2 month after infection were found to be more consistent between the experiments. 4-aminobutanoate (Table 2.3) was found significantly lower in the infected brain samples only in the experiment run 1 (p=0.021) but like glutamate more consistency was observed at 2 month after

infection between the experiments. N-Acetyl-D-Glucosamine (Table 2.3) was significantly increased in the infected brain samples in the experiment run 1 and run 2 (respectively, p values of 0.033, 0.007). At 1 month after infection the levels of Leucine and phenylalanine (Table 2.3) were increased in the infected brains compared to controls in the experiment run 1 and run 2 (respectively p values 0.0013, 0.017369 and 0.00043, 0.023). Levels of serine and tyrosine (Table 2.3) are significantly increased in the infected brain samples compared to controls only in the experiment run 1 (respectively, p= 0.024, 0.00005), these metabolites were detected in the other experimental runs with no significant differences between control and infected mice brain samples. The levels of L-Threonine (Table 2.3) in the infected brain are not consistent between experiment 1 and 3. In fact, in experiment 1 L-threonine was higher in the infected samples compared to controls (p= 0.009) and in the last experiment the levels of L-threonine were lower in the infected samples compared to control (p=0.039). However, consistency was observed in the measurements at 2 month after infection.

At 2 months after infection dopamine (Table 2.4) levels remained significantly lower in the infected samples compared to the controls only in experiment run 1 (p=0.002). Glutamate (Table 2.3) levels in the infected samples were significantly lower compared to controls in experiment run 1 and run 2 (respectively, p= 0.006 and 0.005). 4-aminobutoanate (Table2.3) levels remained decreased in the infected samples in experiment run 1 and run 3 (respectively, p=0.002 and 0.0002). N-Acetyl-D-Glucosamine (Table 2.3) was significantly decreased in the infected samples only in experiment run 1 (p=0.002) and no differences were observed in the other

experiments. At 2 months after infection the levels of Leucine and phenylalanine (Table 2.3) were still higher in the infected samples compared to controls in the experiment run 3 with respectively p values of 0.00056 and 0.026. Levels of serine (Table 2.3) were consistently lower in the infected samples compared to controls in experiment run 1 and 2 with significant p value of, respectively, 0.0007 and 0.004. Tyrosine (Table 2.3) levels were found significantly lower in the infected sample in experiment run 1 (p=0.012) and significantly higher in the experiment run 3 (p=0.02) compared to controls. L-threonine (Table 2.3) levels were consistently lower in the infected samples compared to controls in experiment run 1 and run 2 with significance (respectively, p=0.007 and 0.02).

Purine/pyrimidine matabolites

At 1 month after infection levels of adenine (Table 2.4) were measured and found to be significant lower in the infected mice brains consistently in experiment 2 and 3 with respectively p value of 0.0026 and 0.048 and not detected in experiment run 1. Adenosine (Table 2.4) levels were diminished in the infected brain samples in experiment 1 and 2 (p=0.00027 and 0.01) compared to controls and not detected in run 3. AMP (Table 2.4) levels were lower in the infected samples compared to controls in experiment run 2 and run 3 (p=0.027 and 0.03) and not detected in run 1. Other changes observed in the compounds involved in the purine/pyrimidines metabolism are guanine and guanosine (Table 2.3), both increased in the infected brain samples in experiment run 2 (respectively, p= 0.003 and 0.0031). Uridine (Table 2.3) is instead decreased in the infected samples in experiment run 2 (p= 0.038). Xanthine (Table 2.4), involved in the degradation pathways of the

purine/pyrimidine pathways is significantly increased in the infected brain samples in experiments run 3 (p= 0.003) and not detected in run 1.

At 2 month after infection adenine (Table 2.4) was still consistently lower in the infected samples compared to controls in run 1 and run 2 experiments (p= 0.00000026 and 0.0034), no differences observed in run 3. The levels of adenosine (Table 2.4) in the infected samples were consistently lower compared to control in the experiment 1 and 2 (respectively, p=0.0095 and 0.0076) and not detected in run 3. The levels of AMP (Table 2.4) were consistently lower in all three experiments in the infected samples compared to controls (respectively, p=0.035, 0.00029 and 0.00056). Guanine and guanosine (Table 2.3) levels were significantly lower in the infected brain samples compared to controls only in experiment run 1 (respectively, p=0.03 and 0.034). Uridine (Table 2.3) levels were still found lower in the infected brain samples in experiment run 1 and run 2 (respectively, p=0.005 and p=0.02). Xanthine (Table 2.4) levels remained still significantly increased in the infected samples in the experiment run 3 (p=0.04).

Other metabolites

At 1 month after infection significant differences were also observed in the levels of piperidine and creatine (Table 2.3) compounds between infected and control brain samples. In the experiment run 1 and run 2 higher levels of piperidine were measured in the infected samples (respectively, p=0.00058, 0.023) and vice versa, in the last experiment run 3 significant lower levels were observed (p=0.005). Creatinine significant difference was only observed in the experiment run 3 were significantly

lower levels were measured in the infected brain samples compared to controls (p=0.011319).

At 2 month after infection piperidine (Table 2.3) levels were significantly lower in the infected samples compared to controls in the experiment run 3 (p=0.00007). Creatine (Table 2.3) levels also were significantly lower in experiment run 1 and run 2 (p=0.003 and 0.03).

At 2 month after infection levels of 1-Aminocyclopropane-1-carboxylate, L-Aspartate and L-Glutamine (Table 2.3) are significantly lower in the infected brain samples compared to controls consistently in the experiment run1 and run 2 (respectively, p= 0.010335, 0.002457 and p= 0.003341, 0.031961 and p= 0.011338, 0.001615), no differences were observed in the levels of these compounds at 1 month after infection.

2.3.6 Effects of the changes in gene expression in the Tryptophan degradation Pathway on the tryptphan and serotonin metabolites

In this section an attempt has been made to analyse and correlate the results obtained in a single brain between the levels of gene expression and the compounds detected within the tryptophan degradation pathway. At each time point, 1 month and 2 months after infection, four infected animals were individually compared to the average of the expression and compunds levels detected in the control mice.

At 1 month after infection IDO was raised in most amimals with exception of one, which failed to induce IFN-γ. The mouse that failed to produce IFN-γ also did not have increased level KYNA and KYNAMO. Trypohan levels are higher in the control brains compared to each of the infected mice. However, this does not have an effect on serotonin, which in both aniumal groups, control and infected, are at the same level (Table 2.5).

At 2 month after infection IDO was not raised in all the infected animals. Consequently IFN-γ levels were raised only in the animals with IDO upregulated. Nevertheless, this did not have an effect on levels of expression of KYNA and KYNAMO enzymes. HD expression levels instead are increased in all animals.
In all animals for the exception of one infected mouse the levels of serotnin and tryptophan did not differ from the mean of the controls. In this animal, where trypotophan levels and serotonin levels are higher also IDO and IFN-γ were not expressed (Table 2.6).

Table 2.5: Mean of the tryptophan degradation transcripts and mean of the levels of serotonin and tryptophan present in the control brains, compared with the levels of expression and compunds in the infected brains (shown individually) at 1 month after infection.

Month 1		IDO %	IFN %	HD %	KYNA %	KYNAMO %	Tryptophan	Serotonin
Control	Mean	0.25	30.78	98.77	0.78	27.07	19004011.50	184930.95
Infected	3A	5.73	326.68	2567.66	2.20	58.12	194204.97	194204.97
Infected	6C	0.17	41.33	461.90	4.01	0.00	105434.89	105434.89
Infected	2D	1.56	175.68	333.89	0.69	43.92	223146.75	223146.75
Infected	6F	7.21	132.57	12.48	0.00	0.89	164791.92	164791.92

Table 2.6: Mean of the tryptophan degradation transcripts and mean of the levels of serotonin and tryptophan present in the control brains, compared with the levels of expression and compunds in the infected brains (shown individually) at 2 month after infection.

Month 2		IDO %	IFN %	HD %	KYNA %	KYNAMO %	Tryptophan	Serotonin
Control	Mean	0.01	16.30	54.03	0.70	17.87	2360495.53	181910.82
Infected	5A	16.34	364.79	2007.26	0.00	0.00	2413405.00	170347.84
Infected	1C	3.84	164.85	2432.91	4.84	187.42	1624861.25	151653.48
Infected	1F	0.00	0.00	1075.62	1.71	74.07	9925059.00	498576.28
Infected	4E	0.00	2.03	175.62	0.66	40.63	2413405.00	170347.84

2.3.7 Discussion

In this chapter I have investigated the hypothesis that *T. gondii* has evolved a mechanism to modify host behaviour during chronic infection. This was achieved in a series of rodent behavioural studies, molecular analyses of key molecules in tryptophan metabolism, cytokines and the tryptophan degradation pathway. In addition, the metabolomic profiling of infected brains compared to non-infected provides information regarding the neurochemical balance that can influence behaviour and neurological function.

2.3.8 Behavioural studies

Anxiety levels are a good indicator of behaviour alteration in rodent models and are assessed through the open field test and elevated plus maze task. Although the open field test is considered to be a proxy measure of anxiety levels of the animals challenged, we can conclude that chronically infected male BALB/c mice are less anxious compared to controls. An increased exploratory behaviour, also observed in several previous studies (Hay et al., 1983; Hay, Aitken, and Graham, 1984; Webster, 1994), demonstrates that infected mice are showing less fear to explore open environments. This behaviour is evident by the significant increased number of times that the infected animals enter the central zone of the open field and the time spent roaming around the centre of the field. The diminished anxiety levels in the infected mice are visible at 1 month after chronic infection. This is considered to be the initial phase of chronic disease and at 2 months post infection, once a full state of chronic disease is established we don't observe aby differences. At 1 month post infection mice are also showing an increase in locomotor activity compared to controls,

represented by a longer distance travelled in the field. This could represent a further demonstration that anxiety levels are low in the infected mice compared to non-infected controls. More distance travelled in the open field indicates less time spent in a freeze or immobile posture, a characteristic present in anxious animals. However, the increase in locomotor activity was not present at month 2 of infection and both groups of animals travelled a similar length in the field. Infected mice did show a preference to travel at the centre of the field and the control mice preferred to travel at the side protected by the walls of the box. Together these suggest a difference in the anxiety levels between control and infected mice.

Despite the changes in the open field behaviour, there were no differences in the anxiety levels between the control and infected mice as assessed in the elevated plus maze task. No difference was observed not even in the total travelled distance of the maze between the two groups. This result was not comparable with a previous study were the total distant travelled was increased in the chronic infected group compared to controls in the elevated plus maze (Afonso et al., 2012). Nevertheless, difference of the activity was reported in the open field in this study. This test is considered a more refined test to measure differences of the anxiety levels in the animals compared to the open field test. Alter locomotor activity in open field could relate to non-specific factors such as the environment itself different from the elevated plus maze. Furthermore, the differences observed could be a consequence of the mouse strain chosen for these studies. Notably, BALB/c mice are not always a preferable model for behavioural studies as they present a mutation in the tryptophan hydroxylase 2 (TPH2) enzyme leading lower levels of 5-HT compared to other mice

strains (An et al., 2011). However, they are considered an excellent model for chronic toxoplasmosis and the objective of these studies was to observe the behaviour of the host during an infection that mostly represented the human chronic infection in order to define the interaction of infection, immune response and nervous system.

An inactive response of the rodent in the pre-pulse inhibition (PPI) test is an indicator of behavioural alteration that can be translated to schizophrenia-like symptoms. In these studies no differences were found in the pre-pulse inhibition of the startle response between the control and chronically infected mice. This result is in alignment to a previous study by Kannan et al. (2010), where no PPI deficts were observed in chronic infected mice. However, the parasite strains of this study were different. PPI deficits can be produced by a number of mechanisms, including enhanced dopamine transmission and 5-HT receptor agonists in rodents (Farid et al., 2000). However, as previously mentioned, BALB/c mice are known to have a mutation in the tryptophan hydroxylase 2 (TPH2) that produces serotonin and low level of the neurotransmitter is present in the brain. Nevertheless, deficits are not measured in the PPI of control BALB/c mice. This could be that other mechanisms involved in this process have balanced out the effect of low levels of 5-HT present in the mouse brain. Our hypothesis states that *T. gondii* infection would lead to a further decrease of 5-HT production by the upregulation of the tryptophan degradation pathway and depriving the substrate for serotonin formation. Therefore, in the BALB/c model the mechanism involving 5-HT to obtain deficits in the pre-pulse inhibition circuit is not challenged by the infection and the deficits are not observed.

The timing of infection, the strain of the parasite, the immune system of the host and the genetic susceptibility of the environmental challenges of the host must also be considered. Therefore, I propose that further studies should be performed in order to understand the interrelation of infection and the immune system and the consequences on the central nervous system and on behaviour. The approach to study each mechanism separately is important to clarify the single contribution to the global system. Nevertheless, a multifactorial approach to study the behavioural consequences and the brain environmental of an individual upon the interaction with the external challenges is important. A global vision of the interaction of these mechanisms would possibly lead to a more focused therapeutic approach, which would contribute towards the global biochemical balance of the brain environment rather than the direct intervention on one aspect of the intricate process of the interaction between the systems.

2.3.9 Gene expression analysis

Analyses of the expression of genes involved in tryptophan metabolism, immunological responses and tryptophan degradation were performed to understand whether *T. gondii* infection has an influence on gene regulation that may impact on behavioural changes. Several differences were found in the levels of transcripts of different immunological markers and in the levels of expression of some of the enzymes involved in the tryptophan degradation pathway between controls and chronically infected brain samples. First of all, IFN-γ gene expression was found to be significantly higher at 1 after infection in the infected brain samples compared to controls, as expected. Moreover, the effect of cyst turnover on the immune system in

the brain involving the production of IFN-γ interacting with adhesion molecules involved in the recruitment of peripheral lymphocytes during the chronic infection has already been demonstrated (Wang et al., 2007). However, the up regulation of IDO expression in the chronic infected brain has not yet been experimentally described. In this study, we measured a significant increase of the gene expression of IDO, which is the first step in the tryptophan pathway degradation, at 1 month after infection in the infected brain compared to controls. Interestingly, we also observed differences in the gene expression of some of the other enzymes involved in the tryptophan degradation pathway. In particular, we have observed the up regulation of gene encoding 3-Hydroxyanthranilate 3, 4 dioxygenase (HD) at 1 month and 2 month after infection in infected brains compared to controls. This enzyme is involved in the formation of the quinolinic products from the tryptophan degradation pathway. In addition, the levels of the gene expression of the Kynureninase and Kyna 3-Monooxygenase were also found increased at 1 month after infection in the infected samples compare to controls. The latter two enzymes are also part of the formation of the quinolinic products at previous steps. Quinolinic acid is found to be neurotoxic. High levels of quinolinic acid is found to be in relation to several central nervous system diseases such as AIDS, dementia complex, Alzheimer's disease, Huntington's disease, multiple sclerosis and Parkinson's disease (Zádori et al., 2012). We have observed a significant up regulation of the molecular expression of the tryptophan degradation pathway, which favours the formation of the quinolinic products during *T. gondii* chronic infection. This could interfere with the brain chemical environment and participate in the behavioural changes that we observe in mice during *T. gondii* chronic infection. In contrast, the expression levels of the

genes encoding enzymes involved in the formation of the kynurenic acid are not affected in the same way. The expression of the gene encoding kynurenine aminotransferase I is significantly decreased in the infected brain samples compared to controls. However, this result was not consistent between different runs of the experiments performed during this study. Performing a BLAST gene search we found that there is high percentage of homology between the different aminotransferase enzymes genes present in the brain. Therefore, it may be possible that PCR amplified other genes encoding aminotransferase. Therefore, for future work, other techniques of measuring directly the end product of the tryptophan degradation pathway for the measurement of the kynurenic acid should be evaluated.

The molecular analysis of immunological cytokines in the brain was also performed. The significant up regulation of TNF-α at 1 month post infection in the infected brain samples compared to controls was expected as a previous study that describes the turn over of the tissue cysts in the brain demonstrates the up regulation of the immune system (Wang et al., 2007). However, interestingly in this study we observe the significant up regulation at 1 month post infection of IL-1β in the infected brain samples compared to controls. This is interesting because high levels of this cytokine are present in the cerebrospinal fluid of schizophrenic individuals (Miller et al., 2009; Suvisaari et al., 2011; Westling et al., 2011). In addition, Il-1β and TNF-α have a direct effect on the hypothalamus-pitutary-adrenal axis, which is very important for several regulatory activities within the organism. In particular, the immune, the endocrine and the nervous systems are regulated by the HPA axis. The up regulation of the HPA axis contributes to the down regulation of the immune

response and it has a further effect on the tryptophan pathway by regulating the IDO and TDO enzyme activities (Maes et al., 2011). This further regulation could contribute to the increase of the brain environment imbalance and have a negative outcome on the overall behaviour observed in the infected mice. No differences between the controls and infected samples were observed in the expression of IL-6, also involved in the up regulation of the HPA axis and described during the pathology of schizophrenia (Krause et al., 2012). Herein, a global view is give and future work should involve the analysis of gene expression in specific locations within the brain, in particular where there is an increased number of a tissue cyst.

2.3.10 Metabolomic analysis

The metabolomic profiling of the analysis and the comparison between brains of infected mice compared to controls provides further information of the changes in the chemical network of the brain during *Toxoplasma gondii* chronic infection.

Metabolites involved in the tryptophan pathway

Although tryptophan is an important amino acid involved in the regulation of the immune system a consistent detection of high levels in the brain samples of the infected mice compared to controls at months 1 and 2 post infection were not expected. As previously mentioned, *T. gondii* promotes an inflammatory response characterized by the release of IFN-γ, which directly promotes tryptophan degradation pathway by up regulating the activity of the IDO enzyme towards the production of kynurenine metabolites and therefore a decrease of tryptophan levels in the infected samples was expected. Nevertheless, high levels of kynurenine

metabolites measured by mass spectrometry were still detected at 1 month and 2 months after infection. This, together with the up regulation of expression of the genes encoding the enzymes involved in the tryptophan degradation pathway, supports the hypothesis of the ability of *T. gondii* to alter the host brain environment via the tryptophan/kynurenine pathway with the final outcome of influencing host behaviour. The detection of high levels of tryptophan in the infected brains underlines the consistent action and turnover of the immune system during *T. gondii* chronic infection in the brain.

Neurotransmitters, their precursors amino acids and metabolites

First of all, the up regulation of several amino acids in the brains of infected mice compared to controls clearly indicates the direct involvement of the parasite infection in the host brain metabolism to promote immunological responses against infection. In particular, at 1 month after infection higher levels of tryptophan, leucine, phenylalanine, serine and tyrosine are detected. In fact, amino acids are involved in cell growth, tissue repair and a general immune boost and regulation. Therefore, it was hypothesised that the levels of amino acids would have been elevated at 1 month post infection. This time point of infection coincides with the onset of the asymptomatic chronic infection and the development of tachyzoites into the quiescent bradyzoites form and therefore a more active role of the immune system in the brain would have been expected. In contrast at 2 months post infection the immune response was expected to be less active in the brain because of the establishment of tissue cysts containing bradyzoites. Low levels of aspartate and glutamine were measured in the infected samples at 2 months after infection.

However, metabolomic measurements reveal high levels of several other amino acids, including tryptophan, leucine and phenylalanine in the infected brains also at 2 months post infection suggesting the continuous involvement and influence of the immune system in the brain environment. Furthermore, the inconsistency of the levels measured of threonine at 1 month after infection and the levels measured of tyrosine at 2 month after infection between the experiments demonstrates the continuous activity of several pathways in the brain during infection. This is also confirmed by the molecular analysis of cytokine expression in the brain by real time PCR.

High levels of the amino acid phenylalanine in the infected brain samples compared to controls should correlate with high levels of dopamine in the infected brain samples compared to controls. This would have been also consistent with the recent literature that have demonstrated the ability of *T. gondii* to directly interfere and up regulate the dopamine levels of the infected host by releasing the enzyme tyrosine hydroxylase in the cell host environment (Prandovszky et al., 2011). This was not observed and in contrast lower levels of dopamine were registered in the brain samples of the infected mice compared to controls at 1 month and 2 month after infection. However, the levels of the amino acid tyrosine also involved in the dopamine production were found to be inconsistent between the experimental runs at 2 month after infection, indicating that further studies are needed to clarify this point. Low levels of the neurotransmitter glutamate and 4-aminobutoanate were also observed, at both time points in the infected brain samples compared to controls. These compounds play a major role in the inhibitory brain system. In addition, low

levels of 1-Aminocyclopropane-1-carboxylic acid, involved in the NMDA receptor function were also found in the infected samples compared to controls at 2 month after infection time point. The behaviour of these metabolites clearly shows a direct effect of *T. gondii* infection on the host neurotransmitters and the possible involvement of the drastic neurochemical changes that could influence the host behaviour and lead to pathology. Low levels of dopamine have been associated with boredom and lack of interest type of behaviour (van Nimwegen et al., 2005). Low levels of dopamine caused by dopaminergic neuronal cell death have also been characterized as the principal feature in the neurodegenerative disease of Parkinson and studies have suggested that neuronal cell death could be trigger by viral infection (Toovey et al., 2011). Initial epidemiological studies have associated *T. gondii* with Parkinson disease as a possible environmental risk factor, which would promote a chronic neuroinflammation that could end towards neuro degeneration and the development of pathology (Miman et al., 2010). In this context of the condition of neuroinflammation triggering neurodegenerative diseases *T. gondii* infection has also been associated to Alzheimer's disease through epidemiological studies (Kusbeci et al., 2011). In addition, a recent feature described in the pathogenesis of Alzheimer's disease is the depletion of the glutamate inhibitory neurotransmission (Tiwari and Patel, 2012). Overall, more specific experiments to measure the levels of these neurotransmitters per brain region during *T. gondii* chronic infection are necessary in order to comprehend the specificity and significance on the effects of infection on neurotransmitter production and release.

Purine/pyrimidine matabolites

Another exciting conclusion from the metabolomics analysis is the effect of *T. gondii* infection on the purine and pyrimidines metabolism in the brain. Several of the compounds involved in the purine pathway metabolism were consistently detected and found to be lower in the infected brain samples compared to controls at 1 and 2 months post infection, in particular, adenine, adenosine, AMP and uridine. Vice versa, compounds of the pyrimidine pathway, guanosine and guanine, and involved in the purine production were found to be higher in the infected samples compared to control at 1 month after infection and lower at 2 month after infection. Furthermore, xanthine levels, involved in the purine and pyrimidine degradation pathway were higher both at 1 month and 2 month after infection in the infected samples compared to controls, indicating a clear involvement of the purine and pyrimidine pathway during *T. gondii* infection. First of all, *T. gondii* directly salvages purines from the host for its own metabolism. Therefore, a decrease in the brain environment of purine would be expected. Purines and in particular ATP and adenosine have been now established as important neuronal co-transmitters with glutamate, noradrenaline, GABA, acetylcholine and dopamine (Poelchen et al., 2001). Purines participate in the regulation and the activity of neuronal functions from learning and memory tasks to sleep regulation, anxiety and stress response and appetite (Burnstock et al., 2011). The involvement of an imbalance and dysregulation of the purine metabolites has been described in several pathologies such as neuroinflammation, ischemia, and neurodegeneration, including Alzheimer's disease. In particular, low levels of adenosine are linked to a low anxiety behaviour phenotype. Studies have shown that by blocking the adenosine receptor A2 by using antagonists such as caffeine, the

locomotor behaviour of treated rodents is increased (El Yacoubi et al., 2000). This illustrates the effects of adenosine on anxiety and locomotor behaviour. The decreased levels of adenosine, adenine and AMP in the brain of the infected samples compared to controls mechanistically can explain the low anxiety levels measured by the open field test in the infected mice, behaviour which would increase the likelihood of the infected rodents to be predated by felines and therefore increase the possibility of *T. gondii* life cycle to be completed.

Other metabolites

Piperidine is a constituent of mammalian brain. Its levels are altered in the infected brain samples compared to the controls, although not consistently in all the experiments. However, this could be also an interesting point as Piperidine may have close connections with neuroendocrine activity as well as neuronal functions, and further, with the mechanisms underlying sleep-consciousness and emotional function (Kasé et al., 1989).

Changes of the levels of the N-acetyl-D-glucosamine compound instead indicate differences in the glycolysation process in the infected brain samples compared to controls. Glycosylation in the brain has also been associated with neurodegenerative process. This adds another aspect to the big picture present in the brains of chronic infected mice compared to controls.

Creatine levels are not here discussed, as it is involved in the end reactions of many metabolomic pathways.

2.3.11 Effects of the changes in gene expression in the Tryptophan degradation Pathway on the tryptphan and serotonin metabolites

In the infected brain samples we can observe that the upregulation of gene transcripts involved in the tryptophan pathway directly has an effect on diminishing the levels of serotonin in the brain at 1 month and 2 months after infection.

Instead trypotophan levels do not dimish at 1 month after infection, in contrast high levels are observed in the infected brain. This can indicate that the immune system at 1 month after infection is still active because of *T. gondii* infection and the changes of the tryptophan levels caused by the upregulation of the gene transcripts involved in the tryptophan degradation pathway are only visible at 2 month after infection.

2.3.12 Concluding comments

From these studies we can definitively conclude that *T. gondii* chronic infection is not asymptomatic in BALB/c mice. We have measured several changes that occur in the infected host from the phenotypic behaviour to the brain immunochemical environment. The parasite infection causes a decrease in the levels of anxiety in the host and this could lead to dysfunctional responses towards danger events. A more active immune environment is present in the brain of the chronic infected host, which leads to the imbalance in the pathways of the brain network causing damage to the brain. In particular, we measure changes in the expression of the enzymes involved in the tryptophan pathway degradation during chronic infection leading to the production of kynurenine metabolites, which are neurotoxic and present in high levels in many brain disorders pathologies (Figure 2.24). However, the parasite could

interfere directly with the brain environment and not indirectly through the immune system, by scavenging host molecules and creating chemical imbalance in the brain which could lead to pathology and changes in behaviour. Through the metabolomic analysis we have measured a decrease in the purine metabolites, such as adenine and adenosine, in the brain of the infected host compared to the controls. *Toxoplasma gondii* scavenges these metabolites for its own benefit and by doing so; it also affects host behaviour and the host neuroenvironment. We suggest that *T. gondii* infection affects the host brain immunochemical and could trigger the development of several pathologies affecting the brain, from degenerative disorders to psychiatric disorders, in vulnerable individuals, genetically predispose or subjected to other environmental challenges, such as drug abuse. Therefore, the understanding of the effects of *T. gondii* chronic infection in the host would lead towards the better understanding of the causes involved in the development of many pathologies affecting the brain and possibly provide insight into potential drug treatments and precautionary measures, such as vaccination.

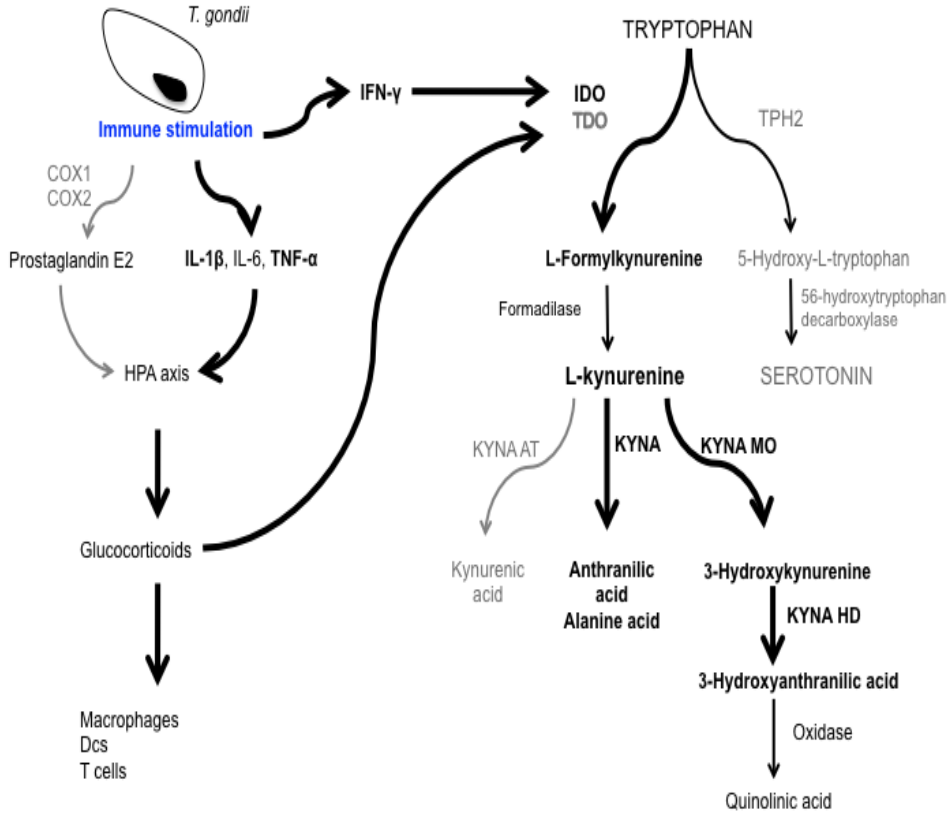


Figure 2.24: In bold black the enymes and pathway that are upregulated in the brain during *T. gondii* chronic infection are highlighted.

3 *T. gondii* congenital transmission: behavioural, immunological and metabolomics consequences

3.1 Introduction

Schizophrenia and other psychiatric disorders have been described as having multi-factorial etiologies (Van Winkel et al., 2010). The genetic contribution to the development of these diseases has been a considerable focus of research (Gejman et al., 2010). However, environmental etiologies including those occurring in utero or postnatal as the brain develops have been subject to an increased interest in recent years (Arnold et al., 2005). In fact, several studies have focused on the possible risk factors during the prenatal and the postnatal period that could intervene and interfere with the healthy development of the central nervous system of the foetus, leading to disruption of the brain neurodevelopment with consequences for behaviour and neuropsychiatric disease (Morgan and Bale, 2011; Haukvik et al., 2012; Sim et al., 2012). These potential effects have been collectively referred to as the 'Neurodevelopmental hypothesis'. Environmental risk factors that have been considered include complications during birth, stress due the exposure of loud noises, nutritional deficiencies, drug abuse and infection (Brown and Derkits, 2010). Infection particularly has recently received increased attention as an environmental risk factor in the development of psychiatric disorders (Arias et al., 2012).

A possible mechanism to explain how environmental risk factors contribute to the development of disease is their ability to induce epigenomic changes in the genome of the individual. These changes include alteration of DNA methylation that affects gene expression and therefore the phenotype of the individual (Gavin and Sharma, 2010).

Aside from epigenetic, other mechanistic hypotheses are also being evaluated and the direct effect of infectious agents or the effects of the immunological mediators released in response to infection might impact on neurodevelopment. These mechanisms are likely to vary according to the time of infection during development, the immunological status of the individual and their genetic susceptibility to the particular infection (Meyer, 2011).

In this study we examine the ability of *T. gondii* infection during gestation to affect the behaviour of the offspring when adulthood is reached. In addition, we also determine if maternal exposure to *T.gondii* during pregnancy affect the behaviour of offspring that do not contract congenital infection. First of all, the ability of *T. gondii* to modify the behaviour in the infected host has been a topic of interest for the past 20 years. It has been shown that the natural fear towards the odour of cat urine in rodents infected by *T. gondii* is inhibited and replaced by an actual attraction towards it (Vyas et al., 2007; House et al., 2011). Therefore, the behaviour consequences of *T. gondii* infection in humans have become a centre of focus and importance. Many epidemiological studies have associated *Toxoplasma* infection with patients affected by schizophrenia (Buka et al., 2001; Conejero-Goldberg et al., 2003; Alvarado-Esquivel et al., 2006, 2011; Torrey et al., 2007; Zhu et al., 2007; Hinze-Selch et al., 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, Torrey, et al., 2007; Niebuhr et al., 2008; Ahmad et al., 2010; Hamidinejat et al., 2010; Okusaga et al., 2011; Pedersen et al., 2011; Tedla et al., 2011; Horacek et al., 2011). A recent meta analysis termed that the odds ratio for schizophrenia was of the order of 2 for *T. gondii*, which is considerably greater than many of the generic risk factors. In

addition, certain behavioural traits through psychological tests have been characterised to be common in individuals infected by *T. gondii* (Flegr et al., 2003; Novotná et al., 2005; Skallová et al., 2005; Lindová et al., 2006; Hodková et al., 2007). Secondly, there is a vast collection of ecological, birth cohort and epidemiological data and *in vivo* laboratory studies that describe an association between maternal infection during pregnancy and schizophrenia (Fatemi et al., 1999; Buka et al., 2001; Shi et al., 2003; Wen et al., 2003; Brown et al., 2005; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, Torrey, et al., 2007; Xiao et al., 2009; Pedersen et al., 2011; Wang et al., 2011). In addition, some of the maternal infection studies resulting in congenital infection also show the same association between timing of infection and psychiatric disorders (Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, and Yolken, 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, Torrey, et al., 2007; Pedersen et al., 2011; Wang et al., 2011). This evidence gives support to the neurodevelopmental hypothesis in the pathology of psychiatric disorders and the importance of infection and the immune status of the mother during pregnancy as a risk factor.

Several mechanisms might account for brain damage during congenital infection. For example, studies have shown that placenta damage caused by an inflammatory immune response of the mother during infection could interfere with the process of brain development (Brown and Derkits, 2010). In addition, the parasite can infect the foetus transplacentally and directly affect the brain or induce immunological mediators that have similar affects as observed in post-natally acquired indection.

In this study we evaluate the consequences of the tryptophan degradation pathway, which is regulated by the immune system. The enzyme IDO is upregulated by the release of IFN-γ and as a consequence, tryptophan, the substrate for 5-HT production is depleted. This also results in the formation of KYNA metabolites, which are found to be present in high concentrations in the brains of schizophrenic individuals and are neurotoxic (Schwarcz et al., 2001). Furthermore, *T. gondii* itself requires host tryptophan and could therefore potentially reduce the availability of this essential amino acid in the brain. Herein, the behavioural traits in rodents infected with *T. gondii* in utero or exposed to the maternal immune response to *T. gondii* from day 12 of gestation are evaluated. The possible involvement of the tryptophan degradation pathway is examined in depth. In addition, the possible involvement of other pathways that could be disrupted directly or indirectly by the parasite infection are investigated through metabolomics.

3.2 Material and Methods

3.2.1 Experimental plan and Infection

A modified Whitten breeding protocol was followed (Dalal et al., 2001). To synchronize and have a successful litter some material from the female BALB/c mice bedding was sprinkled into the BALB/c male cages. The following day single males were paired with 3 to 4 females. Each day the females were checked for vaginal plugs, sign of a possible pregnancy, and if successfully found the females would then be separated from the males. The putative pregnant females with vaginal plugs were weighed daily and subjects with no weight increase were eliminated from the experimental procedure. On day 12 of gestation the dams were infected orally, to mimic human infection, which usually occurs through the ingestion of infected undercooked meat (Sukthana, 2006), by gavage with a concentration of 10 cysts of *T. gondii Beverley* strain (type II) per animal in 200µl of 1X sterile Phosphate Buffer Saline (Invitrogen, UK). The time of infection, the second trimester of gestation, was chosen to diminish the risk of abortion and increase the incidence of parasite transmission to the embryos (Roberts and Alexander, 1992). A number of pregnant dams were not infected in order to have a sufficient number of offspring for the control group. Once the litters were born, both control and the congenital infected group were fostered. New mothers carried out the fostering for all groups. Studies have shown a possible relationship between fostering and the neurodevelopment process as a risk factor in the development of behavioural disorders (Matthews et al., 2011). Therefore, it was important to maintain this factor present in both groups in order to establish that any behaviour differences observed would have been caused only by the additional factor of the parasite infection and not by the fostering effect

of the offspring in adulthood. The infected dams were sacrificed after giving birth according to the Home Office license procedures. This protocol was followed twice, at separate times, in order to achieve the necessary number of animals per group for statistical analysis as the first experimental process did not obtain the usual rate of 50% of infection in the infected litter. The results and analysis were obtained by combining the data from the two experiments.

3.2.2 Animal caging

Animals were housed 5 to a cage. The cages were provided with bedding, and with water and food ad libitum. The rooms where the animals were held were on a cycle 12 hour light and 12 hour dark. The testing was always carried out during the 12 hour light cycle.

3.2.3 Behavioural Studies

The behavioural tests were carried out on each individual mouse in a random sequence between and within the groups at 6, 10 and 14 weeks old in accordance with Home Office regulations. At each time point, the order of the experiments progressed from the least invasive to the most aversive for the animal. Therefore the first experiment involved the observations of the general locomotor activity in the open field test, followed by the measure of anxiety levels in the elevated plus maze.

Open field

This was performed as described in Chapter 2. The dimensions of the black Perspex box were 40cm wide; 40cm deep and 36cm height. Each mouse was placed in the

centre of the arena and allowed to explore for 10 minutes. The Ethovision 3.0 program (Noldus Information Technology, Netherlands) was used to track the movement of each animal. The parameters measured were in the total distance travelled in the box, the time spent in the centre of the box and the number of times the animal entered the centre of the box.

Elevated Plus Maze

This was performed as described in Chapter 2. The elevated plus maze used had two open arms and two closed arms. Each arm of the cross was 30 cm long and 6 cm wide. Each animal was tested in the maze for 10 minutes and the movements were tracked using the Ethovision 3.0 program (Noldus Information Technology, Netherlands). For each animal the total distance travelled in the maze, the number of entries into the open arms and the enclosed arms as well as the time spent in the arms was calculated.

Behavioural Statistics

As the data was not normally distributed the Kruskal-Wallis test selected to compare the three groups: control, congenital infected and the non-infected litter mates. The p value of <=0.05 indicated a significance difference between the groups overall. To identify any significant differences between individual groups the post hoc value Bonferroni-Dunn test was performed.

3.2.4 Antibody ELISA

Tail Bleeding and sera preparation

At the last time point of the behavioural study, mice born from a mother infected during pregnancy underwent tail-bleeding procedure in order to collect serum samples and perform antibody ELISA to distinguish the infected mice from the uninfected. The blood was collected by clipping off the tip of the tail by scissors, using a capillary column and decanting it into a centrifuge tube. The blood samples were then centrifuged at 4000 rpm for 10 minutes. The supernatants were collected and stored at -20°C till further use.

Optimisation of the Antibody ELISA

IgG1 and IgG2a antibodies were tested on blood samples collected from mice born from an infected dam during pregnancy in order to distinguish whether the offspring were congenitally infected with *T. gondii*. IgG antibody production occurs once the individual reaches adulthood and the immune system is fully developed. For this reason, the blood was collected from the animals of the experimental group at the end of the study. In addition, the procedure of blood collecting did not influence the possible behaviour changes in the group of animals.

Toxoplasma Lysate Antigen preparation

Antigen preparation was achieved in vitro by infecting Human foreskin fibroblast cells (HFF). HFFs were grown in IMDM (Invitrogen, UK) supplemented with 125µg pencillin/streptomycin (Sigma, USA), 125µg Amphotericin B and 10% Foetal calf serum (FCS) in 75cm^3 flasks (Corning, UK). Once the HFFs reached confluence they

were infected with *Toxoplasma gondii* RH strain, by directly putting into the flask an aliquot of parasite previously prepared in storage medium (IMDM 70%, DMSO 10%, FCS 20%) and stored at -80°C. When *T. gondii* had multiplied and invaded 95% of the HFFs, the content of the flasks were collected. The preparation was centrifuged at 1200rpm for 5 minutes at 4˚C and the pellet was resuspended in 500µl of distilled H_2O. The preparation was frozen in liquid nitrogen, defrosted at 60°C and then passed through an 25 gauge needle 12 times. This cycle was repeated 6 times, followed by the addition of 4500µl 1X Phosphate Buffer Saline (Invitrogen, UK). The TLA concentration was then measured by protein quantification assay according to manufacturer's instructions (Biorad, USA).

ELISA

96 well ELISA plates (Greiner Bio-One Ltd, UK) were coated the previous night with 100µl per well of 5µg/ml of *T. gondii* antigen diluted in 1XPBS pH 9 and incubated at 4°C over night. Plates were washed 3 times with wash buffer (1X PBS pH 7, 1:20 Tween 20). To each well 150µl of blocking solution (5% (w/v) of dried skimmed milk, wash buffer) was added, followed by an incubation at 37°C for one hour. Plates were then washed a further 3 times in wash buffer. The serum samples, collected from tail bleeding from the mice, were diluted 1/500 in a solution of 2.5% (w/v) milk and wash buffer. 200µl of diluted sample was added to top row. All samples were made in duplicates. On the plate, a series of dilutions of the sample were carried out from top row to bottom by adding 100µl of 1XPBS pH 7.4. The plate was then incubated at 37°C for one hour. The plate was then washed 4 times with wash buffer. This was followed by the addition of 100µl of the conjugate

solution of the two antibodies of interest: IgG1 1/20000 in 1X PBS pH 7.4 and 2.5% (w/v) of dried skimmed milk and IgG2a 1/5000 in 1X PBS pH 7.4 and 2.5% (w/v) of dried skimmed milk. The plates were incubated for one hour Incubation was at 37°C and then washed for 5 times. 100µl of substrate composed by 10ml of sodium acetate pH 5.5, 100µl of TMB, 5µl of H_2O_2 for each plate was added to each well and followed by incubation at 37°C for 30 minutes. To stop the colorimetric reaction 50µl of 10% (v/v) H_2SO_4 was added to each well and the plate was read at 450nm of the spectramax spectrophotometer (Molecular Devices, UK).

3.2.5 Molecular Analysis

The left hemisphere of the brain of the animals from the congenital infected, the congenital non-infected and the control group were stored in TRIzol™ (Life Technologies, Paisley, UK) at -80°C until RNA extraction, cDNA synthesis and Real time PCR on the samples according to the protocols described in chapter 2.

The quantitative gene expression analysis was achieved via real time PCR through the use of standards curves constructed for each considered gene (from 30 to $3x10^6$ gene copy number) with the exception of COX1, the gene copies of which were calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

In fact, because of the high presence of lipids and the high density of tissue in the brain, genomic contamination was frequently present. For this reason, some samples of this study were lost. Therefore, optimisation continued with a number of samples non related to this study. In total the end number of the brains analysed per group were 11 controls, 16 congenital infected and 6 non-infected litter mates. A sufficient

number of brain samples were successfully analysed per group for satisfying statistical analyses.

Molecular statistics

As the data obtained was not normally distributed the Mann-Whitney U test was selected to calculate the presence of a possible significant p value between the groups.

3.2.6 Metabolomics analysis

At the end of each congenital experiment the mice were sacrificed according to the Home Office license and the brain were extracted to carry out molecular and metabolomics analysis. The protocol of sample preparation and data processing is described in chapter 2. At the end of the congenital experiment the brain samples were analysed by mass spectometery using an LTQ-Orbitrap (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) the following day after sacrifice and sample preparation.

Metabolomic statistics

The student t test was performed to establish the presence of possible significance differences between the metabolites detected in control and infected mouse brains.

3.3 Results

3.3.1 ELISA

From the IgG ELISA for seropositive evaluation we found that 22 out of 30 pups born to 8 mothers were congenitally infected with *T. gondii*. These infected pups together with the congenitally exposed but not infected mice and the control mice were assessed in the open field and the elevated plus maze.

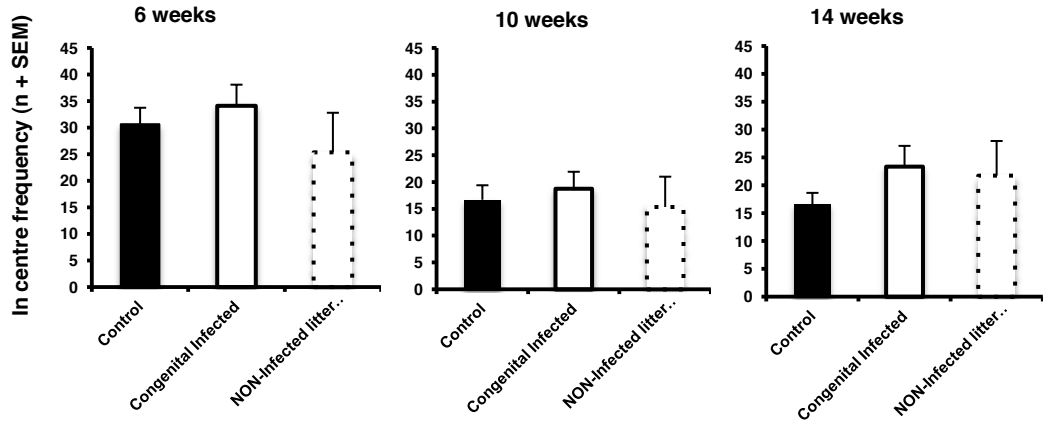
3.3.2 Congenital infection alters general locomotor behaviour in mice as determined by open field

The number of entries (n) into the centre of the open field did not differ between the congenital infected group, the congenitally exposed non infected and the controls at 6, 10 and 14 weeks of age (Figure 3.1 A).

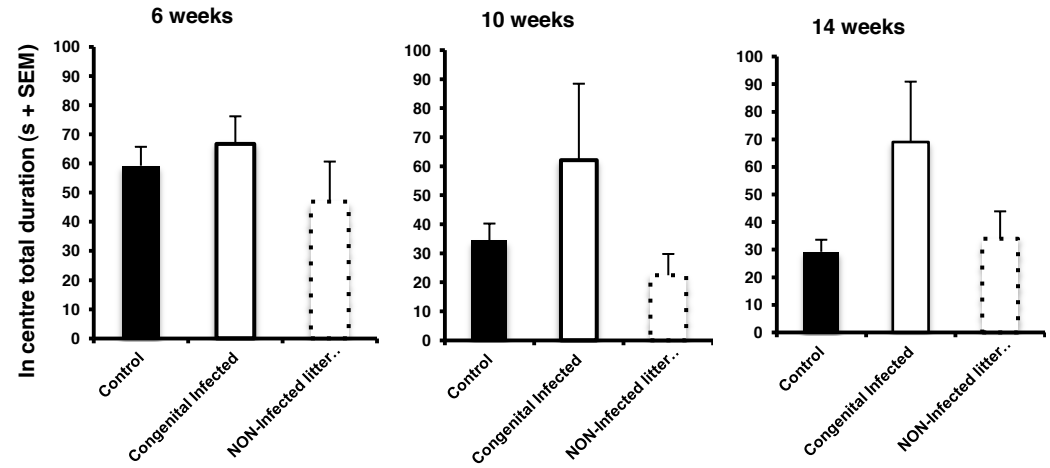
The time spent (s) in the centre of the open field at 6, 10 and 14 weeks also were not significantly different between the three groups taken into consideration, congenital infected mice, congenitally exposed non infected mice and control mice (Figure 3.1 B).

The total distance moved (cm) at 6 weeks and 10 weeks old was not significantly different between the three groups of animals. At 14 weeks of age the total distance travelled of the congenital non infected group was significantly increased compared to the congenital infected group ($p=0.01$) and control group ($p<0.01$). No difference was observed in the total distance travelled in the open field at 14 weeks of age between the mice of the congenital infected group and the control group (Figure 3.1 C).

A


6 weeks
10 weeks
14 weeks
In centre frequency (n + SEM)
45
40
35
30
25
20
15
10
5
0
Control
Congenital Infected
NON-Infected litter..

B


6 weeks
10 weeks
14 weeks
In centre total duration (s + SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Congenital Infected
NON-Infected litter..

C

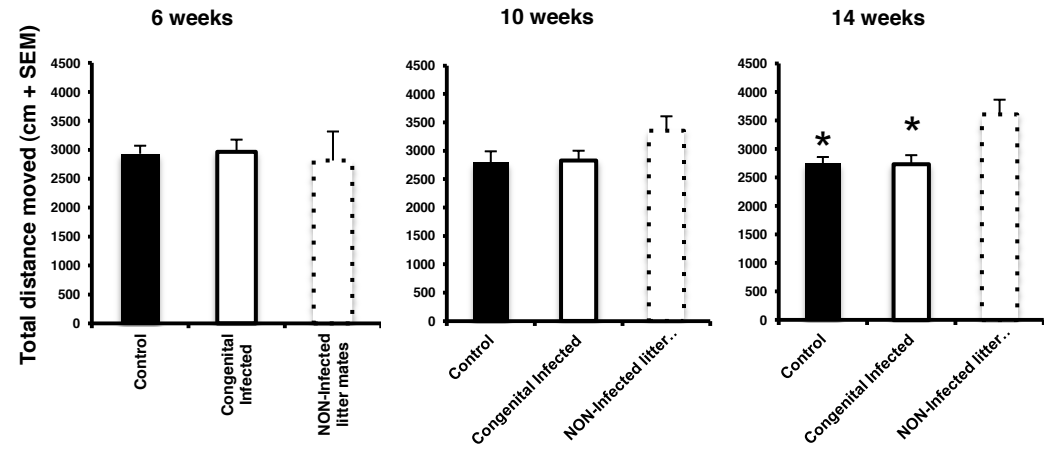

6 weeks
10 weeks
14 weeks
Total distance moved (cm + SEM)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
Control
Congenital Infected
NON-Infected litter mates
NON-Infected litter..

Figure 3.1: Represents the performance of 43 control BALB/c mice (black continuous line), (19 male and 24 female), 22 congenitally infected BALB/c mice (black discontinuous line) with *T. gondii Beverley*, (11 male and 11 female), and 8 non-infected litter mate mice (dotted line), (6 male and 2 female) in the open field task for a 10 minute trial at 6, 10 and 14 weeks of age. Graph A represents the average number of entries (n) at the centre of the field for each group. No differences were observed between the groups. Graph B represents the average of the time spent (s) in the centre of the field for each group. No differences were observed between the groups. Graph C represent the average of the total distance moved (cm) in the field during the 10 minute task. At 14 weeks old the non infected litter mates significantly travelled more distance compared to the controls and the congenitally infected

3.3.3 *T. gondii* congenital infection increases anxiety levels in mouse as determined by the Elevated Plus Maze

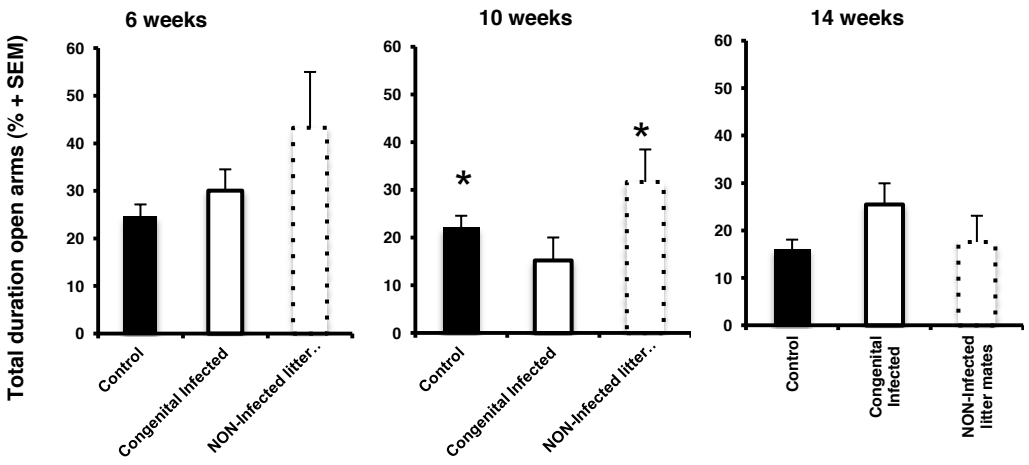
The number of entries and time spent in the open arms were calculated as a percentage of the total number of entries and time spent in all the arms of the maze (Figure 3.2 A-B).

The percentage of the duration of the time spent in the open arm (%) at 6 weeks old was not statistically different between the three groups. At 10 weeks of age old it was observed that the percentage of the duration of time spent in the open arms was significantly lower in the congenital infected group compared to the control group and to the non-infected litter mates with p values of 0.015 and 0.016, respectively (Figure 3.2 A). No difference was observed between the control and the congenital exposed non-infected group at this age. At 14 weeks of age no differences were observed between the groups (Figure 3.2 A).

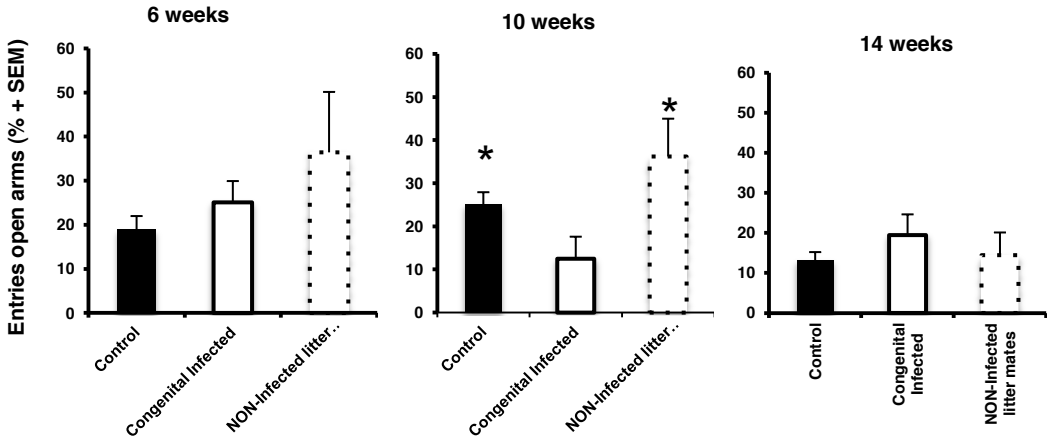
A similar pattern of behaviour was observed for the percentage of the number of entries into the open arm. As with the percentage of time spent in the open arms, there were no differences between groups for the percentage of open arm entries at 6 and 14 weeks of age. Hence at 10 weeks of age the mice in the congenital infected group spent significantly reduced entries into the open arms compared to the controls mice and non-infected litter mice, with p value of 0.0009 and 0.015, respectively (Figure 3.2 B). No differences were observed between the controls and non-infected litter mice.

The overall distance travelled (cm) inside the maze did not differ between the groups, at any of the observed time points (Figure 3.2 C). This indicates that the reduced percentage of entries and time spent in the open arm observed at 10 weeks of age compared to the other groups was related to their anxiety levels, rather than their activity levels.

A


6 weeks
10 weeks
14 weeks
Total duration open arms (% + SEM)
0
10
20
30
40
50
60
*
Control
Congenital Infected
NON-Infected litter..
Congenital Infected
NON-Infected litter mates

B


6 weeks
10 weeks
14 weeks
Entries open arms (% + SEM)
0
10
20
30
40
50
60
*
Control
Congenital Infected
NON-Infected litter..
Congenital Infected
NON-Infected litter mates

C

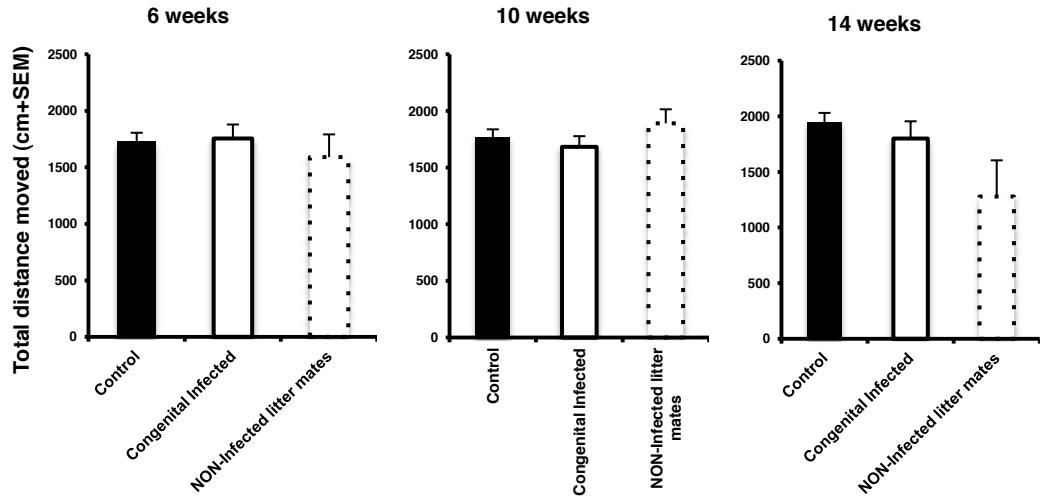

6 weeks
10 weeks
14 weeks
Total distance moved (cm+SEM)
0
500
1000
1500
2000
2500
Control
Congenital Infected
NON-Infected litter mates
Congenital Infected
NON-Infected litter mates

Figure 3.2: represents the performance of 43 control BALB/c mice (black continuous line), (19 male and 24 female), 22 congenitally infected BALB/c mice (black discontinuous line) with *T. gondii Beverley*, (11 male and 11 female), and 8 non infected litter mates mice (dotted line), (6 male and 2 female), in the elevated plus maze open task for a 10 minute trial at 6, 10 and 14 weeks old. Graph A represents the average of the percentage of the time spent in the open arm for each group. At 10 weeks of age the congenitally infected group spent significantly less time in the open arm compared to the control and the congenitally exposed not infected group (*, p=0.0009 and 0.015). Graph B represents the average of percentage of entries in the open arms for each group. At 10 weeks of age the congenitally infected group significantly entered into the open arm a significantly less number of times compared to the control and the congenitally exposed not infected group (*, p=0.015 and 0.016). Graph C represent the average of the total distance travelled (cm) in the maze during the 10 minute task. No differences were observed between the groups. All data are expressed as mean + SEM.

3.3.4 Gene expression analysis

The quantitative real time data analysis was carried out using the standard curve method, as described. The number of copies for each gene was then expressed as the percentage of the relation of the number of copies of the house keeping gene, TBP. This measurement was done for all genes for the exception with COX1, which was analysed using the $2^{\Delta\Delta C}{}_{T}$ method in relation of the house keeping gene TBP.

The analysis of the molecular expression were carried out at the end of the last behavioural time point observed, when the animals were at 14 weeks of age. The number of samples per group was as followed: 11 cDNA samples of the control group, 16 from the congenital infected group, 6 from the non-infected litter mates group.

The order of the figures is as follow: firstly, the effect of IFN-γ on the tryptophan pathway is examined, secondly all other immunological mediators in the brain during chronic infection compared to controls are observed.

IFN-γ expression in the congenital infected group was significantly increased compared to the non-infected litter mates group, (with p values of 0.002) and the control group (with p value of 0.0000003) (Figure 3.3). Similarly this was also observed in the levels of TPH2 in the congenital infected group compared to the non-infected litter and control groups (p value of 0.004 and 0.049, respectively) (Figure 3.6). KYNA expression was significantly increased in the congenital infected group compared to controls, with p equal to 0.015 (figure 3.8). No differences were

observed in the expressions of the other enzymes involved in the tryptophan degradation pathway.

IL-1β expression was found to be significantly increased in the congenital infected group compared to the non-infected litter mates and control groups (p values of 0.0008 and 0.0171, respectively) (Figure 3.13). In addition, the IL-1β expression was decreased in the non-infected litter mates group compared to the control group with a p value of 0.02 (figure 11).

No differences were observed in the expression of the other immunological mediators.

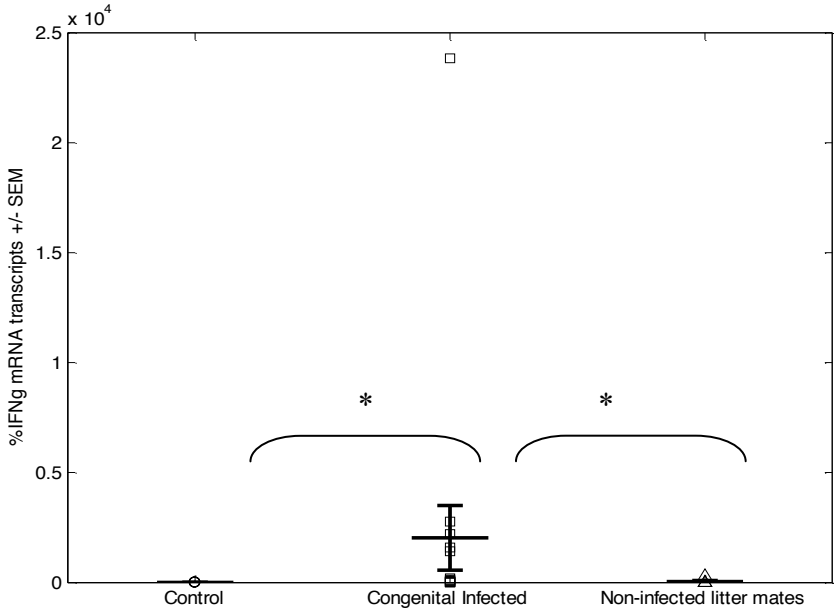


Figure 3.1: represents the expression levels of IFN-γ TPH2 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white traingles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. The expression of IFN-γ in the congenitally infected brain samples was significantly up regulated compared to controls and non-infected litter mates group samples (respectively, p= 0.0000003 and 0.002). This is represented by an *.

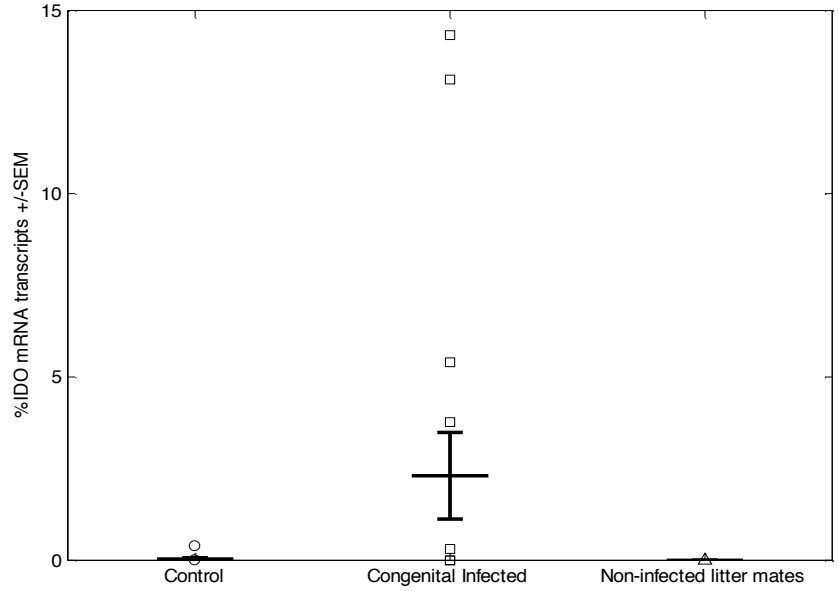


Figure 3.4: represents the expression levels of IDO in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks of age. No differences were observed.

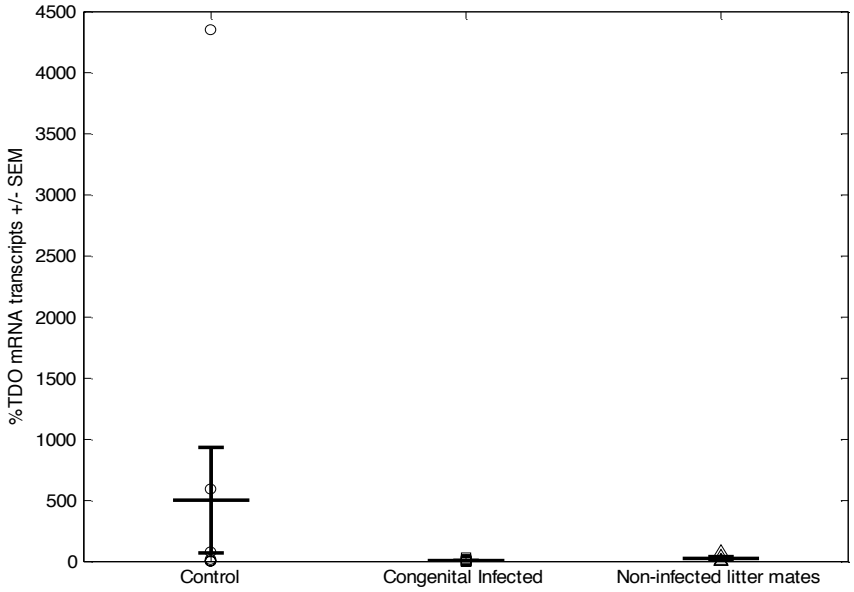


Figure 3.5: represents the expression levels of TDO in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

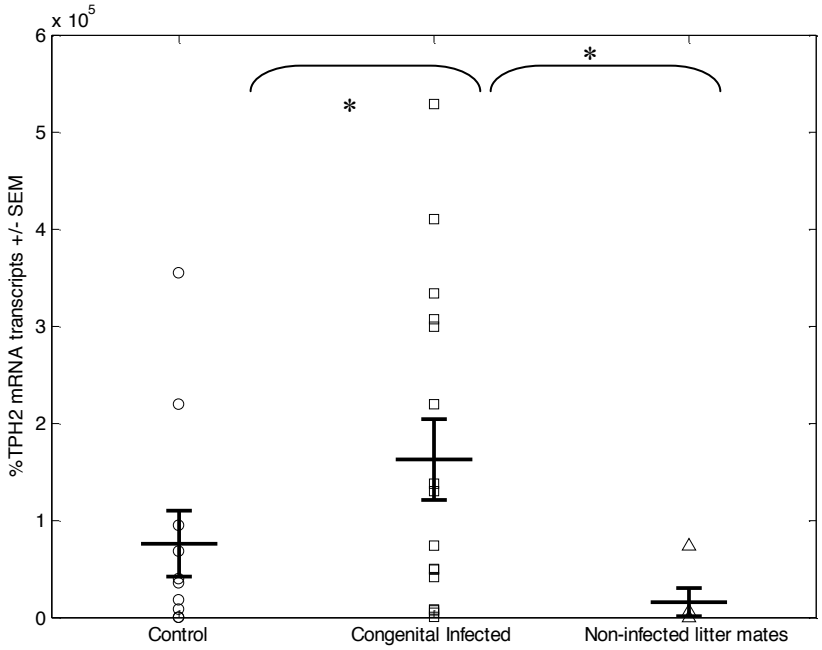


Figure 3.6: represents the expression levels of TPH2 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. The expression of TPH2 in the congenitally infected brain samples was significantly up regulated compared to controls and the non-infected litter mates samples (respectively, p= 0.049 and 0.004). This is represented by an *.

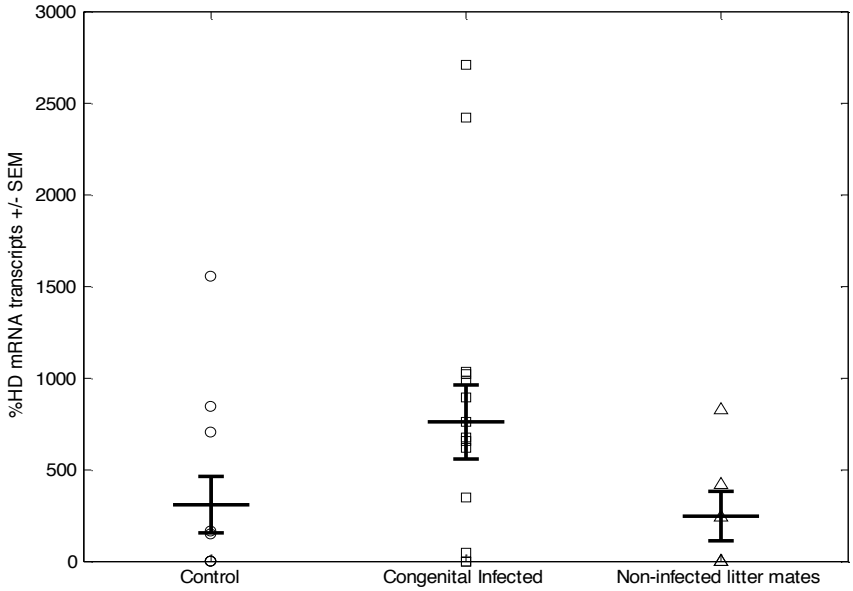


Figure 3.7: represents the expression levels of HD in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

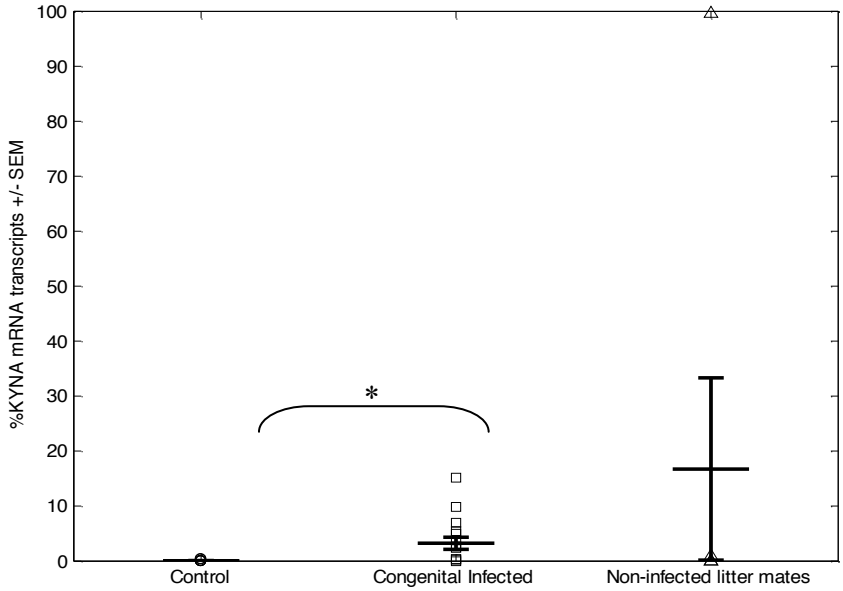


Figure 3.8: represents the expression levels of KYNA in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. The expression of KYNA in the congenitally infected brain samples was significantly up regulated compared to the controls brain samples (p=0.015). This is represented by an *.

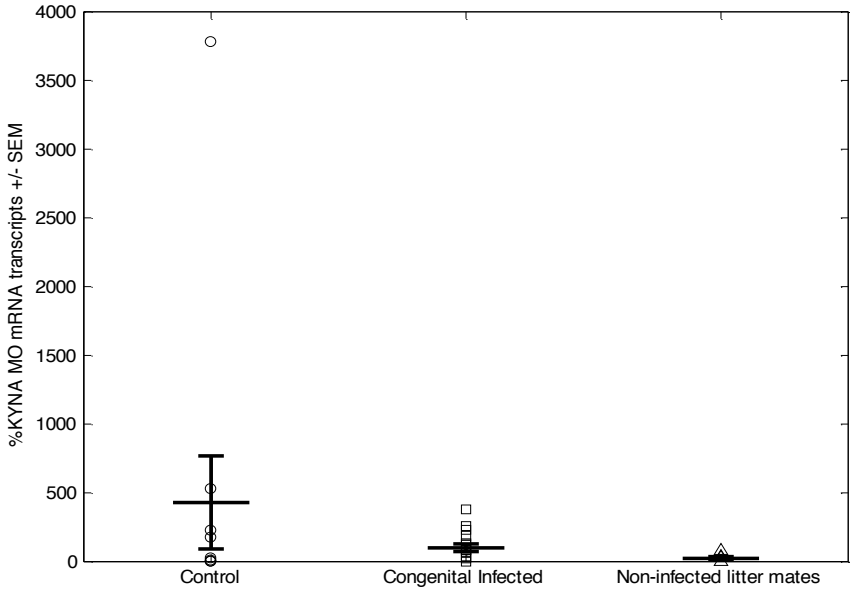


Figure 3.9: represents the expression levels of KYNA MO in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

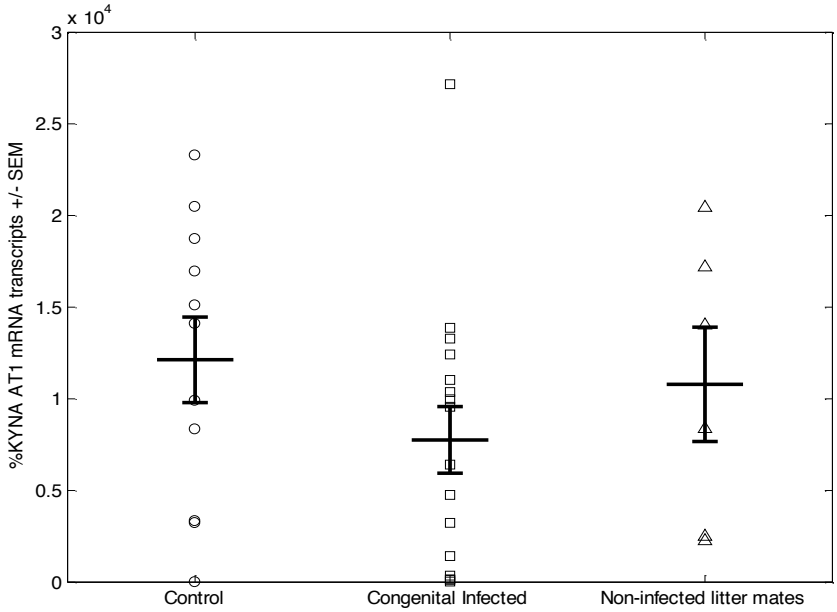


Figure 3.10: represents the expression levels of KYNA AT1 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

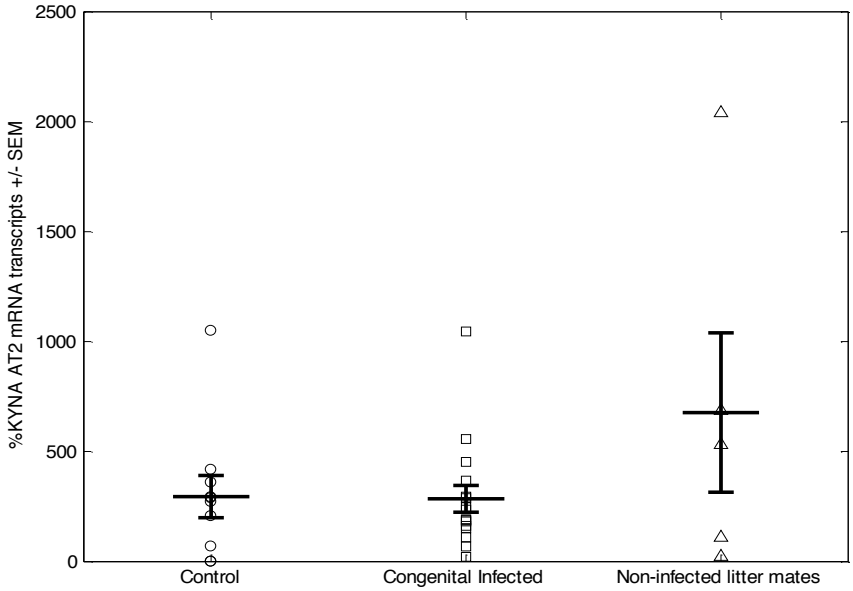


Figure 3.11: represents the expression levels of KYNA AT2 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

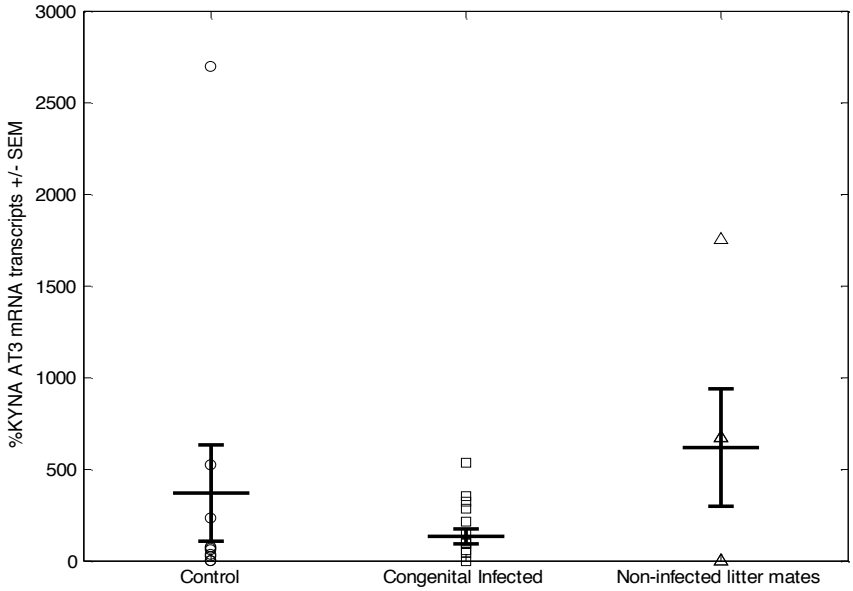


Figure 3.12: represents the expression levels of KYNA AT3 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

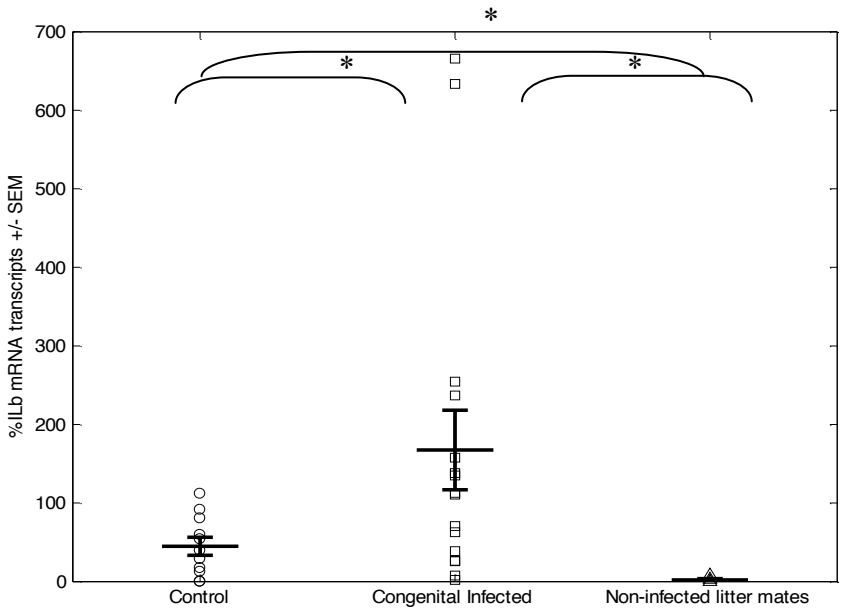


Figure 3.13: represents the expression levels of IL-1β in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. The expression of IL-1β in the congenitally infected brain samples was significantly up regulated compared to controls and the non-infected litter mates samples (respectively, p= 0.0171 and 0.0008). In addition, the non-infected litter mates brain samples had significantly lower expression levels compared to the controls brain samples (p=0.02). This is represented by an *.

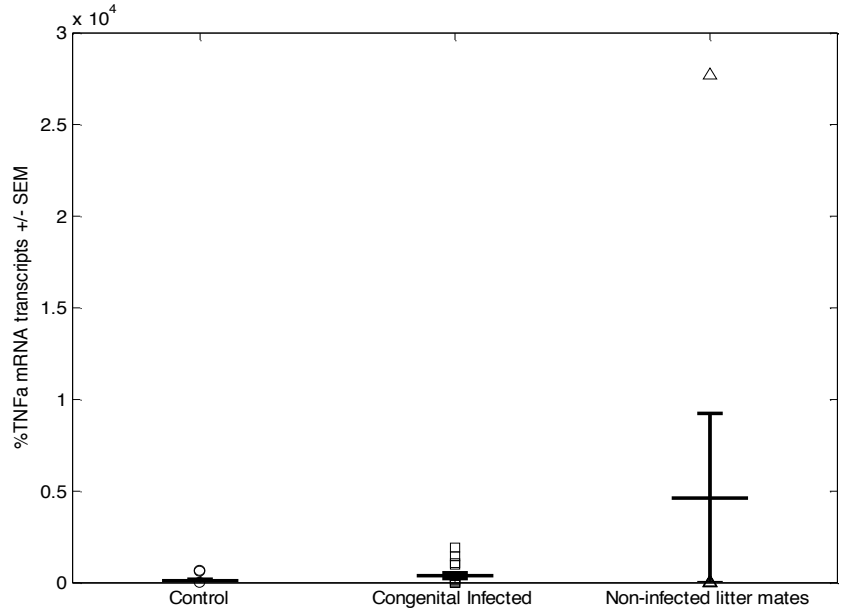


Figure 3.14: represents the expression levels of TNF-α in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

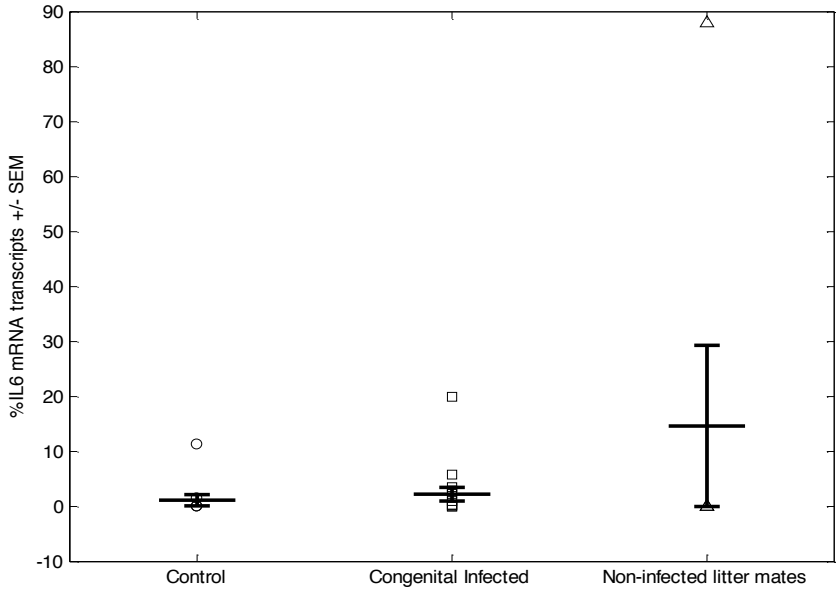


Figure 3.15: represents the expression levels of IL-6 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white square) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

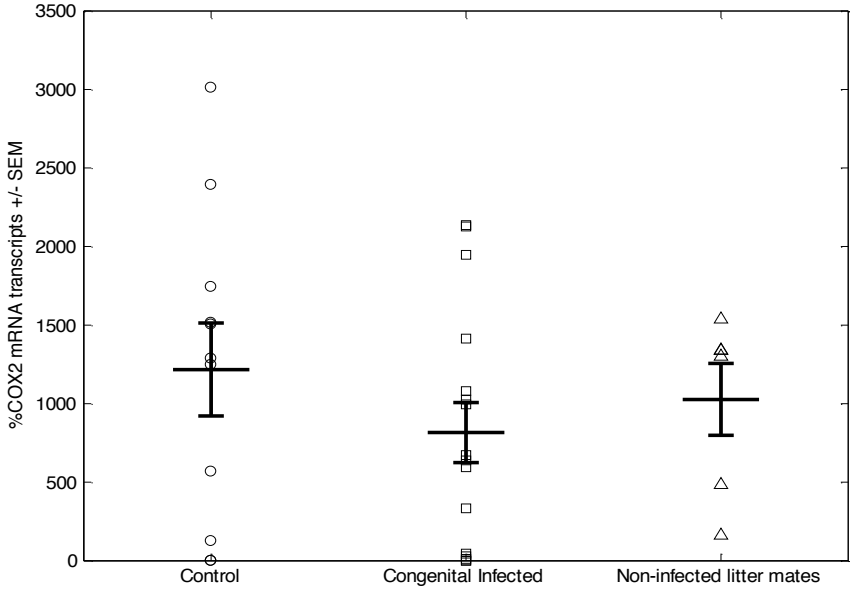


Figure 3.17: represents the expression levels of COX2 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

Figure 3.16: represents the expression levels of COX1 in the controls (white circles) (n=11: 6 males and 5 females), in the congenitally infected (white squares) (n=16: 7 males and 9 females) and in the non-infected litter mates (white triangles) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

Figure 3.3-3.17: the analysis of the molecular expression by quantative real-time PCR of the enzymes involved in the tryptophan degradation pathway and the cytokines present in the brain during T. gondii congenital infection (white squares) compared to the uninfected controls (white circles) and the the non-infected litter mates (white triangles). The number of copies for each gene was expressed in the percentage of the relation of the number of copies of the house keeping gene TBP. This measurement was done for all genes for the exception of COX-1 (figure H), which was analysed using the 2ΔΔCT method in relation of the house keeping gene TBP. The mice were sacrificed after the last time point of the behaviour study at 14 weeks of age and the brain extracted to perform the molecular analysis.

3.3.5 Metabolomics analysis

The analysis of the metabolomic profiling of the brains extracted from the mice of the congenital experiment was carried when the animals were at 14 weeks of age. The analysis reported herein was obtained from brain metabolomic profiling only performed on male mice. The number of samples per group studied was as followed: 6 samples of the control group, 4 from the congenital infected group and 6 from the non infected litter mates group. The metabolomic profiling of the brain of the female mice was performed on a smaller group of samples and the n number was not sufficient for the statistical analysis. In fact, the female brain samples consisted of: 6 samples from the control group, 1 from the congenital infected group and 1 from the non infected litter mates. It was decided retrospectively to carry out the metabolomic profiling on the samples with gender separation. However, it is important to note that many compounds detected were found to be present at different levels between the females and males brain samples of the control group.

The analysis of the congenital metabolomic data was performed by cross-referencing the compounds detected in positive ionization mode with the final results of the adult metabolomic data (appendix Table 5.24). From the 52 compounds detected in the adult experiment only 46 compounds were detected in the congenital experiment (appendix Table 5.24) and among these, significant difference between the experimental groups was observed in 14 compounds (Table 3.1). Amongst the compounds of the congenital data eliminated by cross-referencing (appendix Table 5.24), 35 were found to be significantly different between the experimental groups (Table 3.2).

First of all, the differences measured in the compounds identified in the positive ionization mode also found in the adult experiment will be described (Table 3.1).

Table 3.1: Ratios of the significantly different compounds detected in the congenital, non-infected litter mates and controls brain samples by cross reference with compounds detected in the chronically infected brain samples (see appendix, Table 5.1). Analysis performed with the Proteowizard XCMS and MZMatchR softwares.

			RATIO			
Mass	FORMULA	Identification	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male
85.089232	C5H11N	Piperidine	0.880	1.025	0.858	0.805
105.0426425	C3H7NO3	L-Serine	0.869	0.938	0.926	0.891
115.0634002	C5H9NO2	L-Proline	0.934	0.911	1.025	1.730
117.0790447	C5H11NO2	L-Valine	1.277	1.142	1.118	0.910
125.0147105	C2H7NO3S	Taurine	1.037	1.010	1.026	0.920
131.0695501	C4H9N3O2	Creatine	0.250	589.589	0.000	0.851
132.0535961	C4H8N2O3	L-Asparagine	1.499	1.153	1.300	1.756
133.0375787	C4H7NO4	L-Aspartate	1.303	0.017	75.874	1.175
146.0691643	C5H10N2O3	L-Glutamine	0.891	0.903	0.987	0.770
151.0495019	C5H5N5O	Guanine	0.599	0.809	0.740	1.100
181.0739831	C9H11NO3	L-Tyrosine	1.244	1.248	0.996	1.269
204.089998	C11H12N2O2	L-Tryptophan	1.342	1.134	1.183	1.439
283.0918876	C10H13N5O5	Guanosine	1.130	0.982	1.151	1.380
135.0545969	C5H5N5	Adenine	1.435	1.151	1.247	1.006

TABLE LEGEND	
	Higher in CI (p<0.05)
	Lower in CI (p<0.05)
	Higher in Non infected litter mates (p<0.05)
	Lower in Non infected litter mates (p<0.05)
	Higher in females (p<0.05)
	Lower in females (p<0.05)
	No difference

Amino acids and metabolites

L-Serine is significantly decreased in the male congenitally infected brain samples compared to controls (p=0.043). No differences were observed between the controls brain samples and the non infected litter mates brain samples and between the congenital infected and the non infected litter mates group. A significant difference was observed between the brain samples of control female mice with lower levels of serine compared to the brain samples of the control male mice (p=0.04). Levels of L-valine and taurine were both significantly higher in the congenital infected brain samples compared to controls, (p values of 0.02 and 0.046, respectively). No other difference was observed between the groups. Taurine levels were found significantly higher in the male control brain samples compared to the females control (p=0.0025). Levels of L-Aspartate are significantly increased in the congenital infected group compared to the controls and the non infected litter mates samples (with p values of 0.00019 and 0.00019, respectively). Levels of L-tyrosine and L-tryptophan are both found to be significantly increased in the congenitally infected brain samples compared to the controls brain samples (with p values of 0.009 and 0.02, respectively). In addition, both amino acids were found to be significantly increased in the female brain samples of the control group compared to the male brain sample of the control group (p=0.0019 and 0.0015, respectively).

Purine/pyrimidine metabolites

Levels of adenine are significantly higher in the congenital infected brain samples and in the non infected litter mates brain samples compared to controls (p=0.02 and 0.0150 respectively).

Levels of guanine are significantly lower in the congenital infected brain samples compared to controls (p=0.013).

Other metabolites

Creatine and piperidine also differed between the group samples. In particular, creatine levels were significantly decreased in the congenital infected group compared to controls and the non infected litter mates (p= 0.001 and 0.004, respectively) and significantly higher in the non infected litter mates group also compared to controls (p=0.004). Piperidine levels were significantly decreased in the congenital infected group compared to the controls (p=0.049) and also decreased significantly in the female control brain samples compared to the male controls (p= 0.006).

Glutamine levels are found to be higher in the male control brain samples compared to the female controls (p=0.0023).

Differences between female and male metabolites in the mouse brain

Guanosine, L-asparagine and L-proline levels are higher in the control female brain samples compared to the control male samples (p=0.013, 0.014 and 0.015 respectively).

Herein, the compounds identified in the congenital experiment and not detected in the adult experiment with significantly different levels measured between the groups will be described.

Table 3.2: Metabolomic differences of the congenital experiment brain samples. These compounds were not detected in the adult experiment. The compounds, here listed, are found to be significantly different between the three groups in the male mice: controls (n=6), congenitally infected (n=4) and the non infected litter mates (n=6). Differences found in the metabolomic analysis between the female controls brain samples and the male controls brain samples are also reported.

			Ratio			
Mass	Formula	Identification	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male
97.9769	H3O4P	Orthophosphate	1.090	0.283	3.848	1.020
113.0478	C5H7NO2	(S)-1-Pyrroline-5-carboxylate	1.193	0.907	1.315	0.883
121.0198	C3H7NO2S	L-Cysteine	0.997	0.856	1.164	0.491
129.1155	C7H15NO	4-Trimethylammoniobutanal	0.895	0.908	0.986	0.945
132.0424	C5H8O4	2-Acetolactate	0.814	0.819	0.993	0.934
148.0671	C5H12N2OS	L-methioninamide	1.850	1.486	1.245	1.435
155.0696	C6H9N3O2	L-Histidine	0.944	0.948	0.996	0.821
159.1260	C8H17NO2	DL-2-Aminooctanoicacid	1.282	1.233	1.040	1.265
160.0849	C6H12N2O3	D-Alanyl-D-alanine	1.153	1.005	1.147	2.417
161.1053	C7H15NO3	L-Carnitine	1.207	1.024	1.179	0.833
167.0366	C3H9N3O3S	Taurocyamine	1.173	0.929	1.262	0.901
169.0740	C8H11NO3	Pyridoxine	1.053	1.073	0.982	1.991
174.1005	C7H14N2O3	N-Acetylornithine	0.905	0.761	1.189	2.247
174.1118	C6H14N4O2	L-Arginine	1.136	1.195	0.951	1.278
175.0958	C6H13N3O3	L-Citrulline	1.220	1.211	1.007	1.054
203.1158	C9H18NO4	O-Acetylcarnitine	1.079	0.928	1.163	0.850
204.1111	C8H16N2O4	N6-Acetyl-N6-hydroxy-L-lysine	1.026	1.020	1.005	2.359
205.0952	C8H15NO5	N-Acetyl-D-fucosamine	1.401	1.170	1.198	0.988
220.1060	C8H16N2O5	N-Acetyl-beta-D-glucosaminylamine	1.282	1.065	1.204	1.600
222.0676	C7H14N2O4S	L-Cystathionine	0.851	0.917	0.928	0.713
229.0887	C9H16N3O2S	Ergothioneine	0.856	0.831	1.030	0.613
231.1472	C11H21NO4	O-Butanoylcarnitine	1.152	0.910	1.265	0.686
232.1061	C9H16N2O5	N2-Succinyl-L-ornithine	0.994	1.058	0.940	1.300
245.1629	C12H23NO4	N-(octanoyl)-L-homoserine	0.972	0.844	1.153	0.467
247.1422	C11H21NO5	Hydroxybutyrylcarnitine	1.214	0.962	1.263	0.544
278.1303	C11H22N2O4S	Pantetheine	1.872	1.571	1.192	1.651
278.1520	C16H22O4	2-Ethylhexyl phthalate	0.947	0.912	1.038	0.921
280.0918	C10H12N6O4	adenosine 5'-carboxamide	1.151	1.018	1.131	1.470
297.0898	C11H15N5O3S	5'-Methylthioadenosine	1.292	1.063	1.216	1.440
307.0839	C10H17N3O6S	Glutathione	1.003	1.028	0.975	0.498

TABLE LEGEND	
	Higher in CI (p<0.05)
	Lower in CI (p<0.05)
	Higher in Non infected litter mates (p<0.05)
	Lower in Non infected litter mates (p<0.05)
	Higher in females (p<0.05)
	Lower in females (p<0.05)
	No difference

Amino acids and their metabolites

Levels of L-methioninamide are significantly higher in the congenitally infected brain samples compared to controls (p value of 0.022). Levels of L- citrulline are significantly higher in the congenital infected group compared to controls and the non-infected litter mates (p values of 0.005 and 0.017, respectively). Levels of DL-2-Aminooctanoicacid are significantly higher in the congenital infected samples compared to the non-infected litter mates (p=0.006). Levels of 2-acetolactate are significantly lower in the congenital infected brain samples compared to controls and the non-infected litter mates brain samples (p values of 0.013 and 0.010, respectively).

Purine/pyrimidines metabolites

Levels of orthophosphate are significantly higher in the congenital infected samples compared to the non-infected litter mates (p= 0.034). Levels of taurocyamine are significantly lower in the non-infected litter mates brain samples compared to controls (p= 0.015).

Metabolites involved in the transport of fatty acids

Levels of 4-Trimethylammoniobutanal are significantly lower in the congenital infected brain samples compared to controls and the non-infected litter mates brain samples (p values of 0.016 and 0.045, respectively). Levels of O-Acetylcarnitine are significantly lower in the non-infected litter mates brain samples compared to controls (p= 0.008). Levels of pantetheine are significantly higher in the congenital

infected group compared to controls and the non-infected litter mates (p values of 0.038 and 0.037, respectively).

Other metabolites

Levels of N-Acetyl-beta-D-glucosaminylamine and Tributyl phosphate are significantly higher in the congenital infected and in the non infected litter mates groups compared to the controls (p value of 0.004, 0.043 and 0.015, 0.041, respectively). 3-Butylidene-7-hydroxyphthalide and N-Acetyl-D-fucosamine compounds are significantly higher in the congenitally infected brain samples compared to controls (p values of 0.004 and 0.043, respectively). 12-Hydroxydodecanoic acid levels are significantly higher in the congenital infected group compared to controls and significantly lower in the non-infected litter mates compared to controls (p= 0.031, 0.003, respectively).

Differences between female and male metabolites in the mouse brain

Of note levels of D-Alanyl-D-alanine, Pyridoxine, N-Acetylornithine, L-Arginine, N6-Acetyl-N6-hydroxy-L-lysine, N2-Succinyl-L-ornithine, adenosine 5'-carboxamide and 5'-Methylthioadenosine are significantly higher in the female control brain samples compared to the males (respectively, p= 0.026, 0.020, 0.024, 0.037, 0.017, 0.006, 0.005 and 0.017).

Levels of (S)-1-Pyrroline-5-carboxylate, L-Cysteine, Histidine, L-Carnitine, O-Acetylcarnitine, L-Cystathionine, Ergothioneine, O-Butanoylcarnitine, N-(octanoyl)-L-homoserine, Hydroxybutyrylcarnitine, 2-Ethylhexyl phthalate and Glutathione are

significantly lower in the female control brain samples compared to the male control brain samples (respectively, p= 0.036, 0.008, 0.031, 0.037, 0.013, 0.013, 0.005, 0.004, 0.018, 0.033, 0.002, 0.039 and 0.002). However, these differences have not been discussed it was not the aim of this study.

3.4 Discussion

3.4.1 Behavioural studies

The open field is considered to be a more approximate measure of the anxiety level of the animal than the elevated plus maze test. However, it provides a good estimate of loco motor behaviour in relation to anxiety. From the overall analysis between the congenital infected mice, the non-infected litter mates and the controls, the only difference observed in the behaviour of these mice was the hyperactivity of the non-infected litter mates mice at 14 weeks of age compared to the controls and the congenitally infected mice. Further scrutiny in order to understand the value of the result was carried out to study in detail any differences between the groups of animals caused by the gender. This can be observed on the Appendix at page 164 to page 165. First of all no differences between males and females was observed amongst the groups. However, it was observed that the hyperactivity trend, significant at 14 week of age in the non-infected litter mates mice compared to the controls and the congenitally infected mice, began at 10 weeks of age in the males and at 14 weeks of age in the females. This suggests a real effect caused by the exposure of *T. gondii* during pregnancy on the offspring mice on both genders on general locomotor activity. In the literature the effects of the maternal immune challenge on the offspring is under investigation. In particular, the attention is focused towards the development of new animal models in order to study conditions also present in psychiatric disorders and to create more suitable drug treatments. Many studies differ from one another in the type and in the timing of infection and therefore the consequences on the behaviour of the offspring are not always repeatable. Studies on offspring born to mothers exposed to LPS usually show an

increase in the locomotor behaviour (Fortier et al., 2004; Golan et al., 2005). This is also observed in this study in the congenitally exposed to *T. gondii* but not infected. On the other hand, the exposure to influenza or Poly: IC studies have produced less consistency in the behaviour of the offspring (Shi et al., 2003; Meyer et al., 2008) demonstrating the importance of the time of the immune challenge during pregnancy and amount of time that the challenge continues during pregnancy. In contrast, no differences were observed in the present study in congenitally infected mice. This result was not expected and the reasons for this are unclear. Previous studies have shown a general increased activity in congenitally infected mice by *T. gondii* (Hay et al., 1983; Hay, Aitken, Hair, et al., 1984). However, the parasite strain, the time of transmission and the mode of transmission differ from the experiments described in this thesis. This can confirm the influence and the involvement of many factors in the overall phenotype outcome on the offspring affected by congenital transmission or born by mother exposed to an immune challenge. Alter locomotor activity can result from changes in dopamine neurotransmisson in the mesolimbic dopamine system. Hence, it is possible that congenitally exposed non-infected animals may have resulted in long term changes in dopamine systems. However, it is unclear why this was not apparent in the infected group.

The elevated plus maze is considered to generate a more refined measure of anxiety levels in the animals. A difference in the anxiety levels was observed between the groups at 10 weeks age, where the infected group showed higher levels of anxiety by entering fewer times and spending less time in the open arms of the maze compared to the control mice and the non infected litter mates. In the literature, higher anxiety

levels were observed in the offspring of mothers challenged by LPS (Golan et al., 2005). This consequence could be considered consistent with the previous study, where the maternal immune challenge was *T. gondii* exposure. *T. gondii* has been observed to decrease the anxiety levels in the infected adult rodents (Gonzalez et al., 2007). Nevertheless, the effect of *T. gondii* infection in adult acquired transmission and vertically transmission can clearly lead to different consequences in the subject and still the influence of many factors during the congenital transmission have to be taken into consideration. In fact, from the study by Golan et al. (2005) it was hypothesised that the non infected litter mates group would have been affected by an increase in the anxiety levels, which in this study was not observed. Some consideration of the gender differences in the elevated plus maze test must be taken into account. Differences in the behaviour between the male and female mice from the control group are observed at 6 and 10 weeks of age. At 6 weeks of age female mice appear to be less anxious compared to the males and at 10 weeks of age the opposite behaviour is observed, although in general, congenitally infected male appear to be less anxious compared to the controls males and the non infected litter mates males. This is also observed in the congenitally infected female group compared to the control female and the non-infected litter mates. This further detailed analysis is shown in the Appendix. Therefore, the effect of *T. gondii* congenital transmission appears to be similar in both male and female mice with respect to increasing the level of anxiety in the infected offspring. However, in future experiments the number of mice per group should be increased.

3.4.2 Gene expression analysis

These molecular analyses of gene expression were carried out in order to verify the hypothesis of the ability of *Toxoplasma gondii* infection to modify behaviour via the interaction of the nervous and the immune system. As expected, the expression levels of IFN-γ were significantly increased in the brain samples of the congenitally infected mice compared to controls. So far, the particular involvement of IFN-γ during the chronic phase and the overall challenges of the immune system by the cyst turnover in chronic infected mice have only been studied in the adult acquired infection (Wang et al., 2007). Nevertheless, this could be expected even in the congenital transmission model. In fact, the clinical manifestation of disease is often an event that occurs later on in life of a newborn affected by congenital transmission and studies have shown that this is the case for ocular toxoplasmosis manifestation (Melamed et al., 2010). This implies that *T. gondii* converts into the active tachyzoite stage of with the consequential involvement of the immune response. This has been successfully measured in this series of experiments by both the increase levels expression of IFN-γ and IL-1β. The expression levels of IL-1β in the brains of the congenitally infected mice are significantly increased compared to the brain samples of the control mice. Previous studies of the immunological brain status of offspring born from mothers immunologically challenged by LPS or Poly: IC have shown differences in the levels of expression of cytokines. In general, from LPS studies an increase in the levels of gene expression of several cytokines, such as TNF-α, IL-6 and IL-1β was found (Ling et al., 2002; Kumral et al., 2007; Yesilirmak et al., 2007). From the Poly: IC studies results are not as clear; some studies show an increase of the levels of cytokine in the brain and in others a decrease is observed (Boksa, 2010).

In this study, an increase of the cytokine inflammatory gene expression levels in the brain samples of the congenitally infected mice is observed. However, this is not seen in the congenitally exposed non infected mice leading to the suggestion that the timing of infection is essential in the development of chronic consequences on the brain environment. Furthermore, a decrease of the levels of expression of IL-1β compared to the controls is measured in the brain samples of the congenitally exposed non infected mice, suggesting the requirement for further investigation into the possible consequences of the timing of congenital transmission during the development stages and the interaction of the immune system and the central nervous system.

We also measure differences in the expression levels of genes encoding enzymes involved in the tryptophan degradation pathway. We found a similar result to the adult acquired chronic infection regarding the expression levels of KYNA (see chapter 2), where there is an increase of expression in the brain samples of the congenitally infected mice compared to control. This suggests that the tryptophan degradation pathway is affected in the brain of the congenitally infected mice even though IDO levels are not. Most probably some of the differences present between the expressions of the molecules were not obtained because of a type 2 error with insufficient numbers of male and female subjects. More samples are certainly required for further investigation and the gender separation of the analysis even though the expression levels of the control male mice and female did not differ (see appendix) infection is different between the genders due to hormones regulation, which could lead to different consequences between the genders.

Another result that needs further investigation is the unexpected increase of the expression levels of the TPH2 enzyme in the congenitally infected brain samples compared to control. In fact, if an increase of the tryptophan degradation pathway is measured surely a decrease of serotonin production will be expected. However, these are measurements of the global effect on the brain and single changes in particular regions should also be investigated in further studies. Given the changes in anxiety related behaviours the hippocampus and the amygdala would be areas of interest to target.

3.4.3 Metabolomics analysis

Metabolomic analysis was performed on the brain samples of the male mice between the three groups of this congenital experiment. However, future experiments should increase the number of samples for an optimal evaluation of the data. Nevertheless, interesting points of discussion can be deduced from these results. An increase in the levels of a number of amino acids was detected in all the brain samples of the congenitally infected mice compared to controls. These amino acids are: tyrosine, which could also lead to an increase of the dopamine levels in the brain (difference not measured in these brain samples), citrulline, valine, taurine (which is not an amino acid in the biochemical terms but an organic acid, the term is widely used to incorporate this compound in this group for its functionality) and tryptophan, all involved in the up regulation of the immune responses mechanisms, from antimicrobial activity to wound healing and repair processes. This is likely a consequence of *T. gondii* presence in the brain, involving the up regulation of the immune response and therefore the increase of amino acids involved in the

biosynthesis of proteins and cell growth. The relationship between cytokines and the upregulation of pathways linked to amino acids synthesis has been previously described in several in vitro and vivo studies (Galic et al., 2012). However, this is the first in vivo study that observes this relationship during *T. gondii* chronic infection. Furthermore, increase of tryptophan could also lead to an increase in the metabolites involved in the degradation pathway, such as KYNA and quinolinic acid that are found to be neurotoxic (Müller et al., 2011). On the other hand, tryptophan upregulation could also signify an increase of serotonin production, which then could lead towards melatonin formation, involved in sleeping processes and the decrease in motor activity. High levels of aspartate are also found in the brain samples of the congenitally infected mice compared to the non-infected litter mates. Aspartate is an excitatory amino acid that stimulates the NMDA receptors (Krystal et al., 1999). Studies have shown that chronic high levels of excitatory amino acid, including glutamate, are detrimental for brain function, leading to cell death. Long-term exposure to excitatory amino acids has been linked to the development to several pathologies, including neurodegenerative diseases such as Alzheimer's disease and Parkinson disease (Sultana and Butterfield, 2008; Caudle and Zhang, 2009). In addition, NMDA receptor stimulation also leads to altered synaptic plasticity and it is implicated in changes in cognitive function (Coyle et al., 2003). A decrease in the levels of serine and 2-acetolactate is measured in the congenitally infected brain samples compared to controls and in the case of 2-acetolactate also compared to the non-infected litter mates. These molecules are both involved in the production of other amino acids, such as valine and methiotine, which are found to be upregulated. Also, L-methioninamide, an aminopeptidase, and DL-2-aminooctanoicacid, involved

in peptide synithesis, are increased in the congenital infected brain compared to controls and the non-infected litter mates also for the latter. Therefore, it appears that there is hyper activity of the amino acid metabolism in the brain of the congenitally infected mice compared to controls and the non-infected litter mates. This indicates that congenital *T. gondii* infection and not exposure to a maternal infection is responsible for these observations. In fact, exposure to maternal infection only affects the levels of aspartate. Most probably, congenital infection affects different metabolomic routes in the brain compared to the exposure to maternal infection.

The differences observed in the purine/pyrimmidines metabolism are not the same between the two experimental groups compared to controls. In particular, levels of adenine are found to be higher and guanine lower in the congenitally infected brain samples compared to controls and the non-infected litter mates. This was expected as purine metabolism, involved in the upregulation of the inflammatory responses during brain infection, contributes towards brain pathology and dysfunction (Haskó et al., 2005). Also, levels of orthophosphate, involved in the oxidative phosphorylation are higher in the congenital infected brain samples compared to the non infected litter mates. Instead, levels of taurocyamine, involved in the production of ADP, were lower in the non-infected litter mates compared to controls and no differences observed in the congenital infected group. However, it is interesting to observe the levels of this metabolite as it has been reported that it is present in high levels in the cerebrospinal fluid of neurological and psychiatric patients (Wiechert et al., 1986)

Differences in the metabolites of the carnitine pathway and involved in the transport of fatty acid were also observed. First of all, lower levels of 4-trimethylammoniobutanal, involved in the carnitine biosynthesis were measured in the congenital infected brain samples compared to controls and the non-infected litter mates. Carnitine as mentioned is involved in the transport of fatty acids and it has been reported to prevent dementia and Alzheimer. Nevertheless, no differences of this metabolite were observed between the groups. However, O-Acetylcarnitine, the acetylated form of L-carnitine was significantly lower in the non-infected litter mates compared to controls. Higher levels of this compound are present during intense exercise to act as an antioxidant. It has been hypothesised that carnitine have neuroprotective benefits in the treatment of Parkinson's disease (Zhang et al., 2010) and the lower levels observed in the non-infected litter mates might indicate a possible route towards the development of brain disorders caused by an immune challenged during gestation. Higher levels of Pantetheine in the congenital infected brain samples compared to controls and the non infected litter mates also could indicate an affect of the fatty acid system in the brain caused by the parasite itself.

The presence of higher glycolysation reactions in the brain of the congenital infected and the non infected litter mates compared to controls measured by the increased levels of the N-Acetyl-D-fucosamine and N-Acetyl-beta-D-glucosaminylamine compounds in the brain samples of these groups is also observed. Glycolysation in the brain is known to be an indication of nutrient sensing, gene expression and neurodegeneration (Arnold and Hart, 1999; Arnold et al., 2005). Therefore, this

could indicate another possible metabolomic route disrupted during congenital infection and possibly contributing towards the development of brain pathologies.

3.4.4 Concluding comments

From this study we can observe that the largest effect on behaviour was seen in the congenitally infected mice with higher anxiety levels measured in the elevated plus maze test compared to the non-infected litter mates and controls. Differences in gene expression and metabolites levels were also observed mainly in the congenitally infected group. Nevertheless, additional behaviour tests such as conditional emotional responses, which measure impulsivity, must be carried out to further investigate any possible phenotypical behaviour consequence from the immunochemical changes seen in the brain of the congenitally infected mice. In addition, it can be hypothesised that different interactions and pathways to those observed in the infected brain, such as tryptophan degradation pathway, may be contributing to the behavioral differences observed in the congenitally exposed non infected mice. These changes can be explained perhaps only at the neurodevelopment stage where the challenged immune system develops together with the central nervous system, which could compensate the overall chemical imbalance in the brain but not change this particular behaviour. Nevertheless, changes, in particular of tryptophan degradation pathway, can be minimum and confined in a particular brain region, which could overall result in different behaviour but not measurable in the whole brain.

4 Discussion

In summary, we now compare the results between the adult acquired *T. gondii* infection, the congenital infection and the non-infected litter mates groups.

Behavioural studies

Comparing the analysis of mice behaviour infected either by the adult acquired mode of transmission or by congenital, the ability of *T. gondii* to manipulate behaviour in the infected host is clearly observed (Table 4.1). However, the effect on the behaviour was not similar: in the adult chronically infected mice lower anxiety levels were reported through the increase of exploratory behaviour in the central zone of the open field. This observation is in linear with past studies where *T. gondii* infected mice showed lower anxiety levels (Hay et al., 1983; Webster et al., 1994). In contrast, higher anxiety levels were observed in the congenital infected mice as they entered and spent less time in the open arms of the elevated plus maze compared to the non-infected litter mates and the control animals. Activity levels, measured by the total distance travelled in the open field test, were increased in the adult acquired infected mice and in the non-infected litter mates. Hyperactivity has previously been described during *T. gondii* infection, in both mode of transmission, adult acquired and congenitally (Hay et al., 1983; Hay, Aitken, Hair, et al., 1984). However, in this study the hyperactivity of the congenital infected group was not observed. Moreover, the maternal immune response challenged by *T. gondii* infection during pregnancy had a greater effect on the activity levels of the litters once they reached adult hood. The effect of the maternal immune response was observed in previous studies (Fatemi et al., 1999; Shi et al., 2003) in laboratories models mimicking the effect of

an infection, through administration of LPS or Poly:IC. However, here for the first time we observe this behaviour during a parasitic congenital model.

T. gondii is directly responsible for the effect on the behaviour in the infected host. However, the timing of infection on the effect on the mouse behaviour is clearly an important factor, which can contribute towards the development of different phenotypes. In addition, the maternal immune challenge contributes towards the hyperactivity of the offspring without affecting the anxiety levels. This indicates the additional unique role of *T. gondii* in the brain network of the intermediate host.

Table 4.1: Summary of the behavioural results in the three experimental groups: Adult acquired chronic infected mice, congenitally infected mice and non-infected litter mates compared to their controls.

<table>
<tr><th></th><th>Behaviour Trait</th><th>Infected</th><th>Congenital Infected</th><th>Non-Infected litter mates</th></tr>
<tr><td rowspan="2">Open field</td><td>Anxiety</td><td>Low Vs Control</td><td rowspan="2">N.D. Vs Control</td><td>N.D. Vs Control</td></tr>
<tr><td>Activity</td><td>Higher Vs Control</td><td>Higher Vs Control and Congenital Infected</td></tr>
<tr><td rowspan="2">EPM</td><td>Anxiety</td><td rowspan="2">N.D Vs Control</td><td>Higher Vs Control and Non-infected litter mates</td><td rowspan="2">N.D Vs Control</td></tr>
<tr><td>Activity</td><td>N.D. Vs Control</td></tr>
</table>

Gene expression studies

Differences between the molecular analyses results of the three experimental groups between their respective controls were also observed (Table 4.2). The neuro-immuno environment of the infected mice altered in relation to the different modes of transmission and by the maternal immune challenge. In particular, during adult acquired chronic infection gene transcript levels of the tryptophan degradation pathway were upregulated compared to controls by the direct effect of the upregulation of IFN-γ and IDO immunological mediators. This was previously described during acute infection of *T. gondii* in several other tissues, such as lungs (Däubener and MacKenzie, 1999). Here, we describe this relationship for the first time in a chronically infected mouse model. The visible effect on the tryptophan degradation pathway it is not so noticeable during congenital infection. In fact, IFN-γ upregulation does not directly influence IDO levels. However, KYNA expression levels, one of the key middle enzymes belonging to the tryptophan degradation pathway is upregulated in the congenital infected brain samples compared to controls. However, also TPH2 enzyme expression levels are also upregulated, suggesting also an increase of 5-HT formation as well as kynurenine metabolites. We can also observe that the immune challenge during gestation by *T. gondii* infection has no effect on the tryptophan degradation pathway in the neuro environment of the offspring. This indicates how the presence of the parasite influences directly the activity of the tryptophan degradation pathway, perhaps acting with the immune response in a synergistic manner towards the effect of this metabolic route.

Also, the effect on the other immunological mediators in the brain is different between the modes of transmission and the maternal challenge during gestation. In

the adult chronically infected mice several cytokines are upregulated. In addition to IFN-γ, mentioned earlier, also IL-1β and TNF-α are upregulated in the chronic infected brains compared to controls. However, less immune activity is observed in the congenital infected brains, where IFN-γ and IL-1β are found to be upregulated, and in the non-infected litter mates brains, where a decrease of the immunological mediator IL-1β was observed. Also, this indicates that the presence of *T. gondii* contributes differently to the host neuro-immuno environment.

Table 4.2: Summary of the gene expression analysis of the brain of the three experimental groups: adult acquired chronic infected mice, congenitally infected mice and non-infected litter mates compared to their controls.

Gene expression analysis	**Adult acquired Infection**	**Congenital Infection**	**Non-Infected litter mates**
IFN-γ	Higher Vs Control	Higher Vs Control and Non-Infected litter mates	N.D.
IDO	Higher Vs Control	N.D.	N.D.
TPH2	N.D.	Higher Vs Control and Non-Infected litter mates	N.D.
HD	Higher Vs Control	N.D.	N.D.
KYNA	Higher Vs Control	Higher Vs Control	N.D.
KYNAMO	Higher Vs Control	N.D.	N.D.
AT1	Lower Vs Control	N.D.	N.D.
IL-1β	Higher Vs Control	Higher Vs Control and Non-Infected litter mates	Lower Vs Control
TNF-α	Higher Vs Control	N.D.	N.D.

Metabolomic studies

Metabolomic profiling of the experimental brain samples from adult acquired chronic infection, congenital infection and the non-infected litter mates, clearly indicates several important changes present compared to their controls (Table 4.3). Several pathways are affected in the brains of all the experimental groups. In particular, amino acids levels are mostly increased compared to controls, indicating a very active metabolic, immunological and neuro environment. Several amino acids are precursors or act directly as neurotransmitters. Interestingly, aspartate levels are decreased in the adult acquired infected mice compared to the non-infected litter mates, whose levels were higher compared to controls. Aspartate is the precursor of other amino acids and as well an agonist of the NMDA receptor. This is an indication of different pathways being affected by the same parasite transmitted at different time points during life. Another example is the effect of *T. gondii* infection on purine/pyrimidine metabolism where higher levels of guanine and lower levels of adenine are observed in the adult acquired chronic infection brain samples compared to controls. This is in contrast to what is observed in the congenital infected brain samples compared to controls.

Table 4.3: Summaty of the metabolomic brain profiling of the three experimental groups: adult acquired chronic infected mice, congenitally infected mice and non-infected litter mates compared to their controls.

Metabolites	**Adult acquired infection Vs Control**	**Congenital Infection Vs Control**	**Non-Infected litter mates Vs Control**
Dopamine	Low	N.D.	N.D.
Tyrosine	High	High	N.D.
Phenylalanine	High	N.D.	N.D.
Glutamate	Low	N.D.	N.D.
Aspartate	Low	N.D.	Low
Glutamine	Low	N.D.	N.D.
Serine	Different between time points	N.D.	N.D.
Threonine	Different between time points	N.D.	N.D.
Tryptophan	High	High	High
Kynurenine	High	Not present	Not present
Uridine	Low	N.D.	N.D.
Guanine	Different between time points	Low	N.D.
Adenine	Low	High	High
Pipiredine	High	Low	N.D.
N-acetyl-D-glucosamine	High	N.D.	N.D.

The metabolic and neur-immune environment of the brain has been clearly affected by the direct presence of *T. gondii* and indirectly, through the immune challenge during gestation. Future studies should focus on the single effect and involvement of the *T. gondii* infection on the brain microenvironment, which will involve the analysis of single regions of the brain. I find this a very important aspect to investigate due to the complex network of the relationship between the immune and nervous systems and the challenges caused by infection leading to the predisposition and the development of neurodegenerative and psychiatric disorders. This will contribute to the further understanding of the relationship between the nervous system and immune system and their network when challenged by the environmental factors such as infection. This will further develop new possible therapies targets for many neurodegenerative disorders as well as psychiatric disorders and even possible prevention policies or medical screening.

5 Appendix

CHAPTER 2 : Appendix 5.1

		CONTROL 1 MONTH				INFECTED 1 MONTH			CONTROL 2 MONTH			INFECTED 2 MONTH			1 month	2 month	1 month	2 month
Mass	Formula	Identification	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	RATIO	RATIO	Ttest	Ttest
75.032	C2H5NO2	glycine	3694896.55	3216024.68	928386.36	3617019.67	2187276.25	606641.28	5783142.45	2121560.16	547784.48	5655418.33	2433170.16	674839.98	0.98	0.98	0.47	0.44
85.053	C4H7NO	2-Pyrrolidinone	601449.04	570922.02	201851.42	575951.75	520993.78	144497.68	3796461.21	4701832.24	1214007.86	3232108.13	4022299.35	1115585.12	0.96	0.85	0.45	0.37
85.089	C5H11N	Piperidine	5806033.73	6378502.16	2255141.07	7556386.81	9278993.88	2573529.86	6554068.40	7084956.30	1829327.85	7059164.83	8873465.53	2461056.54	1.30	1.08	0.30	0.43
101.048	C4H7NO2	1-Aminocyclopropa	1348006.08	1254412.72	443501.87	1235766.65	1178381.90	326824.33	8648429.61	10784135.80	2784451.89	6055332.11	7566090.17	2098455.85	0.92	0.70	0.41	0.24
103.063	C4H9NO2	4-Aminobutanoate	36283610.42	17458487.62	6172507.49	31321158.00	13488802.66	3741120.74	91228807.73	64277011.68	16596253.05	57289585.89	70700215.57	19608711.72	0.86	0.63	0.22	0.10
105.043	C3H7NO3	L-Serine	8808012.14	8171240.00	2888969.61	6734705.41	4417252.52	1225125.42	11256755.73	4794756.73	1238000.87	10240691.46	5933760.79	1645729.14	0.76	0.91	0.22	0.31
115.063	C5H9NO2	L-Proline	11852251.73	9117496.00	3223521.62	17143054.12	15988526.66	4434419.44	9772286.08	5233829.81	1351369.05	11119136.45	5586099.39	1549305.21	1.45	1.14	0.16	0.26
117.054	C3H7N3O2	Guanidinoacetate	238192.33	138976.58	49135.64	328324.77	226038.84	62691.89	523228.44	287614.29	74261.69	552810.91	324233.61	89926.22	1.38	1.06	0.12	0.40
117.079	C5H11NO2	L-Valine	14868074.69	9490947.49	3355556.66	21544429.96	16951657.53	4701543.88	17353171.97	12444581.00	3213177.00	17363218.19	11321006.01	3139882.13	1.45	1.00	0.12	0.50
119.058	C4H9NO3	L-Threonine	9051652.14	5017150.66	1773830.63	10481043.12	6731506.53	1866984.00	12444766.77	4138563.31	1068572.45	11465334.19	3647001.97	1011496.36	1.16	0.92	0.28	0.26
122.048	C6H6N2O	Nicotinamide	11627019.65	8668374.21	3064733.09	12294662.17	8753595.05	2427810.45	16989100.81	21796933.61	5627944.06	18856402.47	23291250.74	6459830.68	1.06	1.11	0.42	0.41
125.015	C2H7NO3S	Taurine	31032491.25	18575652.22	6567484.82	27533673.69	13066683.87	3624046.05	53403135.60	21688613.91	5599976.03	55766087.69	19491972.34	5406100.44	0.89	1.04	0.29	0.38
131.069	C4H9N3O2	Creatine	281063557.33	56944844.35	20133042.80	240732382.77	59535053.83	16512053.02	594827698.13	435333175.01	112402542.46	461251170.46	259123998.40	71868066.38	0.86	0.78	0.05	0.17
131.095	C6H13NO2	L-(iso)Leucine	73011521.58	74602108.85	26375828.53	105017632.00	99879802.33	27701672.98	41141812.07	37603226.68	9709111.38	51302016.00	39847649.29	11051749.44	1.44	1.25	0.19	0.25
132.054	C4H8N2O3	L-Asparagine	2804189.67	2038606.16	720756.12	3339936.33	2219670.35	615625.79	3153077.60	1307798.71	337672.18	3327679.62	1759071.09	487878.54	1.19	1.06	0.27	0.38
133.038	C4H7NO4	L-Aspartate	39205424.17	17915106.22	6333946.55	29106723.85	10793496.79	2993577.39	52691825.87	20881967.32	5391700.78	45741338.46	18121750.68	5026069.33	0.74	0.87	0.05	0.18
136.039	C5H4N4O	Hypoxanthine	20926797.92	6549191.71	2315488.93	23997840.38	4976567.59	1380251.51	54384620.93	59900717.16	15466298.66	62635535.62	58074281.46	16106907.66	1.15	1.15	0.10	0.36
145.085	C5H11N3O2	4-Guanidinobutano	309992.73	118067.61	41743.20	335372.80	103959.37	28833.14	569485.23	482407.14	124556.99	581290.14	491733.54	136382.35	1.08	1.02	0.29	0.47
146.069	C5H10N2O3	L-Glutamine	112836631.67	74863555.57	26468263.90	86682856.31	33068205.84	9171470.13	175227038.40	38020595.22	9816875.47	135437221.54	47759097.88	13245990.48	0.77	0.77	0.13	0.01
147.053	C5H9NO4	L-Glutamate	272233529.33	78947281.07	27912078.90	203748018.46	36430699.04	10104057.95	434665298.13	221272195.61	57132235.24	351267414.77	174793916.24	48479109.82	0.75	0.81	0.00	0.14
151.049	C5H5N5O	Guanine	1720626.79	721212.91	254987.27	1855622.21	777127.37	215536.35	4550487.59	3705258.05	956693.52	5125847.36	3073850.69	852532.79	1.08	1.13	0.33	0.33
165.079	C9H11NO2	L-Phenylalanine	54431733.92	39401473.77	13930524.64	83432019.23	62401822.45	17307151.58	31019191.60	20555750.03	5307471.84	40764471.15	23075529.29	6400000.31	1.53	1.31	0.09	0.12
167.058	C8H9NO3	Pyridoxal	545527.86	237989.21	84141.89	456392.69	172662.05	47887.84	516729.02	195252.42	50413.96	506612.67	168189.56	46647.39	0.84	0.98	0.15	0.44
176.095	C10H12N2O	Serotonin	389781.16	317672.96	112314.35	327213.35	289752.27	80362.82	267884.31	124077.40	32036.65	281567.82	169267.56	46946.37	0.84	1.05	0.31	0.40
181.074	C9H11NO3	L-Tyrosine	21032869.46	13394432.00	4735646.85	32514826.85	21433991.34	5944719.60	16242597.53	10877250.28	2808493.95	18820433.27	10467640.47	2903201.11	1.55	1.16	0.06	0.27
188.080	C7H12N2O4	N-Acetylglutamine	151613.75	68688.36	24285.00	166806.79	56883.36	15776.61	267458.25	211265.22	54548.45	229539.32	181572.92	50359.27	1.10	0.86	0.28	0.31
204.075	C7H12N2O5	Ala-Asp	1307543.69	1483245.44	524406.45	1517787.28	1345985.88	373309.32	275167.66	65084.17	16804.66	559990.51	264241.02	73287.27	1.16	2.04	0.36	0.00
204.090	C11H12N2O2	L-Tryptophan	11091610.13	7366186.61	2604340.25	15396185.96	10390866.53	2881907.85	4851061.06	4030765.80	1040739.25	5764899.30	4440402.14	1231545.97	1.39	1.19	0.12	0.29
206.090	C7H14N2O5	Thr-Ser	778074.60	737258.94	260660.40	843272.92	565453.33	156828.54	644709.01	646295.07	166872.67	1097658.13	981014.92	272084.58	1.08	1.70	0.40	0.08
218.127	C9H18N2O4	N2-(D-1-Carboxyeth	20023287.52	15602270.94	5516235.79	22406641.73	15388622.75	4268036.03	9823411.15	6885142.00	1777736.02	13581993.48	8393095.86	2327825.96	1.12	1.38	0.35	0.10
221.090	C8H15NO6	N-Acetyl-D-glucosa	808999.62	276641.81	97807.65	1157616.27	450902.65	125057.89	675742.28	348523.94	89988.49	860892.50	381689.50	105861.62	1.43	1.27	0.02	0.10
222.101	C11H14N2O3	Phe-Gly	3349180.60	3325075.23	1175591.62	6257922.92	5653324.76	1567950.18	1164441.03	976594.86	252155.71	1580141.51	1236184.52	342855.90	1.87	1.36	0.07	0.16
231.122	C9H17N3O4	Ala-Ala-Ala	951485.91	343886.54	121582.25	1096968.40	368442.49	102187.56	1055824.23	846316.16	218517.89	951993.99	694515.58	192623.96	1.15	0.90	0.16	0.36
232.142	C10H20N2O4	Leu-Thr	6916132.04	6410249.12	2266365.31	11273192.44	8301327.94	2302374.12	2725340.50	1626064.37	419848.01	3788489.03	1583908.97	439297.31	1.63	1.39	0.08	0.05
244.070	C9H12N2O6	Uridine	6673999.15	5678022.66	2007484.16	5791494.52	4587173.15	1272252.92	2995293.17	2437972.73	629481.85	2249869.23	2040517.48	565937.72	0.87	0.75	0.34	0.20
245.138	C10H19N3O4	Val-Ala-Gly	4361654.30	3865691.84	1366728.46	7049336.77	5518917.51	1530672.31	1702519.41	1422612.11	367316.87	2346025.01	1739260.76	482384.14	1.62	1.38	0.09	0.15
252.111	C12H16N2O4	Phe-Ser	3273946.84	2278842.94	805692.65	10171427.94	7135607.46	1979061.43	3250098.75	3143456.78	811637.05	3767928.18	3503245.37	971625.45	3.11	1.16	0.00	0.34
259.153	C11H21N3O4	Val-Ala-Ala	5618858.47	3898251.83	1378240.15	8285048.46	5103644.20	1415496.22	3974876.47	3955060.88	1021192.33	4021208.76	3759186.22	1042610.67	1.47	1.01	0.08	0.49
268.081	C10H12N4O5	Inosine	117012880.67	73431414.45	25961925.56	124232743.38	95238395.92	26414378.45	82362266.93	33909905.05	8755499.84	86921551.69	31041246.49	8609292.76	1.06	1.06	0.42	0.36
275.127	C14H17N3O3	Trp-Ala	1188150.62	1406631.97	497319.50	1710391.48	1567818.01	434834.48	259547.04	281579.15	72703.42	418045.76	319773.23	88689.14	1.44	1.61	0.20	0.09
277.110	C10H19N3O4S	Met-Gln	692187.82	481435.02	170212.99	1007964.32	526584.97	146048.39	457577.04	382508.76	98763.34	592346.93	413157.81	114589.36	1.46	1.29	0.07	0.19
279.122	C13H17N3O4	Phe-Asn	4359914.45	2535022.21	896265.70	5414710.81	3064416.93	849916.34	2427290.46	1408423.32	363653.34	2990620.99	1425628.31	395398.15	1.24	1.23	0.18	0.15
280.142	C14H20N2O4	Val-Tyr	2164222.14	1881559.43	665231.72	3785944.19	2956070.13	819866.34	893428.09	668806.76	172685.16	1317298.17	903148.97	250488.46	1.75	1.47	0.06	0.08
283.092	C10H13N5O5	Guanosine	18220515.13	8816526.33	3117112.78	21643722.42	15075074.49	4181073.39	17256866.27	10397385.72	2684593.45	18899114.88	8552232.03	2371962.39	1.19	1.10	0.25	0.33
296.120	C14H20N2O3S	Met-Phe	2363287.68	1488931.78	526416.88	2571322.42	2261206.57	627145.86	2071331.38	1122220.87	289756.18	2904546.46	1377813.99	382136.84	1.09	1.40	0.40	0.04
301.200	C14H27N3O4	Leu-Leu-Gly	790024.40	421350.18	148969.79	1124666.61	597685.65	165768.17	421351.30	278607.39	71936.12	528996.38	245088.91	67975.43	1.42	1.26	0.06	0.15
312.148	C18H20N2O3	Phe-Phe	908937.02	306728.26	108444.82	1033796.65	484860.22	134476.03	688860.42	369680.82	95451.18	1072896.20	469668.89	130262.71	1.14	1.56	0.23	0.01
317.174	C17H23N3O3	Leu-Trp	910827.29	552809.26	195447.59	1169280.92	667837.21	185224.72	349835.51	168546.33	43518.48	535391.60	233179.56	64672.37	1.28	1.53	0.15	0.01
328.142	C18H20N2O4	Phe-Tyr	960143.43	371096.32	131202.36	1236905.00	832390.70	230863.64	638420.03	263080.83	67927.18	1003824.34	412659.84	114451.25	1.29	1.57	0.15	0.00
335.131	C16H21N3O3S	Met-Trp	489123.65	437372.19	154634.42	561961.19	608023.35	168635.34	360921.13	207939.33	53689.70	475208.84	254195.55	70501.16	1.15	1.32	0.37	0.10
344.137	C18H20N2O5	Tyr-Tyr	232681.11	137801.39	48720.15	293099.54	243946.00	67658.45	201706.84	109576.36	28292.50	253865.16	134048.24	37178.29	1.26	1.26	0.23	0.13
357.263	C18H35N3O4	Leu-Leu-Leu	351040.34	130954.21	46299.31	499624.67	262964.02	72933.10	300873.20	165941.99	42846.04	517049.09	252310.80	69978.42	1.42	1.72	0.05	0.01
376.138	C17H20N4O6	Riboflavin	210157.47	59465.59	21024.26	230661.31	79740.14	22115.94	173863.02	60201.86	15544.05	198745.38	74363.88	20624.83	1.10	1.14	0.24	0.17

TABLE LEGEND

	Sign Different
	HIGHER IN INFECTED
	LOWER IN INFECTED
	compund different in > 1 RUN
	No Difference

Appendix 5.2

Mass	FORMULA	Identification	RUN1 Pvalue		RUN 2 Pvalue		RUN 3 Pvalue		RATIO RUN1		Ratio RUN 2		RATIO RUN 3	
m/z			1 month	2 month	1 month	2 month	1 month	2 month	1 month	2 month	1 month	2 month	1 month	2 month
136.062	C5H5N5	adenine		0.000	0.003	0.003	0.049	0.187		0.419	0.447	0.541	0.426	0.751
153.041	C5H4N4O2	xanthine			0.092		0.003	0.039			1.763		2.204	1.585
154.086	C6H3(OH)2-CH2-CH2-NH2	dopamine	0.004	0.002	0.018		0.024	0.312	0.410	0.374	0.493		0.277	0.833
209.092	C10H12N2O3	kynurenine	0.017		0.012	0.027			6.252		3.525	4.332		
268.104	C10H13N5O4	adenosine	0.000	0.010	0.010	0.008			0.204	0.032	0.022	0.050		
348.070	C10H14N5O7P	AMP		0.035	0.027	0.000	0.032	0.001		0.203	0.441	0.353	0.103	0.378

Samples Number	RUN 1		RUN 2		RUN 3	
Time Points	1 Month	2 Month	1 Month	2 Month	1 Month	2 Month
Control	5	5	5	5	4	6
Infected	5	5	5	5	4	6

TABLE LEGEND	
	Sign Different
	HIGHER IN INFECTED
	LOWER IN INFECTED
	compund different in > 1 RUN
	No Difference
	Compound not detected

CHAPTER 3

Behavioural analysis

Open field

A-MALE B-FEMALE

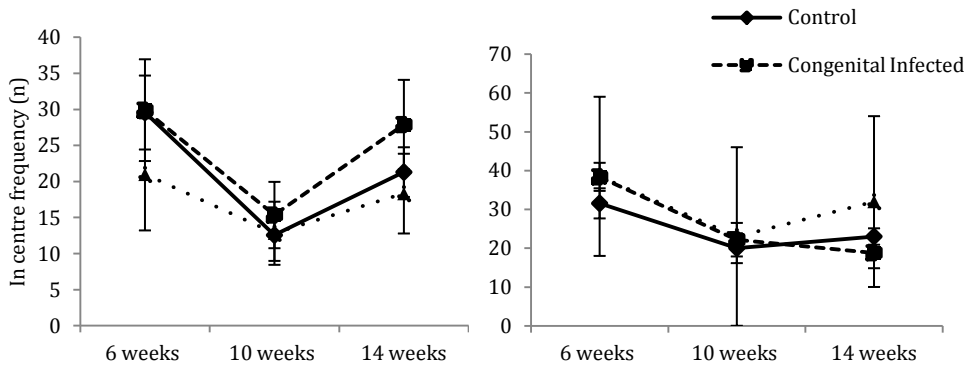


Figure 5.3: Analysis of the centre frequency (n) parameter performed in the in the 10 minute trial of the open field test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). No difference in behaviour was observed at any time point between the groups of the same gender and between males and females of the same group.

A-MALE B-FEMALE

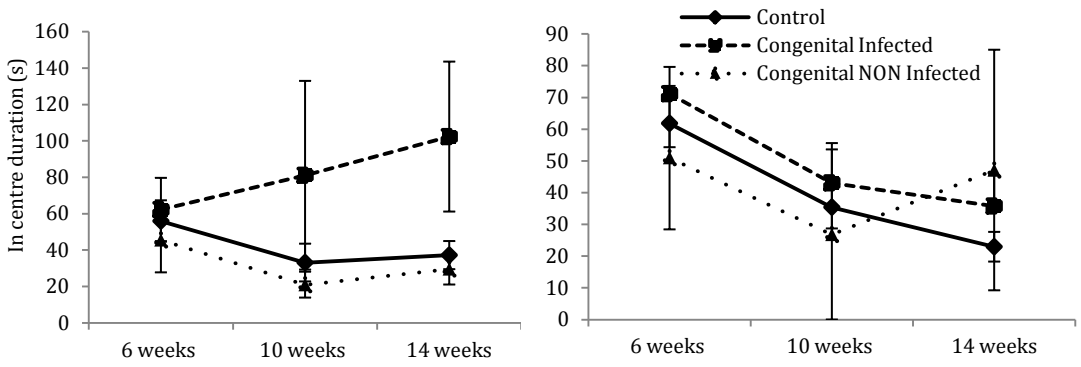


Figure 5.4: Analysis of the centre total duration (s) parameter performed in the in the 10 minute trial of the open field test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). No difference in behaviour was observed at any time point between the groups of the same gender and between males and females of the same group.

A-MALE B-FEMALE

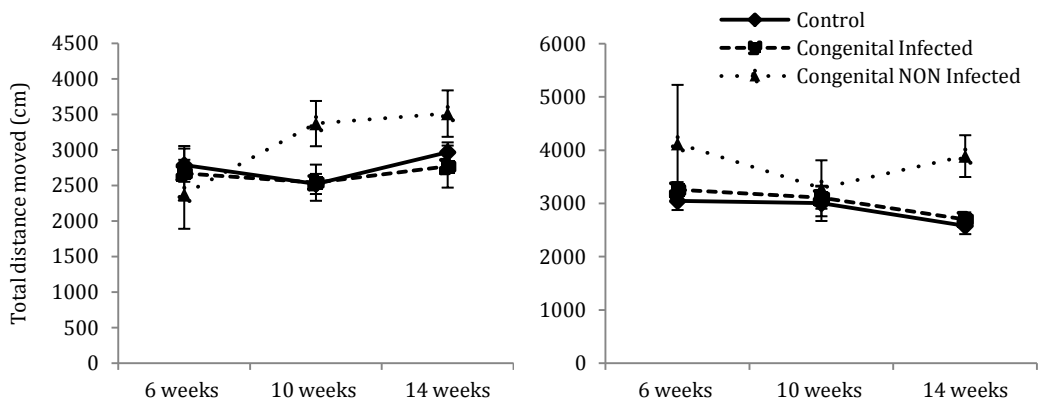


Figure 5.5: Analysis of the total distance moved (cm) parameter performed in the in the 10 minute trial of the open field test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). At 10 weeks differences were found within the male groups. In particular, the difference was found between the control and the non-infected litter mates group ($p=0.02$) and a minor difference was found between the non-infected litter mates and the infected group ($p=0.049$). At 14 weeks differences were found within the female group in the total distance moved (cm) parameter, between the control group and the non-infected litter mates group ($p=0.0123$) and between the non-infected litter mates and the infected group ($p=0.0128$). No differences were found between males and females mice.

Elevated Plus Maze

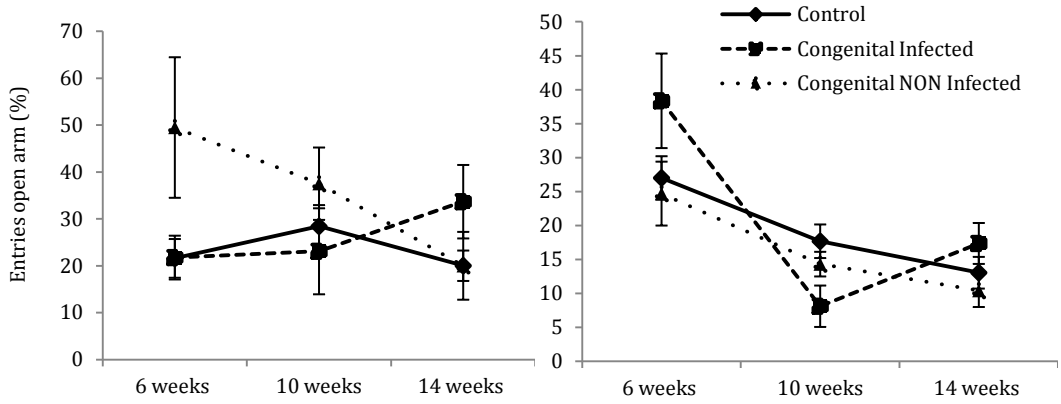


Figure 5.6: Analysis of the number of entries in the open arm (%) parameter performed in the in the 10 minute trial of the elevated plus maze test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). Differences in the male groups were found: at 6 weeks between the controls and the non-infected litter mates group (p=0.039), at 10 weeks between the congenitally infected and the non-infected litter mates group (p=0.036) and at 14 weeks between the controls and the congenitally infected group (p=0.038). Differences in the female groups were found at 10 weeks between the control and the congenitally infected group (p=0.013). Differences were also found between the genders: at 6 weeks between male and females of the congenitally infected group (p=0.032), at 10 weeks parameter between male and females of the control group (p=0.009) and at 14 weeks between male and females of the congenitally infected group (p=0.0278).

A-MALE B-FEMALE

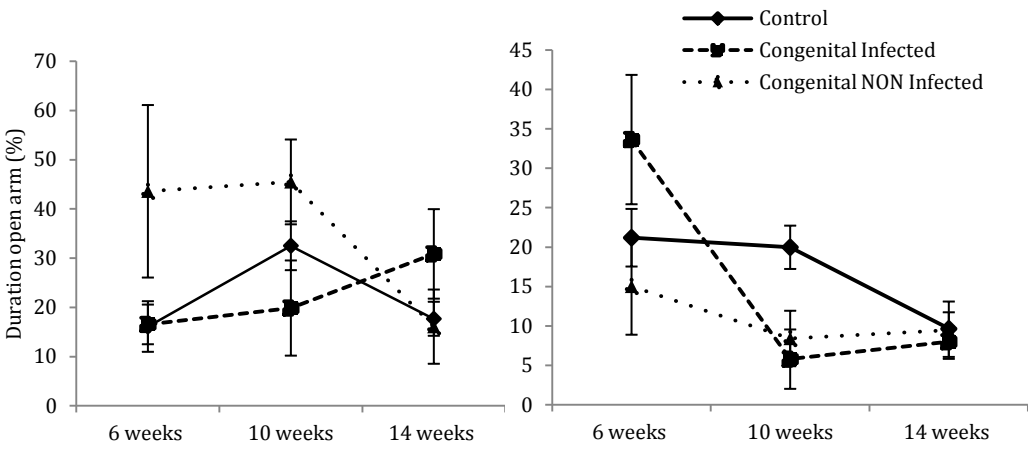


Figure 5.7: Analysis of the time spent in the open arm (%) parameter performed in the in the 10 minute trial of the elevated plus maze test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). Differences in the male group were found: at 10 weeks between the control and the congenitally infected group ($p=0.022$) and the non-infected litter mates and the congenitally infected group ($p=0.0156$). Differences in the female group were found at 10 weeks between the control and the congenitally infected group ($p=0.00376$). Differences were also found between the genders: at 6 weeks between male and females in the control ($p=0.041$), congenitally infected ($p=0.049$) and non-infected litter mates $p=0.035$), at 10 weeks male and females in the control ($p=0.041$), congenitally infected ($p=0.049$) and non-infected litter mates ($p=0.035$) and at 14 weeks between male and female of the congenitally infected group.

A-MALE B-FEMALE

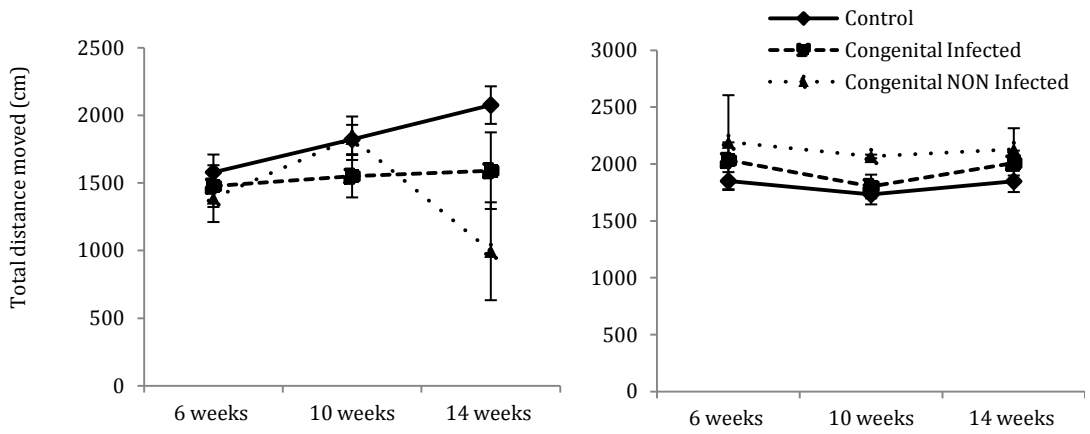


Figure 5.8: Analysis of the total distance moved (cm) parameter performed in the 10 minute trial of the elevated plus maze test during the congenital experiment dived by mice gender at 6, 10 and 14 weeks old. The N number for the male mice (A) per each group was as follow: 19 controls (continuous black line), 11 congenitally infected (non continuous black line) and 6 non-infected litter mates (dotted line). The N number for the female mice (B) per each group was as follow: 24 controls (continuous black line), 11 congenitally infected (non continuous black line) and 2 non-infected litter mates (dotted line). Differences in the male group were found at 14 weeks between the controls and the non-infected litter mates (p=0.00357). Differences in the female group were found at 6 weeks between the control group and the congenitally infected (p=0.049). Differences were also found between the genders: at 6 weeks between male and females of the congenitally infected group.

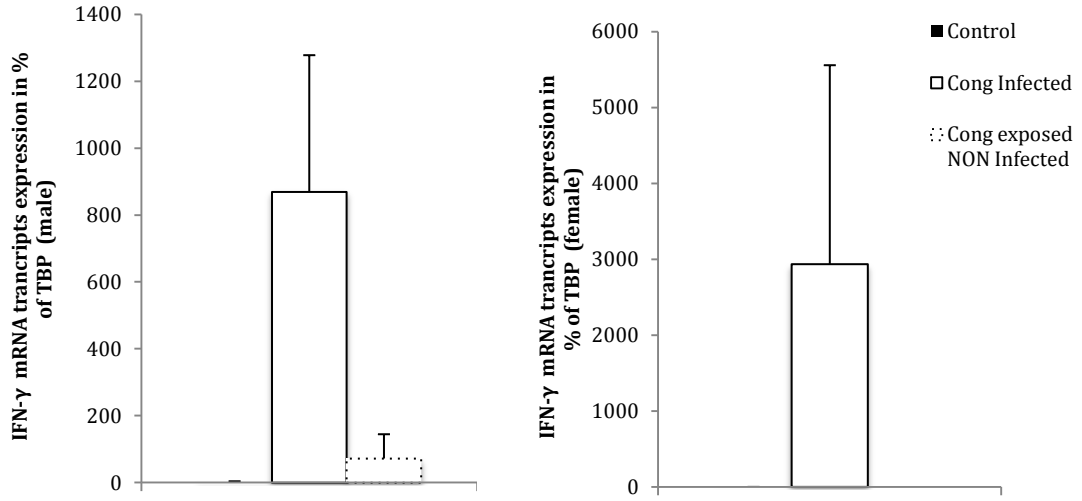


Figure 5.9: represents the expression levels of IFN-γ in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

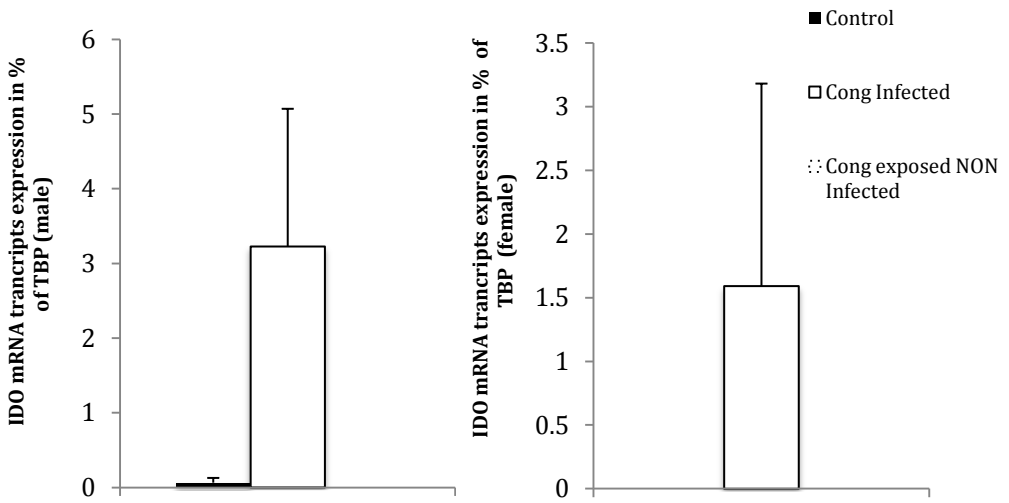


Figure 5.10: represents the expression levels of IDO in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

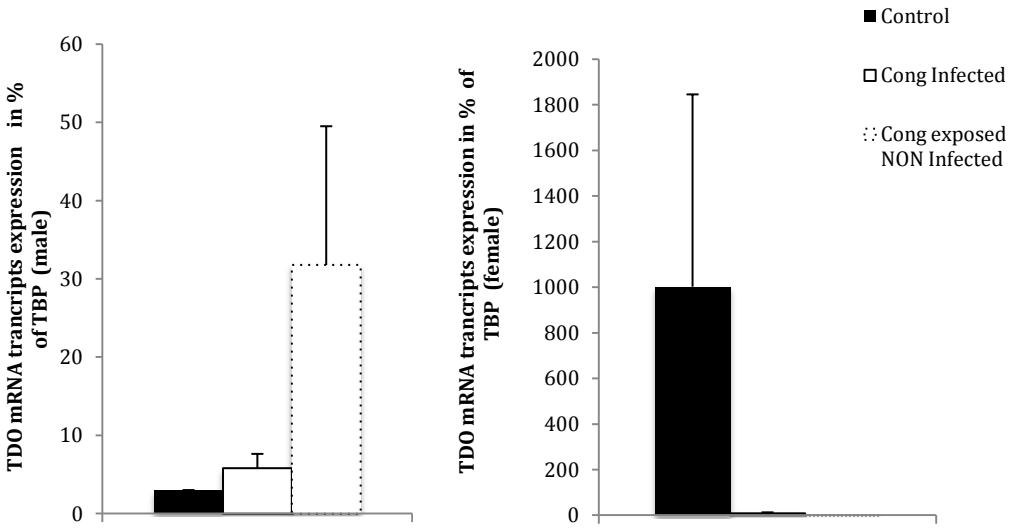


Figure 5.11: represents the expression levels of TDO in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

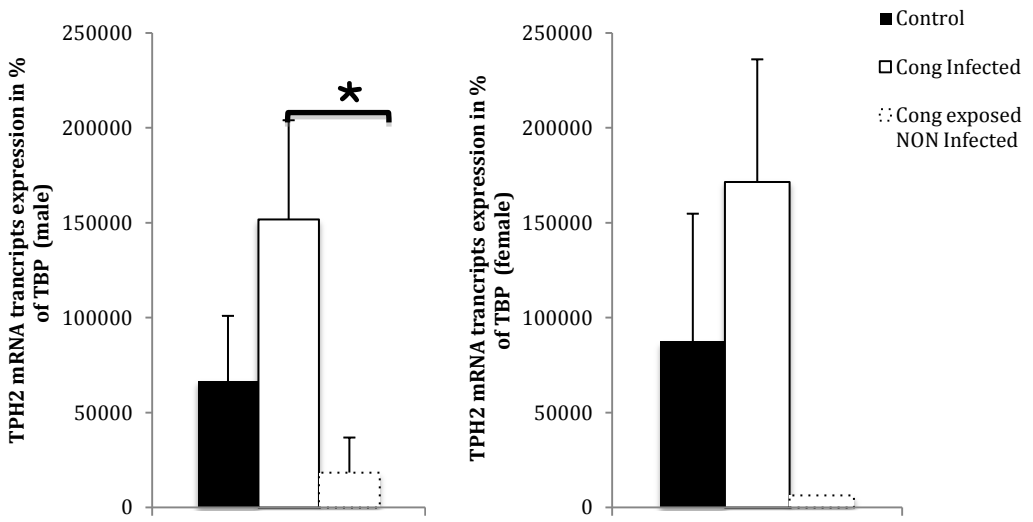


Figure 5.12: represents the expression levels of TPH2 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. Differences were measured in the male group between the congenitally infected and the non-infected litter mates group (p=0.045). This is represented by an *.

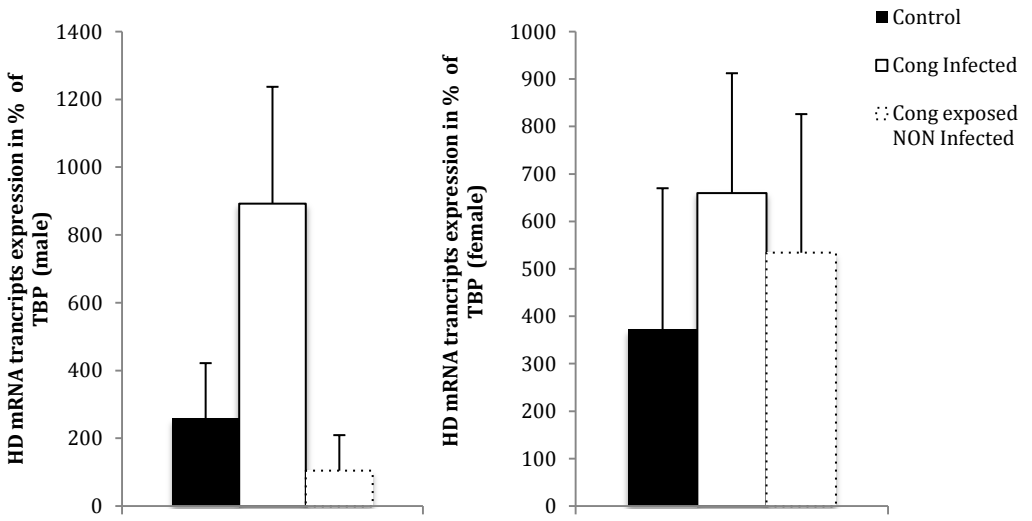


Figure 5.13: represents the expression levels of HD in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

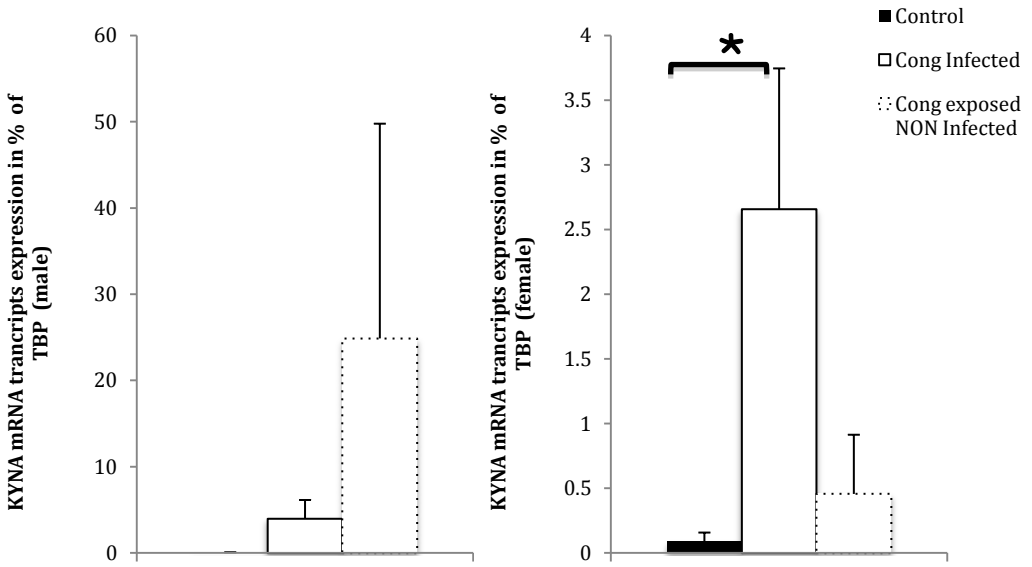


Figure 5.14: represents the expression levels of KYNA in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. Differences were observed in the female group between the congenital infected and the control group (p=0.045). This is represented by an *.

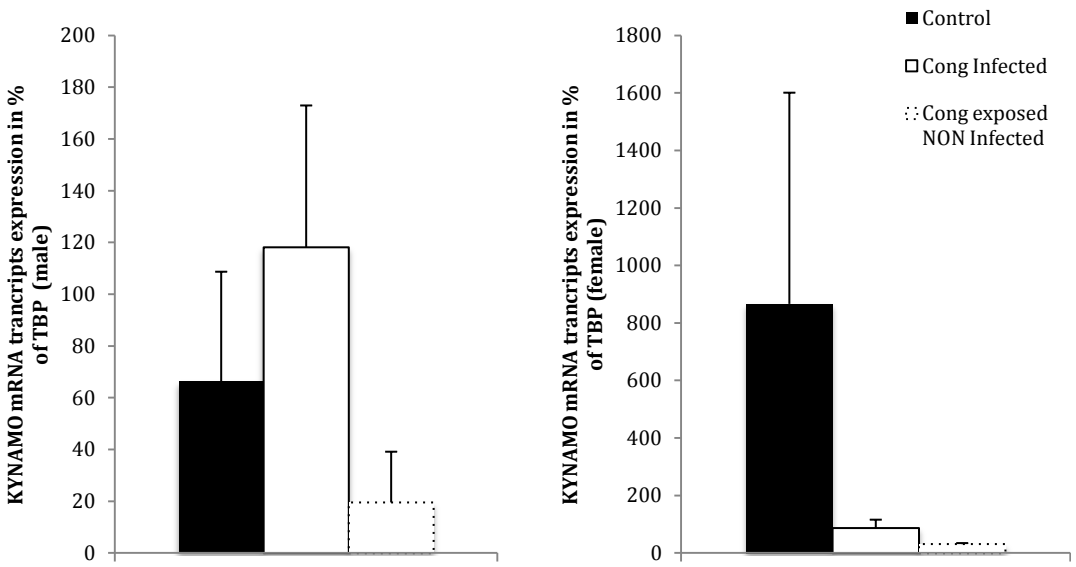


Figure 5.15: represents the expression levels of KYNA MO in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

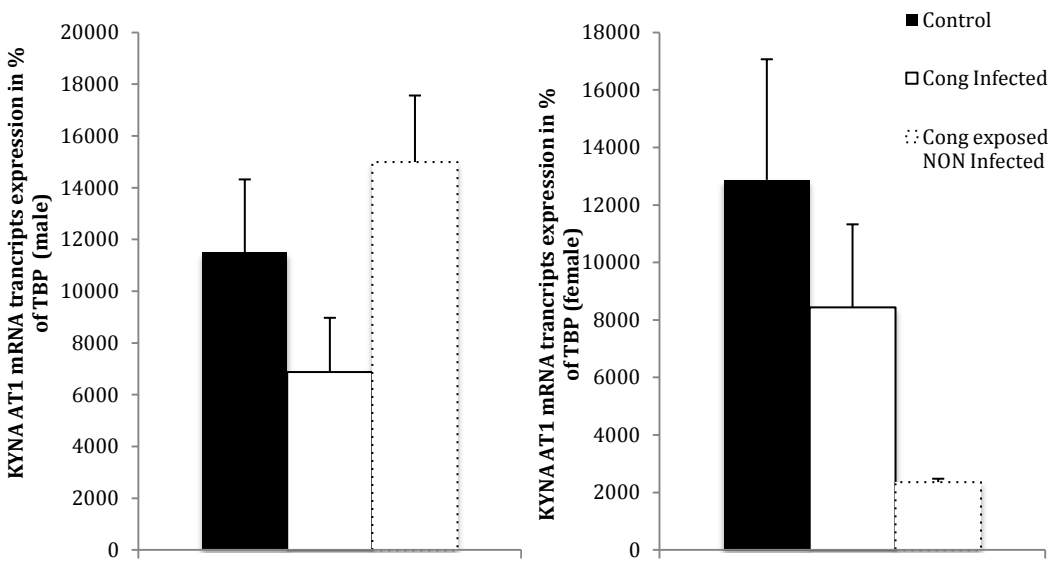


Figure 5.16: represents the expression levels of KYNA AT1 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

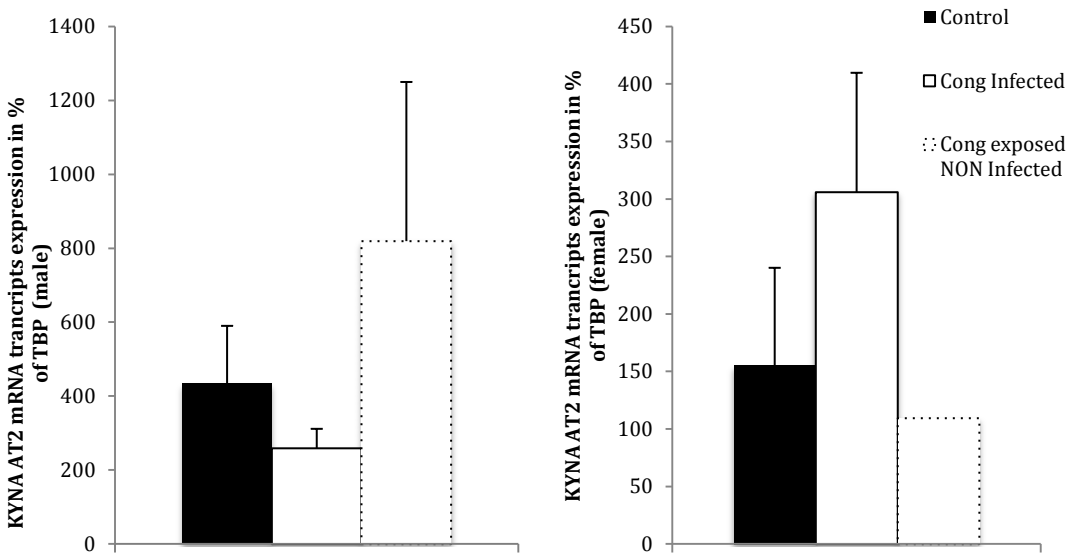


Figure 5.17: represents the expression levels of KYNA AT2 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

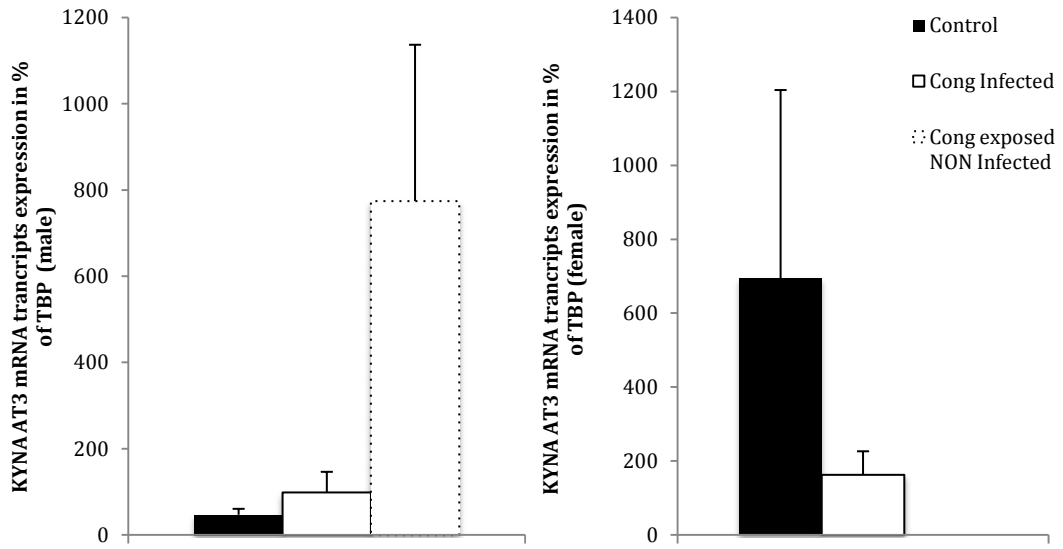


Figure 5.18: represents the expression levels of KYNA AT3 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

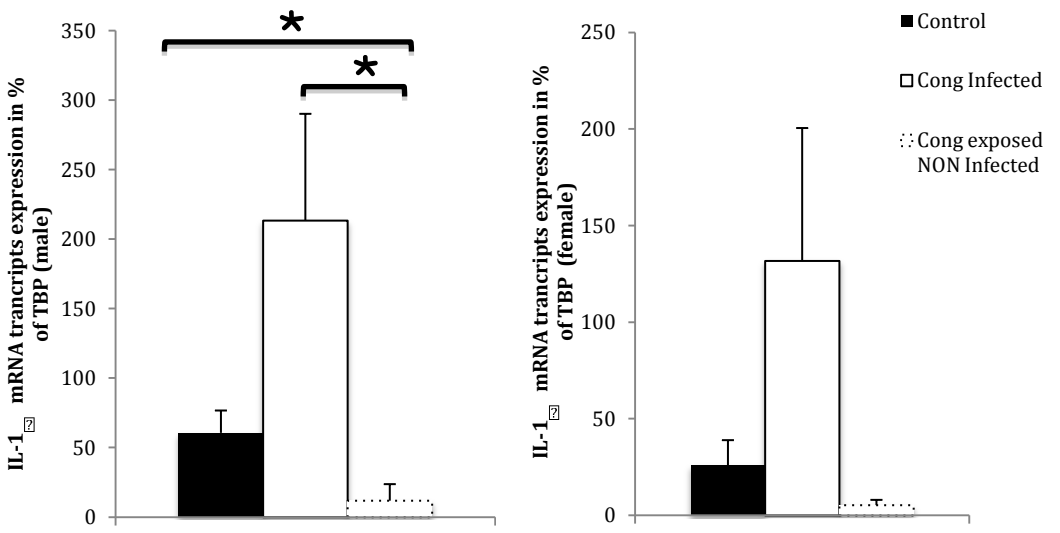


Figure 5.19: represents the expression levels of IL-1β in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. Differences were observed in the male group between the controls and the non-infected litter mates (p=0.040) and the congenitally infected and the non-infected litter mates (p=0.039). This is represented by an *.

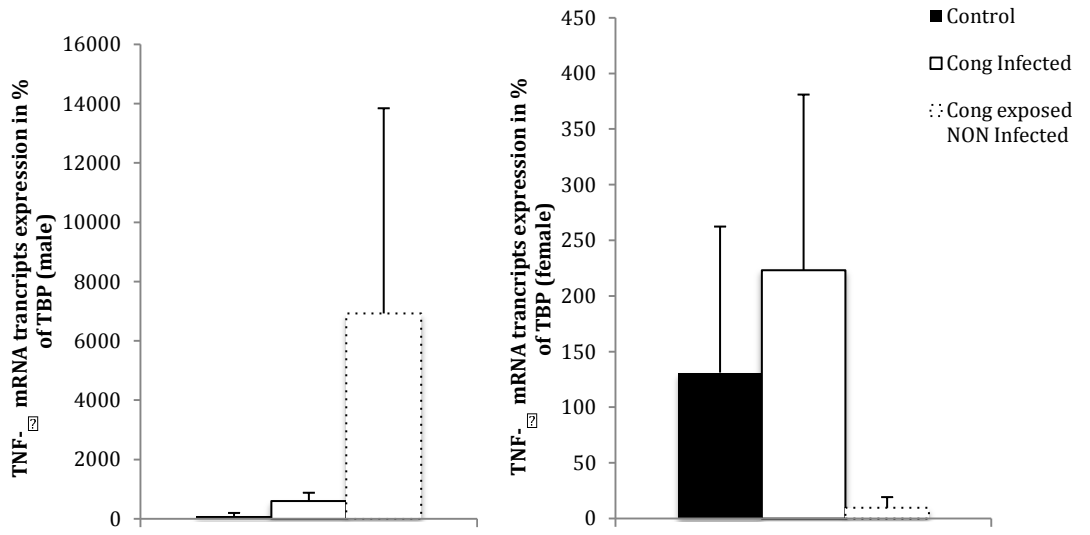


Figure 5.20: represents the expression levels of TNF-α in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

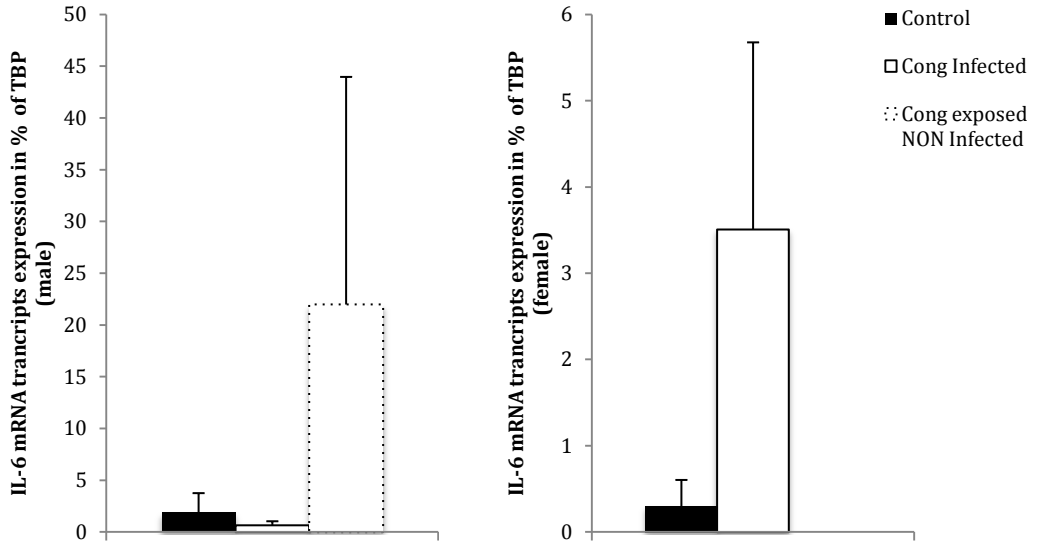


Figure 5.21: represents the expression levels of IL-6 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

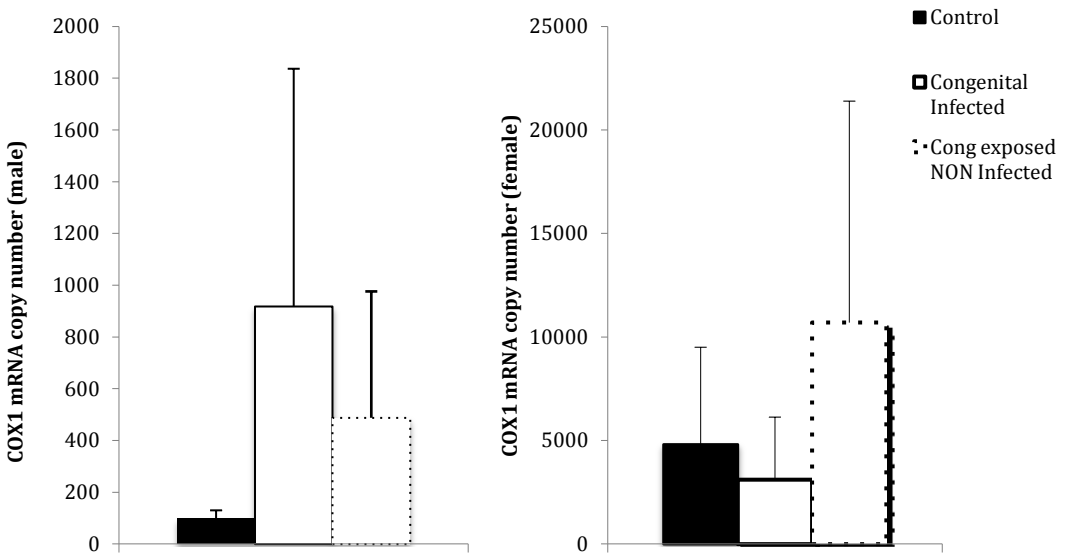


Figure 5.22: represents the expression levels of COX1 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

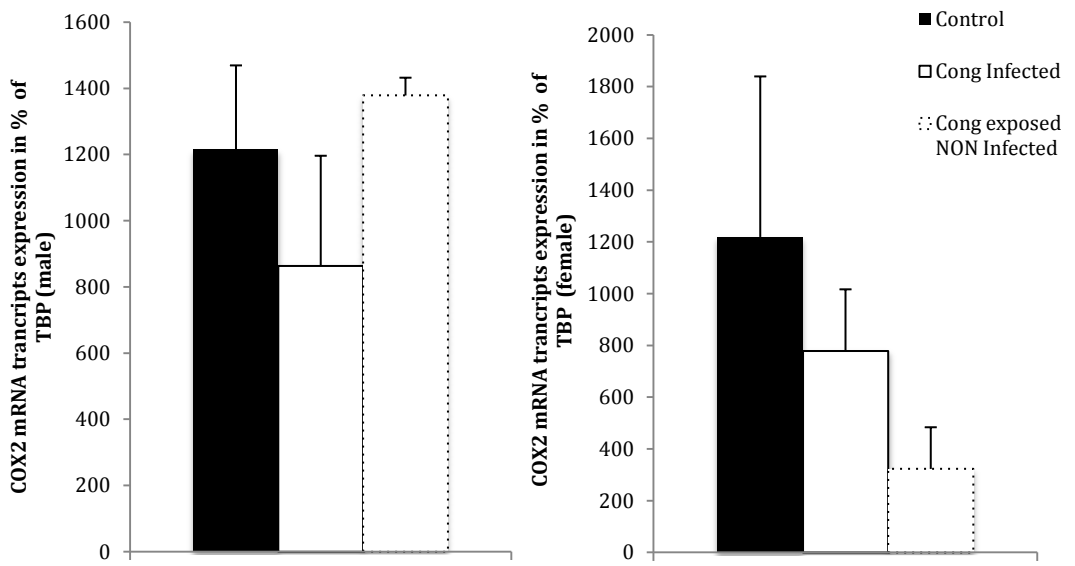


Figure 5.23: represents the expression levels of COX2 in the controls (black columns) (n=11: 6 males and 5 females), in the congenitally infected (white columns) (n=16: 7 males and 9 females) and in the non-infected litter mates (white columns with dotted borders) (n=6: 4 male and 2 female) brain samples at 14 weeks old. No differences were observed.

Metabolomic analysis Appendix 5.24

			Control		Congenital Infected		Non Infected litter mates		Ratio			P Value		
Mass	Formula	Identification	Mean	SE	Mean	SE	Mean	SE	Congenital Infected/ Control	Non Infected litter mates/Control	Congenital Infected/ Non infected litter mates	Congenital Infected/ Control	Non Infected litter mates/ Control	Congenital Infected/ Non infected litter mates
75.0321	C2H5NO2	Glycine	174870.391	20072.892	168879.309	21287.340	161852.780	21259.382	0.966	0.926	1.043	0.843	0.666	0.822
79.0421	C5H5N	Pyridine	30522.188	3288.477	33327.946	2737.972	30071.080	2128.729	1.092	0.985	1.108	0.531	0.911	0.382
84.0212	C4H4O2	3-Butynoate	138934.790	6347.981	140233.256	3412.151	126175.410	8277.780	1.009	0.908	1.111	0.862	0.251	0.163
85.0528	C4H7NO	Acetone cyanohydrin	36407.652	3278.977	28198.184	2363.348	34809.979	1996.836	0.775	0.956	0.810	0.077	0.688	0.071
		2-Pyrrolidinone												
85.0892	C5H11N	Piperidine	37717.533	1702.664	33186.030	858.745	38659.193	2185.346	0.880	1.025	0.858	0.049	0.741	0.056
87.0684	C4H9NO	4-Aminobutanal	60849.356	1403.388	63258.765	1583.584	65592.171	3594.125	1.040	1.078	0.964	0.292	0.262	0.572
89.0477	C3H7NO2	L-Alanine	4978250.708	239580.951	4815071.250	283867.726	4781387.833	213219.352	0.967	0.960	1.007	0.674	0.553	0.927
97.9769	H3O4P	Orthophosphate	22979.595	5138.556	25058.701	8911.235	6512.354	4265.398	1.090	0.283	3.848	0.848	0.034	0.127
99.0685	C5H9NO	N-Methyl-2-pyrrolidinone	124655.879	26839.234	736601.086	391506.768	109196.401	15396.185	5.909	0.876	6.746	0.216	0.631	0.207
100.0525	C5H8O2	Tiglic acid	56114.160	11675.723	84935.751	10127.532	73616.783	3822.226	1.514	1.312	1.154	0.100	0.204	0.357
		1-Aminocyclopropane-1-carboxylate												
101.0841	C5H11NO	Betaine aldehyde	13460.028	3439.884	15880.036	2715.173	18771.612	1455.017	1.180	1.395	0.846	0.596	0.200	0.393
103.0634	C4H9NO2	4-Aminobutanoate	16434223.000	724358.526	16529480.500	1086306.734	16842313.500	392237.797	1.006	1.025	0.981	0.944	0.634	0.801
103.0998	C5H13NO	Choline	9617555.000	527719.881	10931905.750	427667.865	10038553.667	411136.063	1.137	1.044	1.089	0.089	0.544	0.174
105.0426	C3H7NO3	L-Serine	5727842.833	220990.759	4974846.250	217921.341	5375072.250	236180.711	0.869	0.938	0.926	0.043	0.301	0.249
106.0631	C4H10O3	Diethylene glycol	25273.145	2166.719	32435.623	3514.774	27038.172	3068.111	1.283	1.070	1.200	0.140	0.650	0.286
111.0433	C4H5N3O	Cytosine	393471.297	26478.221	459861.430	22043.051	427205.099	17605.165	1.169	1.086	1.076	0.090	0.317	0.288
112.0273	C4H4N2O2	Uracil	253753.956	29418.997	384879.227	50628.467	284654.664	28289.656	1.517	1.122	1.352	0.075	0.466	0.146
113.0478	C5H7NO2	(S)-1-Pyrroline-5-carboxylate	19802.828	682.256	23619.932	8181.426	17961.821	774.249	1.193	0.907	1.315	0.673	0.105	0.540
114.0430	C4H6N2O2	5,6-Dihydrouracil	74134.790	3781.601	84658.883	7914.347	70114.447	3697.282	1.142	0.946	1.207	0.291	0.465	0.166
115.0634	C5H9NO2	L-Proline	5221897.167	309576.356	5191588.875	254251.652	5251262.417	160404.016	0.994	1.006	0.989	0.942	0.935	0.850
115.0998	C6H13NO	Trimethylaminoacetone	41819.422	1122.763	42020.207	1109.739	40938.191	2530.261	1.005	0.979	1.026	0.902	0.760	0.708
116.0838	C6H12O2	Hexanoic acid	49782.105	1660.764	53228.504	2071.482	51093.495	2269.569	1.069	1.026	1.042	0.239	0.652	0.507
116.0950	C5H12N2O	5-Aminopentanamide	43289.235	3258.320	44332.025	687.949	50060.563	3664.818	1.024	1.156	0.886	0.766	0.198	0.181
117.0539	C3H7N3O2	Guanidinoacetate	262359.070	16393.085	400526.988	118555.517	284585.042	36194.323	1.527	1.085	1.407	0.329	0.593	0.409
117.0790	C5H11NO2	L-Valine	3353917.083	142764.490	4282991.438	255814.917	3830544.792	204317.064	1.277	1.142	1.118	0.026	0.088	0.213
118.0532	C7H6N2	Benzimidazole	235423.232	11072.100	205529.063	14074.650	214489.664	9586.136	0.873	0.911	0.958	0.143	0.184	0.619
118.0783	C9H10	alpha-Methylstyrene	190463.247	11513.134	212183.895	14884.969	223991.633	41261.992	1.114	1.176	0.947	0.290	0.465	0.797
119.0583	C4H9NO3	L-Threonine	4355472.750	344274.514	3989546.375	406096.254	4028883.208	105057.567	0.916	0.925	0.990	0.515	0.400	0.931
121.0198	C3H7NO2S	L-Cysteine	518728.323	63649.519	517292.984	25624.524	444268.682	33046.221	0.997	0.856	1.164	0.984	0.331	0.119
122.0480	C6H6N2O	Nicotinamide	38262909.667	1576936.113	36076830.000	1706636.188	35351309.333	1567833.102	0.943	0.924	1.021	0.377	0.220	0.763
123.9926	C2H5O4P	Phosphonoacetaldehyde	106289.428	5555.400	98169.576	4459.757	94661.382	5386.008	0.924	0.891	1.037	0.287	0.164	0.629
125.0147	C2H7NO3S	Taurine	40326472.000	524955.980	41804394.000	337235.093	40729563.333	652134.056	1.037	1.010	1.026	0.046	0.641	0.186
125.0954	C6H11N3	N-Methylhistamine	38474.974	17804.078	79474.548	8256.889	69855.540	7114.716	2.066	1.816	1.138	0.076	0.149	0.407
128.0587	C5H8N2O2	5,6-Dihydrothymine	996672.573	29073.038	1058527.172	57518.548	996236.688	32352.266	1.062	1.000	1.063	0.385	0.992	0.389
129.0427	C5H7NO3	L-1-Pyrroline-3-hydroxy-5-carboxylate	90011.520	13518.486	473012.964	420609.747	86862.724	16892.088	5.255	0.965	5.446	0.430	0.887	0.426
129.0791	C6H11NO2	L-Pipecolate	223047.172	36479.434	198153.695	26003.448	193809.497	30840.123	0.888	0.869	1.022	0.594	0.555	0.917
129.1155	C7H15NO	4-Trimethylammoniobutanal	113287.747	1853.279	101414.309	2877.548	102850.389	3878.289	0.895	0.908	0.986	0.016	0.045	0.774
129.1518	C8H19N	Octylamine	356348.219	44274.748	421787.078	59511.978	522799.995	62802.855	1.184	1.467	0.807	0.411	0.059	0.278
131.0584	C5H9NO3	L-Glutamate 5-semialdehyde	314517.026	30403.560	294763.676	31697.358	260471.354	12728.622	0.937	0.828	1.132	0.666	0.147	0.372
131.0695	C4H9N3O2	Creatine	197692408.000	7460726.887	192336444.000	8264438.969	191885810.667	6200080.002	0.973	0.971	1.002	0.645	0.563	0.967
		L-(iso)Leucine												

			Control		Congenital Infected		Non Infected litter mates		Ratio			P Value		
Mass	Formula	Identification	Mean	SE	Mean	SE	Mean	SE	Congenital Infected/ Control	Non Infected litter mates/Control	Congenital Infected/ Non infected litter mates	Congenital Infected/ Control	Non Infected litter mates/ Control	Congenital Infected/ Non infected litter mates
132.0424	C5H8O4	2-Acetolactate	89880.111	3577.788	73125.263	3703.457	73648.622	3667.013	0.814	0.819	0.993	0.013	0.010	0.923
132.0536	C4H8N2O3	L-Asparagine	1710897.417	87182.051	1715425.906	99359.041	1774614.917	107583.698	1.003	1.037	0.967	0.974	0.656	0.697
132.0900	C5H12N2O2	L-Ornithine	101703.029	4715.380	109508.035	5188.845	130050.743	18758.053	1.077	1.279	0.842	0.302	0.196	0.334
133.0376	C4H7NO4	L-Aspartate	22086215.990	4584930.850	28784678.000	1807746.667	379374.570	35688.474	1.303	0.017	75.874	0.220	0.005	0.001
135.0546	C5H5N5	Adenine	36206.340	1416.539	51947.163	4052.993	41670.189	1198.299	1.435	1.151	1.247	0.024	0.015	0.080
136.0386	C5H4N4O	Hypoxanthine	60257165.333	2378469.560	59560881.000	2299538.719	57250432.667	2940564.789	0.988	0.950	1.040	0.839	0.446	0.553
138.0430	C6H6N2O2	Urocanate	48955.092	1411.811	43722.978	3778.609	45531.870	2173.024	0.893	0.930	0.960	0.267	0.221	0.695
140.0587	C6H8N2O2	Methylimidazoleacetic acid	262577.273	42331.203	495246.859	119848.434	348785.120	28611.460	1.886	1.328	1.420	0.146	0.127	0.312
141.0192	C2H8NO4P	Ethanolamine phosphate	4816454.542	302374.305	5437911.500	196867.431	5469232.375	432090.379	1.129	1.136	0.994	0.124	0.247	0.949
143.0947	C7H13NO2	Stachydrine	46446.951	9771.081	58319.279	2240.883	42664.315	8615.812	1.256	0.919	1.367	0.285	0.778	0.132
143.1311	C8H17NO	valpromide	45074.912	2310.571	47380.145	5489.802	41956.578	3293.847	1.051	0.931	1.129	0.718	0.458	0.435
144.0901	C6H12N2O2	L-isoglutamine	64889.585	21544.337	42505.056	18558.507	59865.532	25066.543	0.655	0.923	0.710	0.454	0.882	0.593
145.0852	C5H11N3O2	4-Guanidinobutanoate	666555.323	46183.536	635793.555	54662.559	617781.370	32662.995	0.954	0.927	1.029	0.681	0.411	0.788
145.1104	C7H15NO2	4-Trimethylammoniobutanoate	3758026.292	213622.535	3683497.875	391577.609	3875450.750	217806.179	0.980	1.031	0.950	0.874	0.708	0.687
146.0692	C5H10N2O3	L-Glutamine	91158420.000	4145861.553	81249754.000	1527657.060	82352530.000	3960573.390	0.891	0.903	0.987	0.064	0.156	0.803
146.1056	C6H14N2O2	L-Lysine	1026941.854	57661.025	1068456.219	96003.499	1048871.729	128539.471	1.040	1.021	1.019	0.726	0.881	0.906
147.0532	C5H9NO4	L-Glutamate	3885315.958	334670.652	4314094.000	241951.362	4492777.083	469979.656	1.110	1.156	0.960	0.330	0.320	0.745
148.0671	C5H12N2OS	L-methioninamide	47290.753	6135.941	87479.955	10663.672	70278.747	8154.154	1.850	1.486	1.245	0.022	0.050	0.246
149.0512	C5H11NO2S	L-Methionine	6624854.250	204376.487	5885759.625	288698.950	6000295.500	182059.303	0.888	0.906	0.981	0.083	0.046	0.750
150.0682	C9H10O2	Phenylpropanoate	131230.875	8084.530	128519.230	15738.812	152132.253	17087.296	0.979	1.159	0.845	0.885	0.305	0.340
151.0495	C5H5N5O	Guanine	172122.195	18266.780	103048.160	11591.836	139214.120	44169.421	0.599	0.809	0.740	0.013	0.514	0.460
152.0336	C5H4N4O2	Xanthine	2267030.521	247936.323	3550843.875	612966.947	2562190.313	233534.396	1.566	1.130	1.386	0.124	0.407	0.208
152.0586	C7H8N2O2	N1-Methyl-2-pyridone-5-carboxamide	95802.247	9023.431	158323.439	55576.107	78488.019	4489.769	1.653	0.819	2.017	0.344	0.128	0.246
153.0790	C8H11NO2	Dopamine	103892.461	9018.362	29821.348	29821.348	73634.341	24228.621	0.287	0.709	0.405	0.084	0.284	0.294
154.0032	C3H7O5P	Propanoyl phosphate	84318.677	8082.857	93782.180	6184.462	94799.266	8415.220	1.112	1.124	0.989	0.380	0.390	0.925
155.0696	C6H9N3O2	L-Histidine	1637877.313	97549.968	1546109.281	56842.503	1552440.938	102741.098	0.944	0.948	0.996	0.441	0.560	0.958
157.0376	C6H7NO4	2-Aminomuconate	181492.315	8451.424	234299.344	51778.984	191343.484	13644.358	1.291	1.054	1.224	0.385	0.556	0.474
158.0441	C4H6N4O3	Allantoin	109603.986	11086.919	132865.521	10693.031	93862.667	10255.736	1.212	0.856	1.416	0.171	0.322	0.032
159.0685	C10H9NO	Indole-3-acetaldehyde	269463.857	27122.119	255367.703	9551.682	287711.042	25462.945	0.948	1.068	0.888	0.641	0.634	0.277
159.1260	C8H17NO2	DL-2-Aminooctanoicacid	11220324.833	537497.982	14384353.250	1319531.631	13837460.667	517434.441	1.282	1.233	1.040	0.090	0.006	0.720
160.0849	C6H12N2O3	D-Alanyl-D-alanine	339329.391	70020.780	391252.414	46325.396	340971.500	19793.917	1.153	1.005	1.147	0.554	0.983	0.373
160.1213	C7H16N2O2	N6-Methyl-L-lysine	196716.943	12853.050	193443.699	11108.579	209625.066	23408.646	0.983	1.066	0.923	0.852	0.642	0.552
161.0511	C6H11NO2S	allylcysteine	94463.303	13593.055	60545.907	11111.534	56364.865	15146.119	0.641	0.597	1.074	0.090	0.091	0.829
161.0689	C6H11NO4	L-2-Aminoadipate	15630.678	879.026	38457.741	20320.634	16982.048	1053.169	2.460	1.086	2.265	0.343	0.349	0.368
161.1053	C7H15NO3	L-Carnitine	13338745.500	694664.854	16104562.000	1081931.354	13654379.000	930626.352	1.207	1.024	1.179	0.080	0.792	0.131
162.1158	C10H14N2	Nicotine	271767.768	7522.150	288172.840	16017.341	287939.945	11238.197	1.060	1.060	1.001	0.402	0.263	0.991
165.0791	C9H11NO2	L-Phenylalanine	14527871.333	520681.762	15347561.750	792918.549	14656381.000	504629.111	1.056	1.009	1.047	0.423	0.863	0.493
166.0057	C8H14NO9S2	Glucocapparin	1198790.313	14739.744	1217486.031	16199.903	1172906.104	12124.737	1.016	0.978	1.038	0.421	0.206	0.069
167.0366	C3H9N3O3S	Taurocyamine	114458.030	8307.103	134223.813	6519.022	106388.010	5767.361	1.173	0.929	1.262	0.098	0.446	0.015
167.0583	C8H9NO3	Pyridoxal	343936.706	32717.014	412309.414	20027.314	361848.073	25542.210	1.199	1.052	1.139	0.114	0.676	0.159
167.0947	C9H13NO2	3-Methoxytyramine	119964.371	10231.375	126804.131	9270.941	130895.711	12410.470	1.057	1.091	0.969	0.634	0.513	0.798
169.0740	C8H11NO3	Pyridoxine	103928.314	10416.102	109440.418	19037.458	111499.893	5100.366	1.053	1.073	0.982	0.810	0.534	0.923
169.0891	C12H11N	Diphenylamine	13266.017	505.760	6472.531	3833.071	13336.526	688.503	0.488	1.005	0.485	0.174	0.936	0.171
169.9981	C3H7O6P	Glycerone phosphate	206335.357	22166.181	175870.734	15480.798	165222.648	6436.780	0.852	0.801	1.064	0.293	0.127	0.559
174.1005	C7H14N2O3	N-Acetylornithine	285585.352	42027.618	258510.383	23584.586	217469.865	5135.664	0.905	0.761	1.189	0.591	0.167	0.180

			Control		Congenital Infected		Non Infected litter mates		Ratio			P Value		
Mass	Formula	Identification	Mean	SE	Mean	SE	Mean	SE	Congenital Infected/ Control	Non Infected litter mates/Control	Congenital Infected/ Non infected litter mates	Congenital Infected/ Control	Non Infected litter mates/ Control	Congenital Infected/ Non infected litter mates
174.1118	C6H14N4O2	L-Arginine	1688604.229	90307.289	1918462.906	107428.973	2017622.438	167997.241	1.136	1.195	0.951	0.147	0.124	0.633
175.0480	C6H9NO5	N-Acetyl-L-aspartate	48805513.333	1571634.892	47493701.000	1977298.531	48824844.667	1184841.008	0.973	1.000	0.973	0.621	0.992	0.588
175.0958	C6H13N3O3	L-Citrulline	383718.359	19090.192	468025.531	9789.289	464714.599	20803.511	1.220	1.211	1.007	0.005	0.017	0.890
176.0951	C10H12N2O	Serotonin	166556.193	15655.909	168499.883	12582.437	180396.664	13727.987	1.012	1.083	0.934	0.925	0.522	0.541
181.0740	C9H11NO3	L-Tyrosine	3528538.625	165858.098	4389048.688	180000.024	4404816.125	358317.996	1.244	1.248	0.996	0.009	0.062	0.970
183.0661	C5H14NO4P	Choline phosphate	192544.943	11933.168	175225.457	7093.129	186074.276	6565.458	0.910	0.966	0.942	0.249	0.648	0.298
183.1260	C10H17NO2	Acetylpseudotropine	16968.681	3753.148	23432.034	4292.799	16195.200	3360.146	1.381	0.954	1.447	0.295	0.881	0.230
188.0798	C7H12N2O4	N-Acetylglutamine	174789.081	24635.769	275930.430	88760.547	172853.005	10625.376	1.579	0.989	1.596	0.343	0.945	0.330
189.0638	C7H11NO5	N-Acetyl-L-glutamate	355498.771	16188.196	382543.625	25827.248	385783.188	29640.637	1.076	1.085	0.992	0.413	0.397	0.936
193.1104	C11H15NO2	3,4-Methylenedioxymethamphetamine	67589.758	2116.843	68995.188	1433.035	67704.589	3567.359	1.021	1.002	1.019	0.598	0.979	0.748
194.0803	C8H10N4O2	Caffeine	95668.336	3563.107	112966.396	8654.613	103141.715	1436.450	1.181	1.078	1.095	0.138	0.095	0.340
199.1574	C11H21NO2	2-Hexenoylcholine	17556.221	3677.627	22036.277	1804.999	14949.532	3290.545	1.255	0.852	1.474	0.310	0.609	0.099
199.1937	C12H25NO	Dodecanamide	148803.694	15162.809	134340.619	26808.973	135436.408	6808.848	0.903	0.910	0.992	0.659	0.448	0.971
203.0795	C8H13NO5	N2-Acetyl-L-aminoadipate	188380.727	4697.909	228821.063	25207.057	183865.339	8073.625	1.215	0.976	1.245	0.207	0.642	0.172
203.1158	C9H18NO4	O-Acetylcarnitine	19215846.833	789651.546	20739308.000	365751.545	17829779.333	712883.280	1.079	0.928	1.163	0.124	0.222	0.008
		Ala-Asp												
204.0788	C12H12O3	3-Butylidene-7-hydroxyphthalide	62915.932	2582.039	98241.799	10928.964	69505.309	6037.044	1.561	1.105	1.413	0.044	0.350	0.071
204.0900	C11H12N2O2	L-Tryptophan	1578011.563	105092.420	2118005.063	139127.091	1789789.250	83276.509	1.342	1.134	1.183	0.020	0.147	0.097
204.1111	C8H16N2O4	N6-Acetyl-N6-hydroxy-L-lysine	631207.385	135956.178	647422.133	103079.609	644139.516	66561.685	1.026	1.020	1.005	0.927	0.934	0.980
205.0952	C8H15NO5	N-Acetyl-D-fucosamine	21796.693	1093.364	32778.446	2146.389	27748.956	2489.715	1.504	1.273	1.181	0.008	0.066	0.165
205.1316	C9H19NO4	Pantothenol	226436.690	6594.647	216138.844	5270.843	217600.104	5101.887	0.955	0.961	0.993	0.257	0.316	0.847
206.0904	C7H14N2O5	Thr-Ser	44969.622	7136.767	51155.060	9525.144	41541.334	2292.496	1.138	0.924	1.231	0.621	0.663	0.392
		kynurenine												
211.1574	C12H21NO2	Elaeokanine C	80802.817	16724.766	76101.979	28719.606	52081.291	4087.671	0.942	0.645	1.461	0.893	0.150	0.466
215.0560	C5H14NO6P	sn-glycero-3-Phosphoethanolamine	5318407.000	218858.924	5221311.500	105730.110	5159993.708	229143.270	0.982	0.970	1.012	0.701	0.628	0.815
215.1887	C12H25NO2	[FA amino(12:0)] 12-amino-dodecanoic acid	58308.600	9434.958	47553.254	10171.101	43916.147	5615.282	0.816	0.753	1.083	0.463	0.226	0.767
216.0747	C8H12N2O5	N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoate	32683.969	2581.389	39380.977	3007.273	35401.471	924.364	1.205	1.083	1.112	0.136	0.358	0.282
216.0974	C27H28O5	Aspulvinone H	154790.095	17948.375	172719.254	22705.805	200041.708	37311.676	1.116	1.292	0.863	0.557	0.310	0.550
216.1727	C12H24O3	12-Hydroxydodecanoic acid	16215.457	5316.666	32324.943	2868.768	7774.652	5033.346	1.993	0.479	4.158	0.031	0.276	0.003
217.1316	C10H19NO4	O-Propanoylcarnitine	3080869.542	228540.441	3584580.813	200466.403	3045547.583	132678.351	1.163	0.989	1.177	0.137	0.897	0.070
218.1268	C9H18N2O4	N2-(D-1-Carboxyethyl)-L-lysine	3025606.042	229101.653	3331005.250	190555.725	3073739.875	108523.147	1.101	1.016	1.084	0.336	0.855	0.294
219.0744	C8H13NO6	O-Succinyl-L-homoserine	45634.100	4335.405	53711.878	8969.004	53553.426	5139.471	1.177	1.174	1.003	0.459	0.267	0.988
219.1108	C9H17NO5	Pantothenate	2898726.042	228433.244	3305019.938	358857.167	3313385.792	263729.368	1.140	1.143	0.997	0.380	0.263	0.986
220.0883	C8H16N2O3S	Met-Ala	399085.219	34640.611	537318.297	51658.309	438702.286	26540.661	1.346	1.099	1.225	0.071	0.387	0.155
220.1060	C8H16N2O5	N-Acetyl-beta-D-glucosaminylamine	70726.159	2838.728	90646.627	3542.715	75295.097	5282.513	1.282	1.065	1.204	0.004	0.469	0.043
221.0901	C8H15NO6	N-Acetyl-D-glucosamine	125955.238	11311.630	158616.357	17869.479	129424.725	8152.063	1.259	1.028	1.226	0.179	0.809	0.207
222.0676	C7H14N2O4S	L-Cystathionine	117744.613	7470.308	100247.441	4258.202	108006.983	12525.230	0.851	0.917	0.928	0.079	0.523	0.579
222.1006	C11H14N2O3	Phe-Gly	305522.958	16322.433	369059.945	22019.705	309124.255	8318.248	1.208	1.012	1.194	0.059	0.849	0.066
224.1891	C13H24N2O	Anapheline	5598.689	5598.689	6871.574	6871.574	19028.693	10844.863	1.227	3.399	0.361	0.890	0.305	0.372
226.0955	C10H14N2O4	Porphobilinogen	25178.072	1580.597	51685.738	25817.751	27730.801	1781.240	2.053	1.101	1.864	0.380	0.309	0.422
226.1068	C9H14N4O3	Carnosine	949802.292	189712.944	777712.141	34707.481	1147956.500	162707.500	0.819	1.209	0.677	0.411	0.447	0.072
227.2251	C14H29NO	myristic amide	91075.543	19303.490	132912.388	33109.912	165040.214	76856.064	1.459	1.812	0.805	0.324	0.389	0.713
229.0887	C9H16N3O2S	Ergothioneine	288183.487	23024.296	246575.438	8898.443	239474.836	14280.469	0.856	0.831	1.030	0.140	0.108	0.685
229.1679	C12H23NO3	N-Decanoylglycine	53173.739	15713.948	45815.396	18275.187	32544.535	5221.054	0.862	0.612	1.408	0.769	0.259	0.529
230.0581	C13H10O4	[PK] Visnagin	48066.639	10999.619	59262.917	3461.345	52616.326	10733.525	1.233	1.095	1.126	0.369	0.773	0.577

			Control		Congenital Infected		Non Infected litter mates		Ratio			P Value		
Mass	Formula	Identification	Mean	SE	Mean	SE	Mean	SE	Congenital Infected/ Control	Non Infected litter mates/Control	Congenital Infected/ Non infected litter mates	Congenital Infected/ Control	Non Infected litter mates/ Control	Congenital Infected/ Non infected litter mates
231.1221	C9H17N3O4	Ala-Ala-Ala	91523.615	3490.587	97255.277	11508.132	102367.406	9129.825	1.063	1.118	0.950	0.661	0.307	0.739
231.1472	C11H21NO4	O-Butanoylcarnitine	3083363.583	281131.917	3551673.188	187356.058	2807323.792	265319.647	1.152	0.910	1.265	0.204	0.492	0.051
232.1061	C9H16N2O5	N2-Succinyl-L-ornithine	235522.315	9701.153	234085.102	11045.258	249152.924	14498.937	0.994	1.058	0.940	0.925	0.455	0.432
232.1425	C10H20N2O4	Leu-Thr	99854.799	7101.281	103283.287	5832.531	105594.563	11004.373	1.034	1.057	0.978	0.719	0.672	0.858
240.1224	C10H16N4O3	Homocarnosine	461389.974	37396.798	477074.352	24566.604	533841.964	67855.082	1.034	1.157	0.894	0.735	0.378	0.460
240.1476	C12H20N2O3	Slaframine	1537349.792	73300.875	1556997.688	211595.420	1459557.990	126900.888	1.013	0.949	1.067	0.935	0.610	0.709
242.0193	C6H11O8P	D-myo-Inositol 1,2-cyclic phosphate	98176.605	5983.280	80965.231	10441.180	81318.696	18960.331	0.825	0.828	0.996	0.212	0.429	0.987
243.0857	C9H13N3O5	Cytidine	602039.125	37243.634	660495.125	17106.756	631976.938	21806.186	1.097	1.050	1.045	0.198	0.507	0.334
243.1837	C13H25NO3	N-Undecanoylglycine	257428.375	53251.871	200780.541	58808.247	200259.441	37038.655	0.780	0.778	1.003	0.498	0.401	0.994
244.0697	C9H12N2O6	Uridine	102263.884	4510.374	190280.754	98188.822	95739.603	6951.647	1.861	0.936	1.987	0.436	0.452	0.407
		Val-Ala-Gly												
		Phe-Ser												
		Val-Ala-Ala												
245.1629	C12H23NO4	N-(octanoyl)-L-homoserine	793012.578	144799.923	771146.688	60556.368	669038.500	52815.125	0.972	0.844	1.153	0.893	0.450	0.245
246.0507	C6H15O8P	Glycerophosphoglycerol	460610.104	24335.098	507078.383	29766.143	496989.495	21283.639	1.101	1.079	1.020	0.268	0.287	0.792
247.1422	C11H21NO5	Hydroxybutyrylcarnitine	1110549.219	90624.156	1348406.469	81783.138	1067824.865	100298.019	1.214	0.962	1.263	0.088	0.759	0.062
249.0864	C10H19NO2S2	S-Acetyldihydrolipoamide	262932.510	38561.085	286294.504	48741.142	301625.271	45129.002	1.089	1.147	0.949	0.719	0.530	0.824
254.0905	C11H14N2O5	N-Ribosylnicotinamide	135521.061	7504.297	125418.668	12862.430	133616.979	9052.348	0.925	0.986	0.939	0.527	0.875	0.621
260.0299	C6H13O9P	D-Glucose 6-phosphate	112481.904	13035.014	82398.404	9534.027	97229.417	7926.519	0.733	0.864	0.847	0.100	0.346	0.272
264.1047	C12H17N4OS	Thiamin	156490.090	6879.802	156633.480	10566.257	150627.191	11391.622	1.001	0.963	1.040	0.991	0.671	0.709
266.1649	C12H27O4P	Tributyl phosphate	61222.633	3494.440	79428.943	4297.211	65253.509	3627.218	1.297	1.066	1.217	0.015	0.442	0.041
		Adenosine												
268.0810	C10H12N4O5	Inosine	27971724.000	1329555.669	27898098.000	942955.441	26296660.667	1456822.066	0.997	0.940	1.061	0.965	0.416	0.384
268.1214	C16H16N2O2	Lysergic acid	126604.707	13500.997	93696.467	18749.319	120248.365	18729.444	0.740	0.950	0.779	0.205	0.789	0.348
273.0851	C12H19NO2S2	Brugine	128622.413	8916.759	136333.439	12364.876	126653.475	6077.534	1.060	0.985	1.076	0.631	0.859	0.517
275.1272	C14H17N3O3	Trp-Ala	187691.819	48415.722	170464.660	14192.922	151937.945	4943.902	0.908	0.810	1.122	0.745	0.495	0.290
275.1371	C12H21NO6	Glutarylcarnitine	1078799.021	121121.030	809975.625	166326.692	947327.802	66364.522	0.751	0.878	0.855	0.239	0.370	0.486
		Met-Gln												
278.1303	C11H22N2O4S	Pantetheine	23537.622	3126.992	44072.641	6343.528	36987.474	4522.383	1.872	1.571	1.192	0.038	0.037	0.399
278.1520	C16H22O4	2-Ethylhexyl phthalate	310066.885	4913.171	293551.172	7721.809	282792.031	10526.846	0.947	0.912	1.038	0.127	0.051	0.434
279.1221	C13H17N3O4	Phe-Asn	748379.156	38259.042	819305.484	36995.536	747339.292	15038.518	1.095	0.999	1.096	0.221	0.981	0.146
280.0918	C10H12N6O4	adenosine 5'-carboxamide	87431.313	7327.376	100616.113	8597.725	88999.949	1956.217	1.151	1.018	1.131	0.282	0.843	0.271
280.1313	C15H20O5	Phaseic acid	85874.327	3834.714	103885.143	9280.868	107128.712	19019.616	1.210	1.248	0.970	0.147	0.320	0.883
280.1425	C14H20N2O4	Val-Tyr	219563.672	25139.748	248762.109	27290.502	236263.539	16615.583	1.133	1.076	1.053	0.457	0.593	0.711
281.1126	C11H15N5O4	1-Methyladenosine	238948.927	14247.703	404211.883	137830.442	244666.073	9811.771	1.692	1.024	1.652	0.317	0.749	0.331
283.0919	C10H13N5O5	Guanosine	3646661.458	295564.689	4119374.813	261742.694	3579949.375	192915.783	1.130	0.982	1.151	0.266	0.854	0.148
283.9540	C6H12O4PCl3	Tris(2-chloroethyl)phosphate	124785.384	6079.612	132192.314	6122.395	122253.453	3271.933	1.059	0.980	1.081	0.417	0.724	0.215
286.0989	C12H18N2O4S	4-Hydroxytolbutamide	17776.922	8112.106	23891.603	3697.469	25326.097	4296.202	1.344	1.425	0.943	0.515	0.436	0.807
291.0957	C11H17NO8	2,7-Anhydro-alpha-N-acetylneuraminic acid	48686.085	9816.191	69788.787	6057.934	55941.257	4745.138	1.433	1.149	1.248	0.106	0.526	0.119
294.1106	C15H18O6	Tutin	64083.595	5882.630	76092.826	4348.790	78810.992	13221.663	1.187	1.230	0.966	0.139	0.343	0.852
296.1197	C14H20N2O3S	Met-Phe	2056360.208	217058.361	2063364.031	206709.502	2101210.917	107482.128	1.003	1.022	0.982	0.982	0.858	0.878
297.0898	C11H15N5O3S	5'-Methylthioadenosine	157475.518	6116.789	203520.512	49812.673	167345.188	9302.948	1.292	1.063	1.216	0.425	0.399	0.524
301.2004	C14H27N3O4	Leu-Leu-Gly	137512.072	14888.385	153424.164	8750.163	160728.898	6733.394	1.116	1.169	0.955	0.385	0.199	0.532
304.0909	C11H16N2O8	N-Acetyl-aspartyl-glutamate	6026161.917	542271.049	7024731.750	402483.748	7192244.000	720180.788	1.166	1.194	0.977	0.178	0.227	0.845
307.0839	C10H17N3O6S	Glutathione	38950796.000	3644573.909	39058992.500	2969847.112	40053598.667	4462258.011	1.003	1.028	0.975	0.982	0.852	0.858

			P Value				Ratio			
Mass	Formula	Identification	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male
243.0857	C9H13N3O5	Cytidine	0.198	0.334	0.507	0.118	1.097	1.050	1.045	0.861
243.1837	C13H25NO3	N-Undecanoylglycine	0.498	0.994	0.401	0.619	0.780	0.778	1.003	1.173
244.0697	C9H12N2O6	Uridine	0.436	0.407	0.452	0.569	1.861	0.936	1.987	1.208
		Val-Ala-Gly								
		Phe-Ser								
		Val-Ala-Ala								
245.1629	C12H23NO4	N-(octanoyl)-L-homoserine	0.893	0.245	0.450	0.033	0.972	0.844	1.153	0.467
246.0507	C6H15O8P	Glycerophosphoglycerol	0.268	0.792	0.287	0.305	1.101	1.079	1.020	0.913
247.1422	C11H21NO5	Hydroxybutyrylcarnitine	0.088	0.062	0.759	0.002	1.214	0.962	1.263	0.544
249.0864	C10H19NO2S2	S-Acetyldihydrolipoamide	0.719	0.824	0.530	0.530	1.089	1.147	0.949	0.857
254.0905	C11H14N2O5	N-Ribosylnicotinamide	0.527	0.621	0.875	0.058	0.925	0.986	0.939	0.831
260.0299	C6H13O9P	D-Glucose 6-phosphate	0.100	0.272	0.346	0.189	0.733	0.864	0.847	0.813
264.1047	C12H17N4OS	Thiamin	0.991	0.709	0.671	0.079	1.001	0.963	1.040	0.823
266.1649	C12H27O4P	Tributyl phosphate	0.015	0.041	0.442	0.246	1.297	1.066	1.217	1.117
		Adenosine								
268.0810	C10H12N4O5	Inosine	0.965	0.384	0.416	0.353	0.997	0.940	1.061	1.071
268.1214	C16H16N2O2	Lysergic acid	0.205	0.348	0.789	0.888	0.740	0.950	0.779	1.029
273.0851	C12H19NO2S2	Brugine	0.631	0.517	0.859	0.349	1.060	0.985	1.076	0.900
275.1272	C14H17N3O3	Trp-Ala	0.745	0.290	0.495	0.067	0.908	0.810	1.122	3.723
275.1371	C12H21NO6	Glutarylcarnitine	0.239	0.486	0.370	0.494	0.751	0.878	0.855	0.910
		Met-Gln								
278.1303	C11H22N2O4S	Pantetheine	0.038	0.399	0.037	0.085	1.872	1.571	1.192	1.651
278.1520	C16H22O4	2-Ethylhexyl phthalate	0.127	0.434	0.051	0.039	0.947	0.912	1.038	0.921
279.1221	C13H17N3O4	Phe-Asn	0.221	0.146	0.981	0.001	1.095	0.999	1.096	1.347
280.0918	C10H12N6O4	adenosine 5'-carboxamide	0.282	0.271	0.843	0.005	1.151	1.018	1.131	1.470
280.1313	C15H20O5	Phaseic acid	0.147	0.883	0.320	0.205	1.210	1.248	0.970	1.252
280.1425	C14H20N2O4	Val-Tyr	0.457	0.711	0.593	0.002	1.133	1.076	1.053	1.744
281.1126	C11H15N5O4	1-Methyladenosine	0.317	0.331	0.749	0.445	1.692	1.024	1.652	1.176
283.0919	C10H13N5O5	Guanosine	0.266	0.148	0.854	0.013	1.130	0.982	1.151	1.380
283.9540	C6H12O4PCl3	Tris(2-chloroethyl)phosphate	0.417	0.215	0.724	0.580	1.059	0.980	1.081	1.033
286.0989	C12H18N2O4S	4-Hydroxytolbutamide	0.515	0.807	0.436	0.070	1.344	1.425	0.943	2.235
291.0957	C11H17NO8	2,7-Anhydro-alpha-N-acetylneuraminic acid	0.106	0.119	0.526	0.473	1.433	1.149	1.248	0.762
294.1106	C15H18O6	Tutin	0.139	0.852	0.343	0.421	1.187	1.230	0.966	1.178
296.1197	C14H20N2O3S	Met-Phe	0.982	0.878	0.858	0.028	1.003	1.022	0.982	1.322
297.0898	C11H15N5O3S	5'-Methylthioadenosine	0.425	0.524	0.399	0.017	1.292	1.063	1.216	1.440
301.2004	C14H27N3O4	Leu-Leu-Gly	0.385	0.532	0.199	0.001	1.116	1.169	0.955	2.133
304.0909	C11H16N2O8	N-Acetyl-aspartyl-glutamate	0.178	0.845	0.227	0.439	1.166	1.194	0.977	1.102
307.0839	C10H17N3O6S	Glutathione	0.982	0.858	0.852	0.002	1.003	1.028	0.975	0.498
308.1222	C12H24N2O3S2	S-8-methylthiooctylhydroximoyl-L-cysteine	0.715	0.439	0.561	0.411	1.052	0.940	1.120	0.818
309.1062	C11H19NO9	N-Acetylneuraminate	0.731	0.815	0.896	0.858	0.973	0.990	0.983	1.015
312.1476	C18H20N2O3	Phe-Phe	0.263	0.158	0.866	0.002	1.124	0.986	1.140	1.511
317.1741	C17H23N3O3	Leu-Trp	0.425	0.123	0.681	0.004	1.045	0.978	1.069	1.254
328.1425	C18H20N2O4	Phe-Tyr	0.893	0.153	0.081	0.011	1.009	0.909	1.110	1.394
		Met-Trp								
339.3493	C22H45NO	Docosanamide	0.916	0.914	0.998	0.450	1.168	0.997	1.172	2.747
347.0723	C18H15FNO3Cl	Flumipropyn	0.096	0.273	0.390	0.935	0.961	0.982	0.979	0.998
357.2631	C18H35N3O4	Leu-Leu-Leu	0.436	0.995	0.379	0.000	1.206	1.204	1.001	2.391

			P Value				Ratio			
Mass	Formula	Identification	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male	Congenital Infected/Control	Non Infected litter mates/Control	Congenital Infected/Non infected litter mates	female/male
		AMP								
359.2463	C22H33NO3	Ajaconine	0.843	0.244	0.135	0.718	0.985	0.880	1.119	0.974
361.0946	C21H16NO5	Chelirubine	0.996	0.978	0.979	0.154	0.999	1.005	0.994	1.406
363.0583	C10H14N5O8P	GMP	0.061	0.921	0.138	0.687	1.168	1.179	0.991	0.971
372.2878	C21H40O5	1,2-dioctanoyl-3-methyl-1,2,3-butanetriol	0.354	0.720	0.655	0.607	1.116	1.058	1.055	0.966
376.1385	C17H20N4O6	Riboflavin	0.379	0.519	0.608	0.124	1.130	1.047	1.080	1.160

TABLE LEGEND	
	Higher in CI (p<0.05)
	Lower in CI (p<0.05)
	Higher in Non infected litter mates (p<0.05)
	Lower in Non infected litter mates (p<0.05)
	Higher in females (p<0.05)
	Lower in females (p<0.05)
	No difference
	Compounds also present in the adult experiment
	Compounds not detected present in the adult experiment

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