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**Gender dimorphism in parasitic behavioural
manipulation hypothesis**

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List of Abbreviations

AFR	Animal facility rearing
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AT	Assessment tasks
AVP	Arginine Vasopressin
β -actin	Beta actin
BBB	Blood-Brain Barrier
BNST	Bed nucleus of the stria terminalis
BTB	Blood-Testes Barrier
cDNA	complementary DNA
CSF	Cerebrospinal fluid
Ct	Threshold cycle
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
EPM	Elevated plus maze
GFP	Green fluorescent protein

HFF	Human foreskin fibroblasts
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IgG	Immunoglobulin G
MeA	Medial amygdala
MeApd	Medial Amygdala Nuclei, Posteriodorsal
MeApv	Medial Amygdala Nuclei, Posterioventral
MPOA	Medial preoptic nucleus
mRNA	Messenger Ribonucleic Acid
MUP	Major Urinary Protein
OFT	Open field test
OT	Oxytocin
OTR	Oxytocin Receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVN	Paraventricular Nucleus
qPCR	Quantitative Polymerase Chain Reaction

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SABV	Sex as a Biological Variable
SBN	Social brain network
SEM	Standard error of mean
SON	Supraoptic nucleus
TH	Tyrosine Hydroxylase
Toxoplasma	<i>Toxoplasma gondii</i>

Abstract

Gender dimorphism in parasitic behavioural manipulation hypothesis

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The behavioural manipulation hypothesis states that a parasite will alter those parts of its host behaviour important to its survival and transmission in order to increase its fitness. It has been demonstrated that there is a gender dimorphism in parasitic infections in many hosts. This raises an important question of the role of gender in parasitic behavioural manipulation. On the one hand, it is possible that gender dimorphism in parasite infection and prevalence extends to parasitic behavioural manipulation. In this case, manipulation will only occur in the gender that is preferentially infected. On the other hand, there is a possibility that gender dimorphism occurs on the level of mechanism of manipulation rather than on phenotypic expression of manipulated trait. The aim of my thesis is to explore the possibilities presented by both options using the protozoan parasite *Toxoplasma gondii* as a model. I first demonstrate that *Toxoplasma* is able to alter behavioural response of female rats to cat odour. This is similar to the response that has previously been observed in male rats. I further demonstrate that this behaviour change is not as a result of masculinization of infected female rats by showing that there was no change in plasma testosterone and arginine vasopressin (AVP) transcript levels in the posterior dorsal medial amygdala (MeApd). These are both substrates reported to drive the same behavior in male rats. Alternatively, I propose a role for the oxytocin system in the MeApd which has an innate gender dimorphic expression and function. Based on my

findings, I propose that manipulation by *Toxoplasma gondii* does not occur at the level of the substrates that are implicated in the mechanism. Rather I propose manipulation affects the negotiation of the approach-avoidance conflict, which is an important part of all aspects of social behaviour. Innate social behavioural responses are mainly mediated by mechanisms that are gender dimorphic in the substrates they employ. In other words, *Toxoplasma gondii* is able to manipulate the behaviour of female rats. However, the mechanism underlying this behaviour is gender dimorphic in the substrates involved. Taken together, this forms one more example of the need to factor in gender as a biological variable when designing experiments and interpreting results.

Chapter 1

Synopsis

This thesis is situated within the field of parasitic behavioral change in female rats infected with the protozoan *Toxoplasma gondii*. During the course of this synopsis, I will first examine the effect of *Toxoplasma gondii* infection on the navigation of the approach-avoidance conflict in female rats exposed to cat odour. In subsequent paragraphs I will demonstrate that female rats display an innate aversion to cat odour. This avoidance behaviour is altered once female rats are chronically infected with *Toxoplasma gondii*. I will also demonstrate the role of the oxytocin system in mediating this behaviour.

Several papers had already demonstrated the effects of *Toxoplasma gondii* (*Toxoplasma*) infection on male rat response to the presence of cat odour. Male rats display reduced fear of cat urine when chronically infected with *Toxoplasma*. This behavioral change has been frequently interpreted as a behavioral manipulation of the host by the parasite because *Toxoplasma* requires trophic entry into the cat in order to complete its life cycle and also to infect herbivorous hosts that cannot be infected by asexual carnivorous route. In parallel to reduction in fear, *Toxoplasma* infected male rats exhibited a robust increase in the synthesis and excretion of sexual pheromones called major urinary proteins (MUP). This resulted in more mating opportunities for infected males compared to uninfected controls. This behaviour is in stark contrast to what is observed in multiple host parasite relationships where females assiduously avoid parasitized males. This change is interpreted as another example of parasitic manipulation that could plausibly increase sexual transmission of the parasite. It should be noted here that field evidence of greater predation of the infected male rats by cats has not yet been collected. Similarly it remains

unclear if sexual transmission of *Toxoplasma* represents a substantial route vis-à-vis trophic transmission.

Much of prior research also aimed to delineate mechanistic aspects of two behavioral changes described above. For the loss of aversion, a sequence of biochemical events centered around testosterone and arginine-vasopressin were suggested. For the gain of attractiveness, testosterone-dependent increase in hepatic synthesis of pheromones was postulated. I will not expand on the rationale or support for these hypotheses in the synopsis section; this point is covered in greater details in the later chapters. What concerns us at the present juncture is that these mechanistic hypotheses were built upon a sexually dimorphic interaction between the brain, gonads and liver. All the building blocks of these hypotheses, all the salient molecular players, are more vigorously expressed in male rather than female bodies. The logical extension of these proposed mechanisms is then that *Toxoplasma* infection affects males and females asymmetrically. The balance between approach and avoidance is altered in male rats, but not female, after *Toxoplasma* infection. An alternative view can also be taken. The conflict between approach and avoidance is universal with respect to genders. Different players are recruited, in gender-specific ways, to negotiate conflict. Historical selection, in this view then affects the approach-avoidance negotiation itself rather than on the mechanics underlying the negotiation. Since the approach-avoidance conflict holds true regardless of gender, female rats will then also display this atypical behaviour.

In this context, my first step was to determine if *Toxoplasma* infection in females resulted in a perturbation of the approach avoidance continuum. I exposed chronically infected and uninfected female rats to cat odour. I recorded and compared the time each group spent

exploring the area with the odour. My data showed that *Toxoplasma* infected female rats spent significantly more time exploring the vicinity of cat odour. This is similar to what was observed in the case of male rats. This suggests that the behaviour change post-infection is not a sexually selected trait. Rather it suggests that the infection re-negotiates the balance between approach and avoidance behaviours due to historical non-sexual natural selection. The test animals were also subjected to other tests of anxiety namely the open field test (OFT) and the elevated plus maze (EPM). I demonstrated through these tests that the atypical behaviour observed did not include other forms of anxiety. Both infected and uninfected female rats showed similar levels of anxiety as measured by these tests (**Chapter 3**).

At this juncture in the narrative, I have established that *Toxoplasma* infection is able to perturb approach-avoidance continuum in both male and female rats. The next step was to understand the proximate mechanism underlying this observation. Previously proposed mechanism in males has been demonstrated to be extremely male-centric. In its basic form the proximate mechanism in males translates into an interaction between the peripheral reproductive hormone (in this case testosterone) and a nonapeptide neurotransmitter involved in socio-sexual behaviour (in this case arginine vasopressin, AVP). This basic model served as my guide to determine the mediators of behaviour change in females. Estradiol and progesterone are the two main female reproductive hormones which are also anxiolytic in nature. Both hormones are important in regulating the female estrus cycle which is quite complicated and important for many behaviours other than reproduction or anxiety. I did not observe an increase in plasma estradiol or

progesterone levels in females post-infection relative to control cohorts. Thus causal role of both progesterone and estradiol remained unclear during my experiments.

Subsequently, I decided to measure the transcript levels of the nonapeptide oxytocin (OT) and its receptor (OTR). I selected OT because it is a nonapeptide very similar in structure and function to AVP. The two peptides differ in only two amino acids and are able to bind to each other's receptors albeit with a reduced affinity. OT is additionally responsible for socio-sexual behaviours in females, similar to the role of AVP in males. Both AVP and OT regulate socio-sexual behaviours in a gender dimorphic manner although both genders express both neurotransmitters. With AVP in males it was enough to measure the transcription levels of the actual peptide. Production of extra hypothalamic AVP in the MeA is well documented and is known to be stable and robust. In the case of OT it was necessary to look also at OTR in the OT system. Production of extra hypothalamic OT is not as robust as is the case with AVP. OT is mostly produced in the hypothalamus and transported to other brain regions via volume transfer through the ventricles. Distribution and density of OTR outside the hypothalamus is thus a more reliable measure of OT activity. This necessitated a look at both OT and OTR in order to understand how infection affected this system.

I measured both OT and OTR along nodes of the social brain network (SBN), namely in the MeApd, MPOA, PVN and BNST. I also measured OT in the SON of the hypothalamus. Both PVN and SON are nodes that are important sites of OT production. The SON was chosen to serve as an internal control for each animal because OT produced in the SON is transported to the posterior pituitary and excreted into the periphery. Thus, it was expected that even if the OT system was shown to be altered, OT levels in the SON

would not change. In the MPOA, our data showed no difference in OT or OTR levels between control and infected rats. However, there was a significant increase in OTR transcript levels in infected animals relative to control animals and a borderline significant increase in OT transcript of said animals within the MeApd. There was no significant change in OT transcript levels in the PVN, SON and BNST. However, we observed a significant down regulation of OTR transcript levels in the BNST. This corresponds with the observation that the inactivation of the BNST led to increased approach type behaviour to cat odour stimulus (**Chapter 4**).

At this point in the synopsis, I have successfully demonstrated that infecting female rats with *Toxoplasma* results in a more approach type behaviour. I have also demonstrated that transcript levels of the nonapeptide OT and its receptor OTR are altered post infection. The next step was to show the necessity of OT to mediate this atypical behaviour. I designed a 2x2 experiment where the OTR antagonist L-368,899 was employed to demonstrate necessity. Briefly, I administered L-368,899 to a subset of both control and infected animals 20 minutes prior to exposure to cat odour. I observed that the data from this experiment was opposite to what I had previously observed. Even in animals that did not receive antagonist, control females displayed a more approach behaviour to cat odour compared to infected females. Moreover parallel measurements in a habituation-dishabituation paradigm demonstrated atypical baseline in uninfected females. During this period of time, multiple animal users in the same facility reported shifts in behavioural baselines in a variety of non-infection paradigms. This unfortunate interruption/observation left me flummoxed. A detailed and painstaking analysis later revealed two accidental sources of the variation during this phase, including a massive

construction project in the vicinity of the animal vivarium and a laboratory diet that contained phytoestrogens derived from soybean. My efforts to identify these confounds and to re-establish baselines of the behavior is detailed in **chapter 5**.

Much of animal neurobiology and behavior research is traditionally conducted using male individuals. The female gender has remained grossly understudied. This situation arises because of perceived experimental complexity that could arise from fluctuating hormonal cycles of the females. These cycles could potentially increase variance and thus reduce power of statistical comparisons. It is noteworthy that this assumption has not been frequently tested and has actually been challenged during meta-analysis of published observations. The bias of experiments for a gender complicates the interpretation of results making it extremely tedious to generalize across the gender gap. A similar situation exists in *Toxoplasma* research. This thesis aims to fill that gap.

In spite of my best efforts I am aware of the several shortcomings of this thesis. Importantly, my work demonstrates that infection alters transcript levels of OT and OTR in certain brain regions. I have however been unable to demonstrate the necessity and sufficiency of the OT/ OTR system to drive the behaviour. The role of the two major female reproductive hormones in mediating the behaviour still remains unclear. In order to determine if the proximate mechanism in female rats follows the basic model proposed in males, it will be important to delineate the pivotal endocrine events. In males, the parasite is hypothesized to hijack the sexual arousal pathway which increases the fitness of the animals and plausibly compensates for the increased risk of predation in females. A corollary is presently unclear in the females because attractiveness to mates is a sexually dimorphic behavior under sexual selection.

In summary, my thesis has demonstrated that *Toxoplasma*-induced host behavioural change is not gender dimorphic. Females respond in a manner similar to males when exposed to cat odour after chronic infection with *Toxoplasma*. I have also demonstrated that this observed reduction in anxiety in response to cat odour does not extend to other forms of anxiety as measured by the EPM and OFT. I have further demonstrated that infection alters transcript levels of OT/OTR in certain nuclei of the brain, notably the MeApd and BNST.

Chapter 2

Introduction

Many studies report sexual dimorphism in terms of parasite prevalence and burden in mammals [1, 2] and non-mammalian vertebrates [1, 3-5]. Male hosts generally present with greater parasite prevalence than females. For example, a meta-analysis of 38 published articles showed that male mammalian hosts were more likely to be parasitized compared to females [6]. A closer look at specific parasite classes revealed a gender dimorphism in infection with arthropod but not helminth parasites [6]. This male bias in parasite prevalence was observed both in field samples and in experimental studies. Other studies have observed gender biases in helminth infections. Webster [7] reports a male bias in the prevalence of the sucking louse *Polyplax spinulosa* in wild brown rats. There are also cases where there is a female bias in parasite prevalence. In murine *Schistosoma mansoni* infections, females showed a higher incidence, parasite load and more intense disease presentation compared to males [8, 9]. Interestingly, this female bias is reversed in humans where parasite prevalence and disease presentation is greater in males compared to females [10, 11].

Sexual Dimorphism in Parasite Prevalence

Several proximate mechanisms have been proposed to explain sexual dimorphism. A dominant hypothesis posits the immune-suppressive role of testosterone in male hosts [12]. Testosterone has been reported to effectively suppress both cellular and humoral immune responses. This is supported by the observation that castration significantly lowered male host parasite loads compared to sham operated cohorts. Parasite levels were restored to pre castration levels when hosts were supplemented with testosterone [13-15].

A 20% decrease in parasite prevalence in the liver was observed in castrated mice infected with *Leishmania major* when compared with non-castrated controls. The same study reported a 88% increase in *Leshmania* prevalence in female mice treated with testosterone [16]. This suggests that production of testosterone in male hosts results in a trade-off between immune competence and reproductive advertisement; On the one hand, there is the need to increase testosterone to produce and maintain secondary sexual characteristics. On the other hand, increase in testosterone means increased immunosuppression in these hosts. However, it is entirely possible that gender differences observed are not a result of direct action of circulating hormones. [17, 18]. Males in several species exhibit greater aggression and exploration during mating periods. This could plausibly increase the chances of infection [12, 19]; resulting in observed gender dimorphism in parasite prevalence [20].

The trade-off described above represents a viability cost of sexual selection. It has been argued that testosterone dependent secondary sexual characters are a form of honest and costly sexual communication. Metabolically or ecologically unfit males cannot invest in the cost of maintaining testosterone (and its concomitant immune suppression) [19]. This cost creates honesty in the process of mate choice whereby females choose males with a better immune system [21, 22]. Free-living male house finches infected with coccidia (a natural parasite) have been reported to have lower levels of circulating testosterone compared to uninfected males. Similarly, testosterone-supplementation in male finches was observed to increase the rate of parasite infection compared to castrated and non-supplemented males [23]. This observation suggests that increased testosterone increases susceptibility to infection as a direct result of immune suppression. Males with established

parasite infection then decrease their testosterone production ostensibly to increase immune competence [23]. A similar observation was made in a study using Grant's gazelle (*Nanger granti*). Male gazelles with increased testosterone levels presented with correlatory bigger horn size as well as increased territoriality. This increase in testosterone was negatively correlated with adaptive immunity. This was determined by lymphocyte count. Surprisingly, testosterone was positively correlated with neutrophil count which denotes increased innate immunity. This somewhat surprising data suggests that testosterone's effects on immunity are more complicated than originally envisaged [24]. In both studies cited above, it remains clear that testosterone-dependent display of secondary sexual characteristics remain a good proxy for determining male overall health.

In the preceding paragraph, I have briefly presented the idea that sexual dimorphism is often observed in terms of parasite burden and prevalence. A part of this dimorphism is likely to result from endocrine cost of secondary sexual characteristics. In addition, the conspicuous nature of sexual communication itself provides avenues for parasites to preferentially exploit one sex, thus creating sexual dimorphism.

Secondary sexual characteristics primarily function as signals to attract mates [19]. These signals are by design overt and consequently visible to both parasites and predators (illegitimate receivers) in addition to the intended mates [25-27]. This suggests that the display of secondary sexual characteristics must trade-off between attractiveness and avoidance of detection by these illegitimate receivers [25]. Several examples of predators and parasitoids exploiting host sexual signals have been reported. Parasites have been reported to eavesdrop mainly on acoustic and olfactory signals and to a lesser extent on visual signals [25]. For example tachinid flies, which are parasitoids of arthropods, have

been reported to locate their host by eavesdropping on the mating calls of crickets [28-31] or the olfactory signal from pentatomid bugs [32]. For most species, males bear the cost of sex signal production whilst females receive these signals. This also means that the males of a species are usually more susceptible to parasite infection via eavesdropping compared to females [25].

Field cricket *Teleogryllus oceanicus* with its parasitoid fly *Ornia ochracea* presents a poignant case study of this phenomenon. Field crickets typically attract mates by a “calling song” produced by stridulating their wings. The parasitoid fly eavesdrops on this signal in order to find its host. This parasitoid was absent from Hawaiian island of Kauai. Field crickets in that ancestral scenario were characterized by a high frequency of calling males. The parasitoid has been accidentally introduced in the Kauai in recent years. This has resulted in resurgence of an alternative ‘silent’ phenotype in crickets. [33]. Silent males possess a mutated wing morphology that prevents them from emitting the acoustic advertisement to attract mates. More than 90% of the field cricket population on the Hawaiian island of Kauai has evolved this mutated wing morphology after only 20 generations. It is believed that intense selection against parasitized male crickets and plasticity of behaviour have both contributed to the establishment of this phenotype [34]. This change suggests that parasites can exploit sexual advertisement of the host, creating sexual dimorphism. This also suggests that such exploitation is costly for the host.

Examples presented till now underline a dynamic equilibrium between natural selection and sexual selection. In other words, imperatives of survival and reproduction often conflict with each other. Benefits of sexual advertisement in crickets conflict with survival penalty of these conspicuous displays. Because sexual selection often results in sexual

dimorphisms, this equilibrium also results in sexual dimorphism in parasitism. Both endocrine and eavesdropping costs of sexual signaling represent the cost of sexual selection in this perspective. Also the examples of eavesdropping, represented by case of crickets in Hawaii, pertain to a passive exploitation of pre-existing host communication by parasites. This has been extensively reviewed earlier [25].

A large and parallel body of research also suggests a more intimate relationship between hosts and parasites. This entails behavioural change in parasitized host due to changes in proximate biology of the hosts itself, rather than using a pre-existing host behavior. The ability of a parasite to modify its host behaviour in such a manner that allows the parasite to complete its life cycle is referred to as the “behavioural manipulation hypothesis” [35, 36]. These changes in host behaviour are a means to enhance host to host transmission of the parasite or to ensure that the parasite is released in an appropriate environment. All manipulation by the parasite is aimed to ensure parasite survival [37]. Several examples of this behaviour manipulation have been described.

Behavioural Manipulation Hypothesis

This includes suicidal behavior of crickets infected with hairworm [35] and ants infected with a parasitic fungus [38]. Other parasites like the ichneumonid wasp of spiders alter maternal behaviour of their spider host. The wasp lays its eggs on the spider and induces the spider to build a shelter for its future larvae just before it kills it [39-41]. As widespread as the phenomenon of parasite manipulation is, relatively little is understood about proximate neuronal mechanisms that underlie these behaviours [42].

Parasite-induced behavioural manipulation has often been considered to be a product of natural selection. In other words, host behavioral change is often discussed in terms of survival advantage or disadvantage. Pertinent examples include endpoints like predation, fear and habitat choice. This framework leads an expectation of nil sexual dimorphism in its purest form, because both genders experience the selection pressure equally. An extension of this view can be nonetheless proposed. If parasites preferentially exploited sexually selected parts of the host physiology, like synthesis of pheromones or conspicuousness of display, then one would expect a sexual dimorphism in the host behavioral manipulation itself. This is because selection pressure in this case is experienced by genders in a dimorphic manner. The next few paragraphs will look at possible examples of such dimorphism as it relates to host behavioural manipulation by parasites.

Examples given below were obtained from searching Google scholar database with the terms “behavioural manipulation, parasite-induced behavioural manipulation and virus mediated behavioural manipulation. Reviews and original experimental publications from 1970 to 2016 were considered in this search. A useful reference was defined using the following criteria. Reference describes a behaviour change in a parasitized organism that has no benefit for the host but could benefit the parasites’ transmission and/ or survival. A reference that proposes a probable proximate mechanism underlying a behavioural manipulation was also considered. Any review that raises doubts about an observed change in host behaviour as a behaviour manipulation was also considered useful.

Hantavirus is a negative sense RNA virus that is a common parasite of rodents [43, 44]. Effects of Hantavirus infection are typically age-dependent; infection of neonates is

typically fatal whereas infection of adult rodents does not affect survival or reproduction [45, 46]. Hantavirus has been demonstrated to be sexually dimorphic in prevalence with a greater likelihood of infecting male rats compared to female rats [43]. Virus is present in saliva, urine and faeces and transmitted directly either through saliva deposited in bite wounds (rodent-rodent transmission) or by inhalation of aerosolized virus in rodent excreta (rodent-human transmission) [47, 48]. Infection has also been shown to increase aggression in a sexually dimorphic manner with a higher increase in males [49]. It remains unclear whether increase in aggression is responsible for increased exposure to Hantavirus (host-mediated hypothesis) or infection with Hantavirus increases aggressive behaviour (parasite-mediated hypothesis) [49]. Much about the proximate mechanism Hantavirus employs to mediate an increase in aggression remains unknown. It is however known that both serum testosterone and fecal corticosterone increases in infected males compared to uninfected males [49]. It has been proposed that increase in both testosterone and corticosterone may be important in mediating increased aggression observed in infected males [50, 51]. Serotonin neurotransmission in the central nervous system (CNS) is an indicator of aggressive behaviour [52]. Decreased serotonin and 5-hydroxyindoleacetic acid (a serotonin metabolite) neurotransmission in the CNS correlates to an increase in aggression. Male rats infected with Hantavirus have lower levels of hypothalamic serotonin and 5-hydroxyindoleacetic acid consistent with observed increase in aggression [49]. It is interesting to note that there is no evidence of Hantavirus RNA or proteins in the brain [43, 50, 53], though presence of Hantavirus has been demonstrated in the kidney, lung and testes [43, 49]. A lot of what is currently known about Hantavirus is correlatory evidence. However, it appears Hantavirus is able to

manipulate the aggression pathway in male rats, resulting in increased aggression in male rats [49]. Females infected with Hantavirus do not appear to display a similar increase in aggressive behaviour [43, 48, 49].

Another form of behavioural manipulation is parasitic castration. Certain parasites such as the larval trematodes that infect snails and barnacles that parasitize crustaceans castrate their hosts in an effort to optimize consumption and longevity [54]. The rhizocephalan barnacle *Sacculina granifera* is a parasite of the sand crab *Portunus pelagicus*. The parasite does not affect reaction time, feeding and locomotion of its crustacean host. It however affects stance and burying behaviour as the crab must now account for the parasite attached to its body between the cephalothorax and abdomen [55]. The parasite infects both male and female crabs. Female crabs infected with the *Sacculina* parasite do not appear to be modified morphologically. In males, there is significant morphological change, most prominent of which is the shortening of the chelar propodus to a length similar to that observed in normal females [56]. Maintenance of the externae on crab is necessary for the parasite survival as loss of externae usually leads to death of the parasite. The broad abdomen of female crabs is better adapted to carrying the externae compared to the narrow male abdomen. It is thus in the parasites' best interest to feminize the male in order to increase its chances of survival [57]. Infected males begin to groom their externae and begin to move seaward in a manner similar to what is observed in gravid female crabs [56]. Other species of barnacles are also able to parasitize crabs with slightly different levels of castration though all barnacle-crab interaction results in some form of castration be it partial or complete castration [57]. Much remains unknown about the mechanism underlying castration and subsequent feminization on male crabs. It is

however assumed that barnacles destroy the androgenic gland responsible for masculinization [58].

Host organisms often alter their life histories in order to minimize the costs incurred due to parasitism. Generally, hosts trade off current and residual reproduction in order to maximize fitness [59]. As such, when parasitism threatens future reproductive opportunities (residual reproduction), life history theory predicts host organisms will maximize investment in current reproduction [60-63]. This surge in investment in current reproduction opportunities post parasite infection is referred to as terminal investment or fecundity compensation [64-66]. This effect has been observed in snails parasitized by castrating trematodes [67], parasitized insects [68], birds [69] and crustaceans [70]. This behaviour has been observed in both males and females. An example of terminal investment is observed in crickets (*Acheta domesticus*) infected with the bacterium *S. marcescens*. Female crickets increased their egg-laying in response to the bacteria infection. Females increased current reproductive investment once residual reproduction became uncertain, consistent with terminal investment [68]. The proximate mechanisms underlying terminal investment in both of these host organisms remains unclear. However, there is indirect evidence of a role for host immune factors in cricket terminal investment [68].

In mammals, female deer mice (*P. maniculatis*) parasitized with the blood fluke *S. douthitti* produced litters that were approximately 6% greater in mass than litters from uninfected females. Increased mass of litters is hypothesized to increase offspring fitness by compensating for negative effects of maternal infection and/ or making offspring more competitive. This signifies an increase in reproductive investment concomitant to terminal

investment [71]. Terminal investment is not usually observed in mammals probably due to the long life span of mammals thus infection has a limited effect on residual reproduction [71].

An excellent example of direct manipulation occurs between the parasitic wasp *Ampulex compressa* and its cockroach host [42]. The parasite hunts the cockroach and renders it a living yet immobile food source for its larva. It achieves this by delivering a venomous sting to the head of the cockroach directly into the cerebral ganglia [42, 72]. The venom contains a neurotoxic cocktail that does not paralyze the host nor does it affect motor neurons, sensory neurons or muscles. Rather it places the host in a hypokinetic state which allows the parasite to clip the cockroach's wings and lead it much like one would walk a pet to a pre-chosen burrow [72]. The venom raises the threshold required for initiating and maintaining walking-related behaviours without affecting other behaviours such as flying and grooming [73]. Cockroaches in this hypokinetic state will not respond to aversive stimuli or initiate any form of escape or walking-related behaviour [74, 75]. The parasite lays its eggs on the legs of the cockroach. The larva hatch and burrow into the cuticle of the still living host and begin to feed on its internal organs. After a few days, the cockroach finally dies with the larva now pupating within it and safe from any predators that may attack it [72].

Many more models of behavior manipulation of hosts by parasites exists and has been discussed here [76]. This has consistently been used to support the theory of extended phenotypes advanced by Dawkins in 1982. Briefly, Dawkins argues that genes of one organism, (in this case the parasite), is able to alter the behaviour of another organism (the host). The extended phenotype paradigm argues that the gene is the basic unit of heredity

and is passed on from parents to offspring. Thus, phenotypes and even organisms are simply a way to transfer genetic material. In essence, they represent a means to an end [37, 77-79]. The idea that the expression of a gene is not constrained to the individual that carries the gene has proven useful in understanding a multitude of behaviours including parasite manipulation [80, 81].

The *Toxoplasma gondii*-*Rattus norvegicus* association is one of the best studied examples of behavioural manipulation [82]. *Toxoplasma gondii* is an obligate protozoan intracellular parasite capable of infecting all mammals [83]. It is an apicomplexan parasite similar to *Plasmodium sp.* [84].

***Toxoplasma gondii* life cycle and transmission**

Toxoplasma gondii (*Toxoplasma*) is an obligate protozoan parasite capable of infecting all mammals [83]. *Toxoplasma gondii* was originally studied as a parasite of rodents and other small animals in 1908 (republished [85, 86]). Gradually, its role as a human pathogen was discovered [87]. Its full life cycle was not discovered until 1970 when the sexual stage and oocyst production of the parasite was described [88, 89]. *Toxoplasma* is classified under the phylum Apicomplexa and is similar to the endoparasites *Plasmodium* and *Cryptosporidium parvum*. It possesses a complex apical structure that it uses to penetrate host cells [84]. *Toxoplasma* has evolved a two host life cycle. It is able to survive as fast replicating tachyzoites or slow replicating bradyzoites in cysts via asexual reproduction in all homeothermic animals, including rats [90, 91]. These species all serve as intermediate hosts. Tachyzoites travel throughout the body via the blood stream and are present during the acute phase of infection typically in the first few weeks. Chronic

infection occurs when cysts containing bradyzoites are present in immune privileged organs like the brain [91-93], eyes and gonads [94, 95]. These remain even after circulating tachyzoites have been cleared. Periodically, tissue cysts rupture and re-encyst (recrudescence). In immune competent individuals, recrudescence does not present a risk. It may however lead to reactivation of latent infection in immune compromised individuals with serious and sometimes fatal consequences [96]. Conversely, *Toxoplasma* can only sexually reproduce to form oocysts in the gut of Felines. The oocysts are excreted in the fecal matter of cats and can persist for long periods of time even during adverse environmental conditions. Excretion of oocysts also makes it possible for *Toxoplasma* to infect herbivores [97], which would be immune to a route completely dependent on carnivory.

Figure 2.1: Life cycle of *Toxoplasma gondii* showing the transmission between definitive and intermediate hosts.

Epidemiology and Etiology of *Toxoplasma gondii*

Toxoplasma remains one of the most successful parasites in the world, infecting approximately one-third of the world's population [98]. IgG seroprevalence varies from region to region. Seroprevalence is less than 10% in Korea [99], 13% in China [100], 24% in Nigeria [101] and approximately 47% in both Tanzania and rural France [102, 103]. Available epidemiological data estimates approximately 17% of Singaporean citizens were chronically infected with *Toxoplasma* in 1987 [104]. Another study using an antenatal cohort reported similar prevalence levels [105]. In the USA, *Toxoplasma*, along with Salmonella is responsible for the highest rates of health liability among foodborne pathogens as measured by disability adjusted life years (DALY) [106].

Clinical Manifestations of *Toxoplasma gondii*

Both immunocompetent and immunocompromised infected individuals are capable of developing toxoplasmosis especially inflammation of the eye (retinochoroiditis) during the acute phase of the infection [107]. Common presenting symptoms of acute toxoplasmosis infection mimic symptoms of mononucleosis infection including sore throat, muscular pain, body rash and fever [107]. These symptoms quickly ease in immunocompetent individuals as soon as infection reaches the chronic stages.

Most work on the clinical implications of *Toxoplasma* has focused on immunocompromised individuals primarily due to co-infection with AIDS [108, 109]. *Toxoplasma* remains the leading cause of focal brain lesions, attributed to the preference of parasite for brain and other immune privileged organs during cyst formation [110, 111].

Figure 2.2: Global seroprevalence of *Toxoplasma gondii*. The dark red areas correspond to areas above 60%. Areas with seroprevalence between 40%-60% are shown in light red and areas with 20%-40% are shown in orange. Yellow represents areas with 10%-20% seroprevalence while green denotes areas with <10% seroprevalence. White denotes an absence for seroprevalence data for these regions. The map was generated with data obtained from Pappas *et al.*, 2009 [112].

Pregnant women are also a commonly studied cohort in *Toxoplasma* research. Congenital *Toxoplasma* is a deep concern as it has been established that pregnant women infected with *Toxoplasma* can transmit the parasite to their unborn fetus via vertical transmission [113]. A fetus infected with *Toxoplasma* in the first trimester usually results in severe toxoplasmosis and in some cases even abortion of the fetus. Conversely, infection in the third trimester results in normal appearance of the newborn but later manifests as subdued growth and/ or ocular toxoplasmosis [114].

Toxoplasma was previously thought to be clinically asymptomatic in chronically infected immunocompetent individuals. Recent evidence has resulted in the need to revise this perception. *Toxoplasma* infection has been reported to increase the odds of developing multiple psychiatric disorders [115]. Odds ratios were calculated based on concordance between seroprevalence of *Toxoplasma* infection and presence of the disorder. Infected individuals have a 3.4 odds ratio for developing obsessive compulsive disorder, between a 1.8 to 2.7 odds ratio for schizophrenia and a 1.5 odds ratio for bipolar disorder [115-118]. In the case of Schizophrenia, concordance studies have been confirmed by different prospective studies.

Researchers determined that in US military personnel, there was a positive correlation between rise in *Toxoplasma* IgG antibodies and later diagnosis of schizophrenia [119]. The same study failed to establish a correlation between *Toxoplasma* IgG and influenza or herpes virus. This suggests a correlation specific to *Toxoplasma* and is not simply as a result of general immune response to infection. A second prospective study involving routine screening of pregnant mothers found a positive correlation between seropositivity for *Toxoplasma* and later development of schizophrenia [120]. A third study reported that

Toxoplasma infected patients with schizophrenia exhibit more severe symptoms of psychosis compared to uninfected patients [121, 122]. Aside this, *Toxoplasma* infected human subjects have been reported to display personality changes [123]. Infection with *Toxoplasma* has been shown to increase risky behaviour specifically increasing the probability of being involved in a traffic accident. This has been confirmed using both retrospective case controlled studies [124, 125] and prospective studies [111].

Potential sexual transmission

Toxoplasma undergoes gametogenesis in the gut of cats to form oocysts. Oocysts are shed as part of cat fecal material into the environment where they are ingested by intermediate hosts like ruminants which cannot be infected by the normal route of carnivory (predator-prey interactions) [91]. Humans can also be infected when they accidentally ingest oocysts. Alternatively, humans are also infected when they consume undercooked meat harbouring bradyzoites-containing cysts and through congenital infection of a fetus. In addition to these more common routes, it has been proposed that *Toxoplasma* is a sexually transmitted infection in humans [126].

Toxoplasma has previously been shown to inhabit immune-privileged organs including the brain and eyes where they form bradyzoites-containing tissue cysts. These sites are protected from immune cells that have very limited access to them. This allows cysts to undergo periodic rupture and re-encystment (recrudescence). In addition to the brain and eyes, another immune privileged organ is the male testes. Testes segregate developing sperms behind a tight barrier that shields them from immune cells [127]. Interestingly, *Toxoplasma* has been found beyond the blood-testes barrier (BTB) in a variety of non-

human animals including sheep [128], dogs [129], rabbits [130] and rats [95]. *Toxoplasma* has also been showed to be present in both the ejaculate and female post-copulation in many of these animals.

There exists some evidence of sexual transmission of *Toxoplasma* infection in human males. These are however all correlative studies with no strong cause and effect link. An early study in 1971 reported 3 individuals positive for *Toxoplasma* in semen with two of them also serologically positive [131]. A later post mortem study revealed the presence of the parasite in the testes of 60% of AIDS patients examined [132]. The presence of tachyzoites after diagnosis usually suggests reactivation of long dormant tissue cysts [133]. The presence of the tachyzoites in the testes will then suggest the presence of tissue cysts in testes prior to immune-compromise. This coupled with observed testosterone increase in both rats [134] and humans [135] necessitates a definitive study to determine if sexual transmission is indeed another route of infection.

Behavioural Manipulation by *Toxoplasma gondii*

The atypical behaviour displayed by *Toxoplasma gondii*-infected rats is one of the best studied behaviour manipulations [82]. In 2000, Berdoy reported that rats chronically infected with *Toxoplasma* displayed a “loss of aversion” towards cat odour. This manifested as an increased approach to and increased time spent in areas marked with cat urine [136, 137]. The cat is the natural predator of rats. It is also the only animal in which *Toxoplasma* can sexually reproduce and complete its life cycle. *Toxoplasma*’s need to complete its life cycle is seriously hampered by the strong aversion of rats towards cues of cat presence. It has been proposed that *Toxoplasma* increases approach behaviour to cat

odour as a means to increase predation. This will consequently increase the likelihood of *Toxoplasma* completing its life cycle [92, 136]. Since the initial report, it has been demonstrated that the effect of *Toxoplasma* was specific to cat odour. Infected rats did not show any significant change in learned fear behaviour, olfaction or general anxiety when compared with uninfected controls [94, 136, 138].

There have also been reports of *Toxoplasma* altering behaviour in other animals and in humans. A recent study reported that *Toxoplasma*-infected chimpanzees (*Pan troglodytes troglodytes*) showed a significant increase in number of approaches to areas marked with the urine of their natural predator the leopard (*Panthera pardus*). A similar increase was observed in the investigation of areas marked with leopard urine. These observations were however absent when chimps were exposed to other large felines that were not natural predators (lions and tigers) [139].

Humans have a long history of coexisting with carnivores and were predated upon at rates similar to what is seen with other primates. Extant humans are no longer under feline predation and thus represent a dead end host for *Toxoplasma*. There however remains the possibility that behavior manipulation of humans by *Toxoplasma* evolved when humans were still being predated upon by large felines and thus served as suitable intermediate hosts [139]. Among the effects of *Toxoplasma* infection observed in humans includes an increased risk of developing schizophrenia. Different studies have observed significantly greater portion of people with schizophrenia show seropositivity for *Toxoplasma* antibodies compared to healthy controls [116, 121, 140, 141]. In utero and/ or early life exposure to *Toxoplasma* also increases risk of developing schizophrenia [142-144]. Latent *Toxoplasma* infection has also been associated with higher vigilance and increased

suspicious and jealous behavior in men [145]. Females on the other hand showed increased warmth, moral adherence and conscientious behavior. Both genders demonstrate increased apprehension and decreased novelty seeking behavior in infected cohorts compared to controls [145]. There have also been reports showing reduced concentration, increased reaction time and increased probability of being involved in traffic accidents among infected individuals [124, 146]. A major concern of all behavioural effects reported in humans is that majority of work merely involved association of observed endpoints with sero-positivity in a case-control design. This impedes the ability to determine direction of causation. For example, are some individuals more risk taking because they are infected with *Toxoplasma* or are they infected because they are more risk taking [147]? Some prospective studies have attempted to resolve this dilemma by showing that infection leads to later increase in risk of traffic accidents [111]. Yet human work in toxoplasmosis remains weaker compared to animal models. This is especially true for mechanistic studies akin to those attempted in this thesis.

Many argue that altered behaviour may simply be an incidental side effect of infection and a result of general sickness behaviour or a general disruption of adaptive behaviours [148]. In rodents, sickness behaviour manifests as a general loss of weight and a general appearance of coarse unkempt fur. Sickness behaviour is also likely to disrupt performance of non-aversive memory tasks. Generally sick animals do not allocate resources to maintaining metabolically costly behaviours such as social status, aggression and mate choice. Data from several groups has demonstrated comparable weight gain and normal grooming behaviour between control and infected animals [94, 136, 138]. Hippocampal dependent non-aversive learning has also been demonstrated to be

unaffected in infected animals arguing against a general disruption in adaptive behaviour [94]. Finally, infection does not appear to affect social hierarchy nor does it hinder ability of infected rats to compete for mates [149].

Another argument is that infection disrupts olfaction in rodents. In other words, it is not that the parasite is manipulating behaviour, it is just that rat can no longer smell properly and that is why it spends more time exploring cat odour [150]. Different reports provide evidence that negates this argument. Infected animals do not differ from control animals at very high or low concentration of cat odour arguing for a dose-dependent response. A general disruption in olfaction will not be able to detect different doses in stimuli [137]. Infected mice retain the aversion to food with a novel odour as well as the ability to learn from odour cues provided by conspecifics [94]. It is also noteworthy that increased approach behaviour to predator odour in infected animals does not extend to other predators that are not the definitive hosts of *Toxoplasma* [151].

Another concern is that *Toxoplasma* exerted its effects simply by affecting all types of defensive behaviour. This is a legitimate concern keeping in mind that neural circuits underlying innate defensive behaviour to cat odour sometimes overlaps with circuits of learned fear and anxiety. Specifically, neural circuits involving the hippocampus, amygdala, hypothalamus and periaqueductal gray are important for both innate fear of cat odour and learned fear [152-154]. That these two types of defensive behaviour overlap raises the possibility that *Toxoplasma* interferes with defensive behaviour in general. Surprisingly, *Toxoplasma* infection does not affect conditioned fear [94] and also retains innate aversion to odour of non-feline predators [151]. However, it is worthy to note that there have been reports of *Toxoplasma* decreasing anxiety in an exploration-based but not

a social interaction-based anxiety test [155]. Collectively, observations stemming from the *Toxoplasma* manipulation model argue for a specific effect geared towards a specific behavioural outcome. This has no doubt been shaped by intense selection pressure on parasites to optimize survival and transmission [156].

It has also been argued that it is impossible to fully understand the historical effects of natural selection and the adaptive nature of such behaviours using current behaviour experiments [157, 158]. One way to remedy this is to understand the proximate mechanisms of the altered behaviour. As well documented as these parasite manipulations are, much remains unknown about the pathways that mediate these behaviour changes and their regulation. It is not enough to simply hypothesize that the manipulated behaviour in question has a historical benefit for the parasite. How can we prove this? Unfortunately there is no way to go back in time and replay the metaphorical tape of life as rhetorically proposed by Stephen Jay Gould and demonstrate historical evolution of the manipulation. An alternative is to argue historical selection by demonstrating the fit between extant form and function of the behavior with perceived selection pressures. If the mechanisms of behavioral change are specific and congruent to needs of selection pressure, then it buttresses the idea that behaviour (or behavioural change) was indeed historically selected. A more generalized mode of change with a variety of side-effects alternatively argues for behavioural change to be merely incidental or a byproduct of a wide-spread disruption.

***Toxoplasma gondii-Rattus norvegicus* association**

The *Toxoplasma*-rodent model is particularly suitable for studying behavioural manipulation because both *Toxoplasma gondii* and rats have been used as model organisms in parasitology and neurobiology research respectively. *Toxoplasma* has consistently served as a model organism for delineating the molecular mechanism of protozoan pathogenesis. Rodents have served as model organisms for neurobiology research for a long time. Biological pathways underlying innate aversion to predator odour are relatively well-studied in these species [152, 159, 160]. The joining of these two well-studied models affords researchers a host-parasite model eminently suited to lab experiments. This can allow researchers to study proximate mechanisms of parasitic behavioral manipulation in greater detail in this association compared to other host-parasite relationships.

However, it is not enough to simply demonstrate that there is a behaviour change post *Toxoplasma* infection. It is necessary to establish if this is simply an accidental by-product of infection or if behaviour has evolutionary significance relevant to survival of the parasite [157]. The only way to differentiate between the two is to demonstrate a dedicated proximate mechanism underlying behaviour. In other words, it is important to determine how this atypical behaviour is maintained in nature [161]. Natural selection selects for those alleles that code for traits that improve the “fitness” of an organism. “Fitness” in this case refers to the ability to survive till sexual maturity and production of offspring [22, 162]. Through experimentation, three hypotheses have emerged in an attempt to elucidate the proximate mechanisms of this atypical behaviour [163].

One of the earliest hypotheses regarding mechanism was the tropism model [94, 164]. This model proposes that the *Toxoplasma* parasite inhabits certain specific brain regions post infection [94, 155, 165, 166]. Many studies have proposed that *Toxoplasma* cysts are preferentially found in areas of the limbic system such as the amygdala and nucleus accumbens [94, 155]. The amygdala plays a pivotal role in defensive pathways [154]. Similarly, the nucleus accumbens is responsible for reward-motivated behaviour via the action of the neuromodulator dopamine. The specificity of brain regions infected is believed to be the driving force behind the observed changes [155]. The role of amygdala in defensive behaviour and nucleus accumbens in reward-motivated behaviour makes even a subtle hint of tropism to these regions immensely interesting.

The main argument against this hypothesis is that there appears to be no consistency between brain regions observed to have the most numbers of parasites [164, 165, 167]. Subsequent detailed analysis of cyst burden failed to observe a tropism for the amygdala and nucleus accumbens. Rather, a more probabilistic distribution as opposed to the previous suggested tropism for particular brain regions was proposed [164]. In other words, data from counting parasite numbers within the brain appears to be non-reproducible among researchers. The assertion that cyst burden in a particular brain region is responsible for observed behaviour changes is debatable. Cyst burden in the brain is quite small compared to brain cells. Furthermore, there is no evidence that behaviour change is as a direct result of cyst presence within the brain. In other words, even after parasite clearance, the loss of fear phenotype is still observed in rodents [168]. It is more likely that cysts will secrete a substance that affects the innate fear pathway in the brain [94, 169]. More importantly this model is unable to explain why other amygdala and

nucleus accumbens dependent behaviours like conditioned fear, neophobia to novel food and social status remain intact [94, 149]

The *Toxoplasma* parasite has two genes that are analogous to the human tyrosine hydroxylase gene [92, 170-172]. Tyrosine hydroxylase is the rate limiting enzyme in the synthesis of the neurotransmitter dopamine, an important mediator of reward and motivation-driven behaviour [138]. Tyrosine hydroxylase converts tyrosine to L-Dopa [173]. Increased levels of dopamine have been observed in the brain during rewarding behaviours such as mating [174, 175]. Therefore it is hypothesized that parasite-induced increase in dopamine mediates the observed behavioural phenotype post infection [92, 138, 171, 176]. Although this hypothesis is much more popular than the tropism model, there remains a lot to be determined as far as how exactly the parasite increases dopamine production in the host brain [172]. It is yet unclear whether the *Toxoplasma* parasite excretes tyrosine hydroxylase into host systems, thus facilitating an increase in conversion of tyrosine to L-Dopa or if the tyrosine hydroxylase analogue genes produce metabolically active tyrosine hydroxylase in the first place [170, 177]. Also, because of the perceived effect of *Toxoplasma* infection on dopamine levels it has been proposed that infection increases the probability of developing schizophrenia [138, 173]. Unfortunately, all available data to this end are correlative data with no clear demonstration of causation [122, 123, 145, 178].

On the other hand, it has been demonstrated that anti-psychotic mood stabilizing drugs are able to alleviate the effects *Toxoplasma* infection *in vivo* and replication *in vitro* [179, 180]. Of the three drugs administered, haloperidol, a D2 dopamine receptor antagonist proved superior in alleviating behavioural effects of infection. It is believed haloperidol's

ability to inhibit dopamine as well as inhibit *Toxoplasma* replication accounts for its superior action [180]. Despite all these, there appears to be some disagreement among researchers on the effect of infection on dopamine production both in vitro and in vivo [176, 177, 181].

More recently, a third hypothesis has been proposed. *Toxoplasma* infection has been reported to cause leakiness between the defensive and reproductive pathways [182]. Exposure to cat odour causes increased cFos activation in the posterior dorsal medial amygdala (MeApd), usually activated in response to reproductive cues. Uninfected rats typically show preferred activation of the posterior ventral medial amygdala (MeApv) upon exposure to cat odour [182]. It is noteworthy that the innate defense mechanisms are stringently regulated [153]. These pathways regulate response to intrinsically threatening stimuli perceived to threaten survival. In other words, innate defensive behaviours and their proximate mechanisms are crucial to survival and are hardwired right from birth [152]. Congruently it has also been reported that *Toxoplasma* infected males become more attractive to and receive more mating opportunities from females compared to uninfected rats [95]. *Toxoplasma* invades both the brain and testes [95]. In the testes, it increases testosterone production [134], which increases synthesis of major urinary proteins (MUP) [183]. Testosterone also crosses the blood-brain barrier (BBB) and results in hypomethylation of the arginine vasopressin (AVP) promoter in the MeApd [184]. This results in an increase in AVP production in *Toxoplasma* infected rats [184]. This is similar to what is seen when males are exposed to reproductive cues [185].

Sexual Dimorphism of infection

The testosterone-AVP hypothesis appears to be the most parsimonious model. It accounts for both observations in male rats post infection. It sufficiently explains the increased attractiveness of male rats [95, 183] as well as the increased approach to cat odour [95, 184]. All substrates involved have been shown to elicit similar responses in non-infected male rats when experimentally manipulated [185-187]. As detailed and parsimonious as this model is, it encompasses substrates that are highly gender dimorphic. This raises a very important question: What happens in female rats infected with *Toxoplasma*? Do they show increased approach behaviour post *Toxoplasma* infection? Or is this atypical behaviour restricted to males?

With the important exception of female-specific behaviours such as maternal behaviours, most social behaviour studies that aim to understand the proximate mechanisms underlying these behaviours are hardly ever studied in both genders [188, 189]. Data obtained from these studies in males are simply assumed to hold true for females as well. However, it has been demonstrated, and more so in recent years that the brain, especially what is often referred to as the social brain or social behaviour network (SBN) is gender dimorphic [190, 191]. The amygdala, a very important node in social behaviour, has been shown to be different in size and distribution of neurotransmitters and their receptors [192, 193]. Similarly, other brain regions including the many sub regions of the hypothalamus and bed nucleus of stria terminalis (BNST), all part of the SBN, have been shown to function in a gender dimorphic manner [193]. The notion that it is possible to understand any type of social behaviour that is not gender specific by experimenting with only one gender is erroneous at best and disastrous at worst. Our inability to explore, if

any gender dimorphisms exist in social behaviours between the different genders will hamper any efforts to develop proper therapeutic interventions aimed at treating neuropsychological diseases [193].

Additionally, *Toxoplasma* is capable of infecting humans with fatal outcomes in people with diminished immune systems [97]. However, the behavioural effects of *Toxoplasma* infection in humans are not well understood [194]. Several publications have presented evidence of a correlation between infection and increased reaction times, increased risk taking behaviour and even the development of schizophrenia [111, 124, 145]. There is currently no evidence however, of a direct causation link between *Toxoplasma* infection and any of these conditions. Inability to do the kind of experiments that will demonstrate causation in humans necessitates that in order to fully understand the parasites' effects on behaviour in humans, there is a need to completely understand the effect of post chronic *Toxoplasma* infection in a model animal. This raises several questions. Firstly, does host behaviour manipulation by *Toxoplasma gondii* occur in female rodents? Yes, it does. This has been reported in both mice [94] and rats [195, 196]. The more important question concerns the proximate mechanism in females. Does the parasite function by a parallel mechanism? Does it increase maleness in infected females and employ the same substrates as in males? Or does it employ a completely different pathway from males? All of these questions currently remain unanswered.

The data available makes it difficult to conclusively determine if parasites manipulate sexually selected proximate mechanisms. It is quite clear that certain parasites have a gender bias in their infection and persistence within their host organisms [12]. Consequently, in most cases of parasite manipulation, it is unclear if the parasite is able to

manipulate both genders or not. Most experimentation is usually done only in one gender, usually the male. Several reasons account for this; primarily, males are used due to the notion that they are less variable as well as easier to work with when compared with females. This notion has however been debunked [197-199]. Significant male bias observed in the majority of parasite infections is also another reason most studies on proximate mechanism focuses on males. Most studies on proximate mechanisms related to parasite-induced behavioural manipulations in females are because the behaviour in question is female-specific [188, 189].

One way to address the question of possible sexual dimorphisms in parasitic manipulations is to focus on proximate mechanisms. The first step is to find out if the parasite in question is able to manipulate host behaviour in both genders. Does the parasite employ the exact same pathway in both genders to achieve manipulation? Or does it employ parallel but gender specific pathways to alter host behaviour? Understanding proximate mechanisms underlying host behaviour change post infection may offer insights into the apparent gender dimorphism of parasite infection and prevalence in host organisms. It will also help to understand how parasite infections may or may not affect sexual selection in a species. In this thesis, I take this approach.

Alternatively, convergent and divergent evolution experiments can be used to understand gender dimorphic effects of parasite infection. For example, *Toxoplasma gondii* has been demonstrated to increase attractiveness of infected male rats to uninfected female rats [95]. An experiment using monogamous prairie vole under weak sexual selection and the polygamous montane vole under strong sexual selection could help to understand the link between sexual selection and parasite prevalence. The hypothesis here is that there will be

no effect of *Toxoplasma* infection on the attractiveness of monogamous male prairie voles to females. Attractiveness of male montane voles to females is however expected to increase in a manner similar to what is observed in male rats. In a similar manner, sexual signalling in infected and uninfected populations is expected to differ. For example a population with high *Toxoplasma* prevalence is expected to invest more in sexual signals to demonstrate “fitness” to potential female mates compared with uninfected or low prevalence populations. This approach is promising. Yet, I do not take this approach in my thesis because of unavailability of model species in Singapore.

Understanding effect of *Toxoplasma* infection on behaviour in females as well as underlying proximate mechanisms of any atypical behaviour will provide a better understanding of what occurs post infection. A better understanding in the rat model improves the odds of understanding effects in humans which will hopefully lead to the development of improved therapeutic intervention in *Toxoplasma* infected humans.

Specific Aims

In this report, my aim is to study the behavioural effects of *Toxoplasma gondii* on female rats. Furthermore, I will attempt to determine the proximate mechanism underlying the observed behaviour changes. My approach is to place this within a larger context of the gender dimorphic nature of the brain and the necessity to explore proximate mechanisms of social behaviours in both genders.

I briefly discuss my specific aims in the sections below.

Specific Aim1: Determine the behavioural effects of chronic *Toxoplasma gondii* infection

The most important part of the work is the initial demonstration that similar to what was observed in male rats, *Toxoplasma* infection results in increased approach type behaviour to cat odour. This was especially important given the male-centric nature of the testosterone-AVP hypothesis. It was also important to determine the effect of infection on other anxiety and fear-related behaviours especially given the anxiolytic nature of the two important female reproductive hormones, estradiol and progesterone. I aim to demonstrate the effects of *Toxoplasma* infection in female rats on exposure to cat odour. I further demonstrate the effects of infection on other types of anxiety behaviours (**Chapter 3**).

Specific Aim 2: Investigate a role for oxytocin system in the proximate mechanism of behaviour change

The testosterone-AVP model is a parsimonious model that accounts for both changes in defensive and reproductive behaviours in male rats post infection. It also possesses a distinct maleness that makes it unlikely to apply in the case of females. I propose the model can be reduced to its most basic form. It is essentially an interaction between a peripheral reproductive hormone and a nonapeptide neurotransmitter involved in socio-sexual behaviour. Based on this I explore the role of the oxytocin (OT) system in mediating this behaviour. Oxytocin is a nonapeptide similar in structure and function to AVP, though both neurotransmitters are expressed in a gender dimorphic manner. I measured the transcript levels of both OT and OTR in the MeApd, PVN, MPOA and BNST, all important nodes of the SBN. Transcript levels of OT were also measured in the SON of the hypothalamus. The PVN and SON are important sites of OT production. I hypothesized that much like AVP in males, the OT system will be important in the proximate mechanism of *Toxoplasma* effects in females (**Chapter 4**).

Specific Aim 3: Determine the necessity of the OT system to drive the behaviour change

My next aim was to demonstrate the necessity and sufficiency of OT to elicit similar behaviour changes sans infection. I attempted to use the OTR antagonist L-368,899 to demonstrate necessity OT production in mediating behaviour change. I also exposed experimental females to the habituation-dishabituation paradigm as a test of social recognition prior to exposure to cat odour. Data from this experiment led me to explore

the role phytoestrogens in observed behaviour. I will also report on those findings in this chapter (**Chapter 5**).

Chapter 3

Female rats show loss of aversion to cat odour after *Toxoplasma gondii* infection

Aim

Chronic *Toxoplasma gondii* infection causes loss of innate aversion to cat odors in male rats. This observation has often been interpreted as parasitic manipulation of the host behavior because cats are the only host in which the parasite can undergo sexual reproduction. Prior work suggests a causal role of testosterone in the host behavioral change. Because testosterone is preferentially produced in males, these observations suggest a sexual dimorphism in host behaviour change post-infection. With this backdrop, I present experimental evidence in this chapter that females exhibit loss of aversion after *Toxoplasma* infection akin to males, and that behavioral manipulation itself is not sexually dimorphic.

Background

Many studies in the last decade have reported on sexual dimorphism in neurobiology [190, 191]. Specifically, it is noteworthy that studies are beginning to point out the exclusion of female animals in preclinical research that is not gender specific [189, 197]. This is puzzling especially in light of the growing evidence of sexual dimorphism in rodent behavioural traits [189, 198-200]. Even behaviours that do not appear to be gender dimorphic may be mediated by dimorphic proximate mechanisms [197, 198]. Failure to report sex of animals and/ or cell lines used in research as well as predominant use of males in preclinical research has created a gap in knowledge that affects understanding and subsequent treatment of diseases [197]. Fish (2008) [201] reports that 80% of prescription drugs withdrawn in the US in 2005 were as a result of differential manifestation of side effects and health issues in men and women. The NIH in a bid to address this issue has mandated that from 2016, investigators applying for grants must account for “sex as a biological variable” (SABV) in preclinical research. The hope is that it will inform the development of sex-appropriate medical intervention for both sexes [202].

Previous work in the study of *Toxoplasma*-induced behaviour change has focused almost exclusively on males [36, 94, 95, 136]. As a result of this study, three (3) different hypotheses have been proposed to explain the proximate mechanism mediating this behaviour [92, 163, 171].

The most parsimonious of the three posits that *Toxoplasma* infection in male rats results in an increase in testicular testosterone synthesis [134]. The testosterone then crosses the

blood-brain barrier (BBB) and causes hypomethylation of the arginine vasopressin (AVP) gene within the posterior dorsal medial amygdala (MeApd) [184] which subsequently leads to increased AVP production which then drives the behaviour change [185]. This hypotheses accounts for both the increased approach to cat odour and increased mating opportunities in male rats [95]. Yet this narrative has a significant limitation in that the proposed mechanism is extremely male-centric. Testosterone is a predominantly male reproductive hormone that is produced in relatively smaller quantities in females [203]. AVP is a nonapeptide that is expressed in both genders. Extrahypothalamic AVP is however testosterone dependent [204-206] and has very little expression in females [207]. The extrahypothalamic AVP system particularly in the MeA and BNST, has been demonstrated to have denser projection with approximately three times more AVP positive neurons in males compared to females [208]. In addition AVP is functionally gender dimorphic as well. It is important for male sexual behaviour, inter-male aggressive behaviour and social recognition behaviour in males and not females [208-210]. Even the medial amygdala where this change occurs is also gender dimorphic. The medial amygdala is larger in males than in females. There is also a difference in neurotransmitter receptor distribution, dendritic length and branching, spine density and synaptic connectivity in the MeA between males and females. In addition, MeA together with BNST is known to mediate several gender dimorphic behaviours including aggression and sexual behaviour [211-213]. Thus the current model suggests a proximate mechanism mediated by very gender dimorphic and male-centric nodes. This suggests *Toxoplasma*-induced behavioural manipulation does not occur in the case of females.

The goal of this set of experiments was to determine the effects of chronic *Toxoplasma gondii* infection on behaviour of female rat using the cat odour avoidance assay, elevated plus maze and open field test.

Methods and Techniques

Animals and Infection

Adult Wistar Han female rats approximately 7-8 weeks (49-56days) old were used for all experiments. Animals were procured from InVivos animal rearing facility, Singapore and housed two per cage at the NTU vivarium. Animals were provided food and water *ad libitum* under controlled temperatures (24-26°C) and humidity (60-70%) in a 12 hour light on/ light off cycle (light on at 7am). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technology University. All animals were allowed to acclimatize to the facility for a minimum of two days before being subjected to any experiments. A Prugniald strain of *Toxoplasma gondii* with both Luciferase and GFP inserts (PA7) was used. This strain is similar to the strain used for prior work with male rats ([95, 184, 185]. The parasites were maintained in vitro in human foreskin fibroblast (HFF) monolayers and harvested using syringe lysis. Females were randomly assigned in experimental groups and injected with either *Toxoplasma gondii* (5×10^6 tachyzoites suspended in 500 μ l phosphate buffered saline (PBS); i.p.) or the vehicle (PBS) alone. All rats were weighed weekly. Absence or presence of overt sickness behavior was noted every week. Experiments were conducted from the 7th week of infection onwards.

Determination of estrus

Vaginal lavage was collected under mild restraint using 10 μ l of buffered saline. Stage of the estrus cycle was determined using previously published criteria under 200X (total)

magnification. Specifically, vaginal estrus manifested as preponderance of cornified and epithelial cells.

Cat Odour Avoidance Assay

Aversion to cat odor was quantified in a rectangular arena with two opposite and identical arms (76 x 9 cm each), separated by a central part (9 x 9 cm in size; white Perspex). The entire length of the straight maze was used during testing. Cat odour was presented in one extreme of the arena and rabbit odour was presented in the other extreme (2ml bobcat urine soaked on a gauge of towel; Maine Outdoor Solutions, ME, USA; 2ml rabbit urine soaked on a gauge of towel; Biopolis Vivarium, Singapore; trial duration = 20 minutes). The animal was placed in the centre of the maze and allowed to move freely between the two odours. Animals were habituated to the arena for 3 consecutive days, 20 minutes each day. Exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA). The amount of time spent exploring the bisect with cat odour was used as a measure of innate aversion to cat odour.

Elevated Plus Maze

Intact estrus female rats (control, infected) were tested on the elevated plus maze (EPM) as a measure of anxiety during the dark phase of their light cycle for 300s per animal. Each open arm was kept at a lux of 10. The closed arm was kept as dark as possible. The open arm is 75cm x 11cm, the closed arm is 75cm x 11cm x 26cm. The arms are raised 60cm off the ground. Percentage time spent and percentage number of entries into the open arm was used as measure of anxiety behaviour. Quantification of number of head dips in the open arm was used as a proxy of risk assessment behaviour.

Open Field Test

A rectangular Perspex open field apparatus with dimensions 100 cm x 100 cm x 30 cm was used. Control and infected estrus female rats were tested for anxiety and locomotion during the dark phase of their light cycle. Test females were placed in the centre of the apparatus and allowed to explore the apparatus. Exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA). Quantification of time spent in the centre, walls and corners was used as a measure of anxiety. Total distance travelled served as a proxy for locomotion in test animals.

Quantification of estradiol, testosterone, progesterone and Corticosterone levels

Trunk blood was collected during sacrifice. Plasma concentrations of all hormones were determined using commercially available enzyme-linked immunoassay kits (Enzo Life Sciences, NY, USA). For corticosterone, plasma steroids were extracted using diethyl ether prior to immunoassay. 200 μ L of plasma was added to 400 μ L of diethyl ether and shaken. The mixture was allowed to separate and the top layer containing the extracted steroid was pipetted into a clean test tube. After three successive extractions, diethyl ether was allowed to evaporate to dryness and the pellet was then re-suspended in 1 mL of buffered saline. For all other hormones, the serum displacement reagent provided with the kit was used. The protocol recommended by the immunoassay kit was followed without modification.

Quantitative PCR

Brain tissue was harvested post-decapitation and flash-frozen in liquid nitrogen. The tissue was stored at -80°C till further analysis. Subsequently, mRNA abundance was

quantified for the nonapeptide arginine vasopressin (AVP) in the posterodorsal medial amygdala (MeApd). A standard method based on SYBR green dye was used as described before [134, 184]. PCR cycles needed to reach a pre-determined fluorescence threshold at early linear phase of the amplification were determined (Ct, threshold cycle number). Greater mRNA abundance in this assay results in lower Ct values. Ct values for OT and OTR were normalized by subtracting corresponding Ct values of 3 housekeeping genes (HPRT, β -actin and 18s rRNA). Three technical replicates were used for each determination. The median coefficient of variation between technical replicates was $\leq 15\%$.

Statistics

The student's t-test was used to determine statistical significance between control and infected animals. Analysis of variance (ANOVA) was used to determine main effects and interactions of the infection and the surgical status. In view of non-normal distribution, Mann-Whitney test was used to determine statistical significance.

*Please refer to the Annexe for a more detailed methodology.

Results

Infection reduced aversion to cat odor in gonad-intact female rats.

23 female rats were randomly assigned to groups and infected with five million *Toxoplasma gondii* tachyzoites (n=12) or injected with physiological saline (n=11). Innate aversion to cat urine was quantified during the proestrus/estrus phase (>6 weeks post-infection). Control females did not exhibit significant aversion to cat urine in this assay, reflective of the weak and partial predator cue employed (Figure 3.1A; paired student's t-test between rabbit and cat odor bisect occupancy: $|t|_{10} = -0.604$; $p = 0.559$; statistical power (β) = 0.117; effect size: Cohen's $d = 0.322$). In contrast, females infected with *Toxoplasma gondii* spent significantly more time near cat odor compared to rabbit odor ($|t|_{10} = 2.615$; $p = 0.026$; Cohen's $d = 1.282$). Figure 3.1B represents median and interquartile range for the time spent by control and infected females in the vicinity of cat odour. Figure 3.1C represents the same assay performed on a later cohort. The assay has been performed for different groups of females with different baselines in view of an intervening construction event at the animal vivarium. This duplication was done to ensure the stability of the observed behavioural change. Infected females exhibited increased approach behaviour to cat odour compared to uninfected females ($|t|_{22} = -3.864$; $p = 0.001$; Cohen's $d = 1.577$).

Generality of fear was tested by Elevated Plus Maze and Open Field Test

Anxiety was tested in the elevated plus maze. Control and infected female rats showed similar exploration of anxiogenic open arms. There was no significant difference between control and infected animals with respect to number of entries (Figure 3.2A; control =

19.46 $\pm$ 1.66, Infected = 19.51 $\pm$ 1.66) into the open arm. Independent t-test $|t|_{21} = 0.023$; $p = 0.982$; $n = 11$ control, 12 infected; Power (β) = 0.05; Cohen's $d = 0.01$. Similarly there was no significant difference between control and infected animals with respect to time (control = 29.46 $\pm$ 3.76, infected = 26.14 $\pm$ 4.46) spent in the open arm. Independent t-test $|t|_{21} = 0.563$; $p = 0.579$; $n = 11$ control, 12 infected; Power (β) = 0.054; Cohen's $d = 0.236$. There was no significant effects of infection on entry (control = 36.65 $\pm$ 1.53, infected = 36.00 $\pm$ 1.52; independent t-test $|t|_{21} = 0.302$; $p = 0.766$; Power (β) = 0.05; Cohen's $d = 0.126$), and time (control = 52.47 $\pm$ 3.60, infected = 45.55 $\pm$ 5.50; independent t-test $|t|_{21} = 1.302$; $p = 0.314$; Power (β) = 0.062; Cohen's $d = 0.435$) spent exploring the closed arm.

Risk assessment behaviour was measured by quantifying the mean number of head dips (Figure 3.2B; control = 18.63 $\pm$ 1.86, infected = 19.08 $\pm$ 3.05; independent t-test $|t|_{21} = 0.122$; $p = 0.904$; Power (β) = 0.052; Cohen's $d = 0.052$).

Control and infected animals explored the anxiogenic centre of the open field test in a similar manner. There was no significant difference between control and infected animals in time spent exploring the centre of the OFT. Independent t-test $|t|_{20} = -1.333$; $p = 0.197$; Power (β) = 0.276; Cohen's $d = 0.435$. Similarly, no significant difference was observed when time spent along the wall was compared between control and infected animals. Independent t-test $|t|_{21} = -1.761$; $p = 0.093$; Power (β) = 0.564; Cohen's $d = 0.745$. Conversely, infected females spent significantly less time in the corner of the OFT arena (Figure 3.3A; Independent t-test $|t|_{21} = 2.132$; $p = 0.045$; Cohen's $d = 0.897$) and made significantly more entries into the centre of the arena (Independent t-test $|t|_{21} = -2.176$; $p = 0.041$; Cohen's $d = 0.919$; Figure 3.3B). Infection did not affect locomotion of

female rats. Total distance travelled in the OFT was comparable between control and infected animals (Figure 3.3C; mean $\pm$ SEM' control = 2.9115 ± 0.89701 , infected = 2.7681 ± 0.95777 ; Independent t-test $|t|_{21} = 0.370$; $p = 0.715$; Power (β) = 0.101; Cohen's $d = 0.155$).

*Please see the next page for the legend

Figure 3.1: Infected female rats show increased approach behaviour in response to cat odour compared to control females.

Panel A shows a plot of time spent exploring both rabbit and cat odor. Panel B and C is a box plot representation (with mean and SEM) of the time spent by control and infected female rats in exploring cat odour. (Trial duration = 1200s; t-test $*p \leq 0.05$; $n = 12$ infected + 11 control). Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution.

Figure 3.2: Infection does not result in more anxiogenic behaviour as measured by the EPM

Panel A shows mean percentage number of entries and time spent by both control and infected animals exploring the open arm of the EPM. Panel B is a graph of mean head dips of control and infected animals in the open arm of the EPM. There was no significant difference between the two groups suggesting infection does not affect risk assessment behaviour. Mean $\pm$ SEM data plotted for all graphs. (Trial duration = 300s; n = 12 infected + 11 control).

*Please see the next page for the legend.

Figure 3.3: Infection does not hinder locomotion or increase anxiety in the female rats tested in the OFT

Panel A shows mean time spent by both control and infected animals exploring the centre, corners and wall of the OFT. There was no statistical difference between control and infected animals in time spent in the centre and along the walls of the OFT. Infected females spent significantly less time in the corner of the OFT apparatus, $p = 0.045$. Panel B is a graph of mean number of entries in to the centre of the OFT apparatus by control and infected females. Infected females had significantly more entries into the centre compared to controls, $p = 0.041$. Panel C is a graph of total distance travelled in the apparatus. Infection did not affect locomotion in the OFT apparatus. (Trial duration = 300s; $n = 12$ infected + 11 control).

Measurement of plasma levels of estradiol, progesterone and corticosterone

Plasma concentration of hormones was determined using commercially available EIA kits. There was no statistical difference in the plasma level of estradiol in control and infected females (Figure 3.14A; $|Z| = -1.215$, $p = 0.224$, Mann-Whitney test; Power (β) = 0.05; Cohen's $d = 0.018$). Similarly, control and infected females did not differ in the levels of progesterone in the plasma (Figure 3.4B; $|Z| = -0.635$, $p = 0.525$, Mann-Whitney test; Power (β) = 0.176; Cohen's $d = 0.418$). Measurement of plasma corticosterone showed no difference between control and infected females (Figure 3.4C; $|Z| = -0.927$, $p = 0.354$, Mann-Whitney test; Power (β) = 0.117; Cohen's $d = 0.355$).

Figure 3.4: Infection does not alter plasma estradiol, progesterone and corticosterone levels. Panel A: Infection had no effect on blood plasma levels of estradiol. Panel B: Infection did not alter progesterone levels in females. Panel C: Similarly for corticosterone, there was no statistical difference between control and infected females. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; $n = 18$ sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

Toxoplasma gondii infection did not make infected females more male-like.

Toxoplasma gondii infection did not affect AVP mRNA levels in the MeApd of test subjects (Figure 3.5B; $Z = -0.662$, $p = 0.508$, Mann-Whitney test; Power = 0.097; Cohen's $d = 0.291$). Similarly, testosterone measurement did not reveal a significant difference in plasma levels of testosterone (Figure 3.5A; $Z = -1.127$, $p = 0.260$, Mann-Whitney test; Power (β) = 0.425; Cohen's $d = 0.756$).

Probability of a type 2 error was estimated by calculating the power of the statistical test. Aside from the open field test and the testosterone hormone measurement, all endpoints that were not statistically significant had a β value below 0.2 reflecting a low probability of making a type 2 error. In the open field test and testosterone assay, β values were above 0.2. It is therefore difficult to conclusively determine equivalence between two experimental groups, although type 1 error probabilities remained statistically non-significant.

Figure 3.5: Infection did not result in more maleness in females. Panel A: Infection did not result in an increase in testosterone production in females. Panel B: AVP mRNA abundance did not increase post infection in females. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

Interpretation

Previous reports in both male rats [94, 136] and female mice [94] have shown increased approach behaviour in *Toxoplasma* infected animals compared to uninfected controls. A previous report in female rats infers increased approach to cat odour by demonstrating decreased approach to rabbit odour [195]. This is the first report demonstrating increase in approach behaviour post *Toxoplasma* infection in female rats. Golcu *et. al.*, [195] tested females across all stages of the estrus cycle. Their experiment exposed both control and infected rats to cat odour (predator) and rabbit odour (non-predatory large animal). As expected, uninfected female rats displayed increased approach to rabbit odour. This effect appears to be hormone dependent and manifests only in the proestrus/ diestrus stage of the estrus cycle. Infected female rats lost this preference for rabbit odour. This response was also hormone dependent and occurred in all stages of the estrus cycle except the estrus phase. Congruent to the hormone dependence of observed behaviours, infected female rats had significantly increased levels of progesterone compared to controls [195].

The prior demonstration by Golcu and his colleagues however has several limitations. The paper failed to show a clear preference for predator odour in infected animals as has been demonstrated in males [94, 136]. Rather it shows infected animals display a loss of preference for rabbit odour and infers this to mean an increased preference for cat odour. They also had a very small sample size for measurement of progesterone (n=3 control and 4 infected). Our data demonstrates a direct increase in approach behaviour to cat odour in *Toxoplasma* infected female rats compared to uninfected controls. Infected female rats were also observed to spend significantly more time exploring cat odour than rabbit odour. The reverse is true for uninfected controls that spend less time exploring cat odour

compared to rabbit odour (Figure 3.1). Our paradigm is the first report of a direct increase in approach behaviour to cat odour post *Toxoplasma* infection in female rats.

The central idea of behavioral manipulation is that parasites only alter behaviours that affect their transmission and survival [41, 214]. Some sceptics have raised the argument that increased approach to cat odour observed may simply be impairment in the general defensive behaviour, a side effect of infection or a symptom of general sickness behaviour rather than a targeted parasite manipulation [214].

Several publications have reported conflicting accounts concerning the effect of *Toxoplasma* infection on general anxiety in both males and females. Some publications report an increase in anxiety [215, 216] and locomotion [217] post infection. Other publications report a decrease in anxiety [155, 218] and locomotion [165, 219] in infected animals. Our observations demonstrate that *Toxoplasma* infection in female rats does not affect general anxiety behaviour of the female rats. This is in line with a report that saw no change in generalized anxiety behaviour as measured by the EPM [220] (Figure 3.2). Infected animals demonstrated a reduction in thigmotaxis, manifesting as a decrease in time spent in corners of the open field compared with control animals. This decrease did not manifest as an increase in exploration of the centre of the OFT maze (Figure 3.3). Similarly, infected females recorded a significant increase into the centre of the maze compared with control cohorts. This increase in entries however did not manifest as an increase in time spent in the centre. A holistic consideration of results from the EPM and OFT experiments suggests infection had no effect on anxiety in our paradigm. Similarly, infection had no effect on locomotion suggesting infection does not result in motor impairment.

The substrates that form the testosterone-AVP hypothesis are strongly gender dimorphic with a significant male bias [134, 184, 210, 212, 213]. What this suggests is that behavioural manipulation by *Toxoplasma* is gender dimorphic and only occurs in males. But this is not the case. As has been demonstrated in mice [94] and in this thesis, *Toxoplasma* infection induces approach behaviour in females in response to cat odour. This raises the important question of how the parasite is achieving this. On one hand, it is possible that the parasite is employing a completely different and unrelated pathway in females to drive this atypical behaviour. Alternatively, the parasite may be employing a parallel pathway to the AVP-testosterone pathway in females. In other words, AVP and testosterone may represent the male delimited version of a non-gender problem, like current versus future trade-off. Negotiating approach-and avoidance to stimuli occurs in both sexes. The only difference is found in the substrates that are important for the negotiating. It is clear that *Toxoplasma* is manipulating this negotiation for its benefit. What remains unclear is what substrates are involved in the case of females.

There is always the possibility that *Toxoplasma*-mediated behavioural manipulation in females is as a result of increased “maleness” of infected females. In other words, there is a small likelihood that *Toxoplasma* infection masculinizes female rats and employs the same substrates to manipulate behaviour. In this case, infected female rats will be expected to have significantly increased levels of plasma testosterone as well as increased AVP mRNA transcription in the MeApd. The results presented in this chapter argue against this idea. There was no significant increase in the AVP mRNA levels of infected female rats compared to their control cohorts. Similarly, plasma testosterone did not increase in infected female rats compared to controls (Figure 3.5). Rather surprisingly,

infected females presented with reduced testosterone production compared to control female rats. This decrease was however not statistically significant. This argues for a gender dimorphic mechanism underlying *Toxoplasma*-induced behavioural manipulation.

Another limitation of the data presented by Golcu and colleagues was the failure to discuss the progesterone increase in terms of biological significance. Increase was about 1500 pg/ ml (1.5 ng/ ml) on average from control to infected cohorts. Although statistically significant, it remains unclear if a change of this magnitude is biologically significant. Previous studies have reported serum concentrations of progesterone from about 2 ng/ ml to 40 ng/ ml, across the different stages of the estrus cycle [221-224]. In control female rats, all animals recorded approximately 2.0 ng/ ml. This rose to about 3.5 ng/ ml in infected females. This raises questions about the biological significance of the observed increase. Our data shows no statistical difference in the concentration of progesterone between control and infected females. There was also no statistical difference in estradiol concentration between control and infected female rats (Figure 3.4). This suggests ovarian steroids do not play a role in mediating the behavioural change.

Our data shows no statistical difference in plasma concentration of corticosterone measured in both control and infected females (Figure 3.4). This contrasts with previous reports that noted significantly lower concentrations of corticosterone both at baseline prior to cat odour exposure and after cat odour exposure in male rats [225]. Several factors may account for the discrepancy including time of sample collection and possibly different sensitivities of enzyme immunoassay methods. Alternatively, the above mentioned study was performed exclusively in male rats, making it possible that *Toxoplasma* infection does not alter corticosterone levels in female rats.

We have also demonstrated that infection does not affect general anxiety as measured by both the EPM and OFT. Our data argues for specificity of the infection effect. The constraint of infection effect to the response to cat odour suggests that infection does not result in a deficit in general defensive behaviour. This is similar to observations in males [94] and challenges the idea that atypical approach behaviour is as a result of disruption in general defense mechanisms.

In light of the gender-dimorphism of the testosterone-AVP model to explain effect of *Toxoplasma* infection in male rats [134, 184], it is important to understand the proximate mechanism of *Toxoplasma*-mediated behavioural manipulation in female rats. This will help in developing a unified model of proximate mechanisms underlying the increased approach behaviour and improve our understanding of behaviour manipulation in the *Toxoplasma*-rat association.

Chapter 4

The oxytocin system is atypically activated in female rats exposed to cat odour post *Toxoplasma gondii* infection

Aim

In the previous chapter, I demonstrated that *Toxoplasma gondii* infection causes loss of aversion in female rats, akin to what has been widely observed in male rats. I also presented observations about endocrine milieu of infected and non-infected female rats. The presence of behavioral manipulation in female hosts raises some very interesting questions. Proximate mechanisms of this behavioral change have been historically studied in male rats, where it has led to a framework built upon sexually dimorphic neural nodes. In that backdrop, I investigate if neural mechanisms analogous to those proposed in males are recruited during infection of female rats.

Background

The *Toxoplasma gondii*-male rat association has been extensively studied as an exemplar of the idea that parasites can manipulate the behaviour of their hosts. Three different proximate mechanisms have been proposed to mediate this behaviour manipulation. Namely, the tropism hypothesis, the dopamine hypothesis and the testosterone-AVP hypothesis (see [163] for a review). The most parsimonious of the models proposes that the parasite increases testosterone synthesis after invading the male testes [134]. Increased testosterone and/ or its metabolites then cross the blood-brain barrier (BBB). The steroids subsequently act on the posterior dorsal nucleus of the medial amygdala (MeApd) and result in the hypomethylation of the promoter region of the arginine vasopressin (AVP) gene, increasing its transcription [184]. AVP neurons in the MeApd participate in the perception of sociosexual cues in infection-naïve animals [185, 226]. Thus an increase in AVP post-infection has been proposed to result in an atypical “leakiness” between neural circuits mediating response to defensive and sociosexual odour cues [182]. This leakiness results in a shift from avoidance to approach of predator odours [227].

Interestingly, the mechanism detailed above encompasses substrates that are highly dimorphic between genders. Testosterone is primarily produced by male testes, although small amounts are produced by ovaries and/or adrenals in females [228]. Similarly, medial amygdala in male rodents is almost twice in volume compared to females. Hypomethylation of arginine vasopressin is dependent on male testosterone, as shown by its reversal through castration and reinstatement through exogenous testosterone [229]. The levels of extra-hypothalamic arginine vasopressin itself are dimorphic with greater expression and recruitment in males rather than females [230]. Thus the sexually

dimorphic nature of the proximate mechanism raises the possibility that effect of *Toxoplasma* infection on rodent aversion to cat odour cues is gender specific. Contrary to this prediction, *Toxoplasma* infection has been reported to alter the behaviour of both female rats [195] and female mice [94]. The question then is how *Toxoplasma* effects this behaviour change in females.

I started engaging with this dilemma by asking what the mechanistic niches are, that testosterone and AVP are proposed to fill in the model built during historical work on male rats. It is true that both testosterone and AVP, as individual molecules, are preferential hallmarks of male behavior. Yet a broader view can also be proposed that deemphasizes sexual dimorphism and instead focus on trade-offs and brain-hormone interactions.

At its core, the testosterone-AVP hypothesis represents an interaction between a peripheral reproductive hormone and a nonapeptide (9 amino acid peptide). This also represents the idea that the balance between approach and avoidance, or the magnitude of the fear, is a dynamic equilibrium rather than a fixed point. The peripheral steroid hormone and brain nonapeptide communicate to shift the magnitude of fear. Reducing it to this simple model allows candidate molecules possibly involved in driving the behaviour in females to be determined. Testosterone is the main reproductive hormone in males [203] and is produced in relatively minute quantities in females. In females, estradiol and to a lesser extent progesterone are the major female reproductive hormones [222]. Like testosterone, estrogen and progesterone are anxiolytic hormones that affect female response to stress or fear conditions [221-223]. Estradiol has been demonstrated to interact with oxytocin to mediate social behaviours in female rodents [231, 232].

Oxytocin (OT) is a nonapeptide very similar to vasopressin in structure. The two nonapeptides differ by only two amino acid residues [233]. OT is expressed in the female brain in a gender-dimorphic manner, similar to the expression of related nonapeptide arginine vasopressin in the male brain [94, 203, 222]. Oxytocin, along with vasopressin have been implicated in various social behaviours including social interaction [234], social recognition [235] and anxiety [236, 237]. It is mainly produced by magnocellular neurons in the PVN and SON of the hypothalamus, transported to the posterior pituitary via axonal projections and subsequently secreted into the periphery [238]. In addition, there is also dendritic release of OT from the PVN that acts locally and in other brain regions via volume transfer in the CSF [239]. There is well documented extrahypothalamic production and release of AVP in areas like the medial amygdala and BNST [208, 210]. In the case of OT, there are much fewer extrahypothalamic projections [210]. It is thus generally accepted that the bulk of OT behavioural effects hinge on OT receptor (OTR) distribution and density [240].

I built two specific aims in the backdrop of the model presented above. In view of the necessary role of reproductive steroids in mediation of the behavioral change in males, I investigated if the female reproductive steroids were necessary for host behavioral phenotypes post-infection [11]. In view of the central role of nonapeptides in mediation of the behavioral change, I also investigated if *Toxoplasma gondii* infection enhanced the abundance of oxytocin in the female brain.

Methods and Techniques

Animals and Infection

Adult female rats approximately 7-8 weeks (49-56days) old were used for all experiments. Animals were procured from InVivos animal rearing facility, Singapore and housed two per cage at the NTU vivarium. Animals were provided food and water *ad libitum* under controlled temperatures (24-26°C) and humidity (60-70%) in a 12 hour light on/ light off cycle (light on at 7am). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technology University. All animals were allowed to acclimatize to the facility for a minimum of two days before being subjected to any experiments. A Prugniald strain of *Toxoplasma gondii* with both Luciferase and GFP inserts (PA7) was used. This strain is similar to the strain used for prior work with male rats ([95, 184, 185]. The parasites were maintained in vitro in human foreskin fibroblast (HFF) monolayers and harvested using syringe lysis. Female were randomly assigned in experimental groups and injected with either *Toxoplasma gondii* (5×10^6 tachyzoites suspended in 500 μ l of phosphate buffered saline (PBS); i.p.) or the vehicle (PBS) alone. All rats were weighed weekly. Absence or presence of overt sickness behavior was noted every week. Experiments were conducted from the 7th week of infection onwards.

Ovariectomy and Estradiol Supplementation

Surgery was performed using aseptic techniques under isoflurane anesthesia (2.5% gaseous isoflurane with pure O₂). After placing animals in ventral recumbency, ovaries were approached bilaterally using incisions caudal to ribcage. Ovaries were excised after suturing fallopian tubes. The wound was subsequently sutured. Animals assigned to sham

surgery group were operated in a similar manner, except that fallopian tubes were not tied and ovaries were not excised.

Silastic capsules containing estradiol were prepared by packing 5mm of tubing with pure estradiol (total length of capsule = 15 mm). This length of silastic capsules resulted in constant delivery of estradiol for 60 days, creating constant estrus as determined by the presence of cornified and epithelial cells in vaginal lavage. Animals undergoing estradiol supplementation received one silastic capsule subcutaneously immediately after ovariectomy. Animals receiving placebo treatment received an empty silastic capsule of similar length.

Animals were given pre-operative prophylaxis antibiotic (Baytril 10mg/kg, sc; Bayer) and pain relief (Carprofen 5 mg/kg, sc; Pfizer). After surgery, animals were housed singly for >3 days with supplemental pain relief daily (Carprofen 5 mg/kg, sc). Animals were re-housed with prior cage-mates once wound healing was visually confirmed. At least one week elapsed between surgery and start of further experiments.

Determination of estrus

Vaginal lavage was collected under mild restraint using 10µl of buffered saline. Stage of the estrus cycle was determined using previously published criteria under 200X (total) magnification. Specifically, vaginal estrus manifested as preponderance of cornified epithelial cells.

Cat Odour Avoidance Assay

Aversion to cat odor was quantified in a rectangular arena with dimensions (76 x 9 cm). The entire length of this arena was used during testing. Cat odour was presented in one

extreme of the arena (2ml bobcat urine soaked on a gauge of towel; Maine Outdoor Solutions, ME, USA; trial duration = 20 minutes). Animal was placed at the opposite end of the arena and allowed to move freely within the confines of the arena. Animals were habituated to the arena for 3 consecutive days, 10 minutes each day. Exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA). The arena was divided into 2 halves and the amount of time spent exploring the half containing the cat odour soaked towel was used as a measure of innate aversion to cat odour.

Quantitative PCR

Brain tissue was harvested post-decapitation and flash-frozen in liquid nitrogen. The tissue was stored at -80°C till further analysis. Subsequently, mRNA abundance was quantified for the nonapeptide oxytocin (OT) in the paraventricular nucleus of hypothalamus (PVN), medial preoptic area (MPOA), supraoptic nucleus (SON), bed nucleus of the stria terminalis (BNST) and posterodorsal medial amygdala (MeApd). Oxytocin receptor (OTR) mRNA abundance was quantified in the MeApd, BNST, PVN and MPOA. The standard method based on SYBR green dye was used as described before [134, 184]. PCR cycles needed to reach a pre-determined fluorescence threshold at early linear phase of the amplification were determined (Ct, threshold cycle number). Greater mRNA abundance in this assay results in lower Ct values. Ct values for OT and OTR were normalized by subtracting corresponding Ct values of 3 housekeeping genes (HPRT, β -actin and 18 sRNA). Three technical replicates were used for each determination. The median coefficient of variation between technical replicates was $\leq 15\%$. Please refer to Annexe 2 for primer sequences and associated PCR efficiencies.

Statistics

Student's t-test was used to determine statistical significance between control and infected animals. Analysis of variance (ANOVA) was used to determine main effects and interactions of the infection and the surgical status. In view of non-normal distribution, Mann-Whitney test was used to determine statistical significance.

*Please refer to the Annexe for a more detailed methodology.

Results

Ovariectomy did not rescue the effects of infection

A two-way ANOVA revealed significant effect of infection on time spent exploring the cat odor stimulus $F_{(1,48)} = 5.410$, $p = 0.024$. There was, however, no effect of surgery $F_{(2,48)} = 0.819$, $p = 0.447$ or the interaction of the two factors (infection * surgery $F_{(2,48)} = 1.567$, $p = 0.219$; Figure 4.2). Sham infected animals showed the expected reduced aversion in response to cat odour when they spent 561 ± 56.59 seconds in the vicinity of cat odour. This was greater than the 385.13 ± 24.47 seconds spent by control sham animals in the vicinity of cat odour (* $p = 0.013$, LSD post hoc test; Cohen's $d = 1.426$). Ovariectomised infected animals also spent more time in the vicinity of cat odour 626.56 ± 43.57 seconds, as compared to control cohorts 417.56 ± 101.33 . This however did not reach significant levels. ($p = 0.076$, LSD post hoc; Cohen's $d = 0.893$; Power = 0.474). Ovariectomised and estradiol supplemented control rats spent almost equal time in the vicinity of cat odour as infected cohorts. There was no significant difference between control and infected cohorts in the estradiol-supplemented group (control = 560.80 ± 64.47 , infected = 556.80 ± 70.84 ; $p = 0.967$, LSD post hoc; Cohen's $d = 0.019$; Power = 0.05).

Infection increased abundance of oxytocin receptor (OTR) mRNA but not OT mRNA in posterodorsal medial amygdala (MeApd) of sham animals.

Infected sham animals showed increased expression of OTR mRNA in the MeApd compared to their control cohorts (Figure 4.3B; $|Z| = -2.309$, $p = 0.021$, Mann-Whitney test; Cohen's $d = 1.43$). The effect was so pronounced, the 75th percentile of the infected sham was below the 25th percentile of the control sham animals. OT mRNA abundance

increased in infected females compared to control females. It however failed to reach statistical significance (Figure 4.3A; $|Z| = 1.810$, $p = 0.07$, Mann-Whitney test; Power = 0.527; Cohen's $d = 0.96$).

Infection marginally increased abundance of oxytocin (OT) but not OTR mRNA in paraventricular nucleus (PVN).

Infected sham animals had a marginally higher abundance of OT mRNA in the PVN compared to their control cohorts (Figure 4.4A; $|Z| = -1.898$, $p = 0.058$, Mann-Whitney test; Power = 0.498; Cohen's $d = 0.92$). mRNA abundance of OTR in the PVN did not differ between control and infected females (Figure 4.4B; $|Z| = -1.192$, $p = 0.233$, Mann-Whitney test; Power = 0.231; Cohen's $d = 0.577$).

Infection marginally decreased OTR mRNA but did not affect OT mRNA in the bed nucleus of the stria terminalis (BNST).

Control sham animals had a marginally lower abundance of OTR mRNA in the BNST compared to their infected cohorts (Figure 4.5B; $|Z| = -1.828$, $p = 0.068$, Mann-Whitney test; Power = 0.682; Cohen's $d = 1.17$). Infection did not affect abundance of OT mRNA in the BNST (Figure 4.5A; $|Z| = -0.439$, $p = 0.661$, Mann-Whitney test; Power = 0.154; Cohen's $d = 0.48$).

Infection does not affect the abundance of OT mRNA in the supraoptic nucleus (SON) and both OT and OTR mRNA abundance in the medial preoptic area (MPOA).

mRNA abundance of OT did not differ between control and infected females in the SON (Figure 4.4C; $|Z| = -0.486$, $p = 0.627$, Mann-Whitney test; Power = 0.069; Cohen's $d = 0.19$). Similarly, mRNA abundance of OT and OTR in the MPOA did not differ between

control and infected females (OT: Figure 4.6A; $|Z| = -0.927$, $p = 0.354$, Mann-Whitney test; Power = 0.105; Cohen's $d = 0.32$; OTR: Figure 4.6B; $|Z| = 0.000$, $p = 1.000$, Mann-Whitney test; Power = 0.075; Cohen's $d = 0.23$).

Measurement of animal weight

Animal weight was monitored once a week for 6 weeks starting from the day of infection.

A repeated measures ANOVA showed surgery $F_{(2,50)} = 46.145$, $p < 0.0001$ but not infection $F_{(1,50)} = 0.081$, $p = 0.777$ or an interaction between infection and surgery $F_{(2,50)} = 1.218$, $p = 0.305$ had an effect on weight gain (Figure 4.1).

Figure 4.1: Mean weights of females. Panel A: Repeated measures ANOVA did not show any difference between any of the groups used. A post hoc planned comparison did not show any difference between control and infected sham cohorts. Y-axis represents the mean weight in grams (g) of animals. Each point in the line graph denotes the mean weight of all females in that group for that week. Panel B: Mean weight gain (week 6 – week 1) for animals in each group.

Figure 4.2: Ovariectomy did not rescue the effects of *Toxoplasma gondii* infection. Y-axis represents the percentage time spent in the vicinity of cat odour. x-axis represents control and infected cohorts of sham, ovariectomised and estradiol supplemented groups. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham and ovariectomised (9 control, 9 infected), 20 estradiol supplemented (10 control, 10 infected); trial duration = 1200s).

Figure 4.3: OT/ OTR expression in MeApd. Panel A: *Toxoplasma gondii* infection marginally increased oxytocin (OT) abundance. mRNA isolated from the posterior dorsal medial amygdala (MeApd) of infected sham females required fewer cycles to reach a predetermined threshold for OT. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Panel B: *Toxoplasma gondii* significantly increases OT mRNA abundance in the MeApd. Ordinate represents relative number of cycles required when using OTR primer to when using multiple housekeeping genes primers. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

*Please see legend on next page.

Figure 4.4: OT/ OTR expression in hypothalamic nuclei. Panel A: *Toxoplasma gondii* marginally increases OT mRNA abundance in the PVN. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Panel B: *Toxoplasma gondii* infection does not alter OTR mRNA abundance in the PVN. Ordinate represents relative number of cycles required when using OTR primer to when using multiple housekeeping genes primers. Panel C: *Toxoplasma gondii* infection does not alter OT mRNA abundance in the SON. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping gene primers. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

Figure 4.5: OT/ OTR expression in BNST. Panel A: *Toxoplasma gondii* infection does not alter OT mRNA abundance in the BNST. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Panel B: *Toxoplasma gondii* infection marginally decreases OTR mRNA abundance in the BNST. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

Figure 4.6: OT/ OTR expression in MPOA. Panel A: *Toxoplasma gondii* infection does not alter OT mRNA abundance in the MPOA. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Panel B: *Toxoplasma gondii* infection does not OTR mRNA abundance in the MPOA. Ordinate represents relative number of cycles required when using OT primer to when using multiple housekeeping genes primers. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution; n = 18 sham (9 control, 9 infected) animals each; * denotes $p \leq 0.05$; Mann-Whitney test).

Interpretation

The experiment pertaining to ovariectomy was built up on the premise that similar to male rats, behavioral change with females will be dependent on gonadal steroids. I viewed this hypothesis as worthy of experimental follow-up due to the extensive data on the anxiolytic properties of estradiol [221, 223, 241, 242]. Ovariectomy however failed to rescue this behaviour. Infected ovariectomised female rats still spent more time in the vicinity of cat odour compared to controls. Inter-group differences in ovariectomised females did not reach low probabilities of type 1 errors in order to term it as statistically significant. Yet, the overall trend and effect size closely mirrored gonadally-intact sham-operated females.

Figure 4.2 shows that supplementing ovariectomised females with supraphysiological levels of estradiol did not in any way enhance the reduced aversive response post infection. Both control and infected females from the estradiol group spent a lot of time in the vicinity of cat odour. It is believed that since estradiol in itself is a well-known anxiolytic, administering supraphysiological levels may have caused the females to hit a ceiling effect in their loss of aversion making it difficult for infection to move this loss of aversion any further. Another limitation of the design was failure to supplement progesterone prior to testing. In light of the anxiolytic nature of progesterone and its complementarity with estrogen, it might have potentiated steroid effects if supplemented. In short, we assume that a simple ablation of ovaries along with its corresponding steroids is not enough to rescue parasitic behavioural change. This assumption is tinged with the limitation that p values in operated groups were close to 0.05 but were not less than 0.05. A caution is thus required in this interpretation. This data also shows that supplementation

with estradiol reduces aversion to cat odor. It is not possible to answer if such supplementation reinstates infection-like behavioural change because high reduction in fear observed in both experimental groups might have masked inter-group differences due to ceiling effect. These limitations are common and frequent in lesion/supplementation studies in the prior literature.

The interaction between the gonads (testosterone from testes) and the brain (AVP in the MeA) [184] is shown to be responsible for the change in behaviour observed in male rats post infection. Having been unsuccessful in teasing out the mediators from the peripheral point of view in the case of females, we sought to approach the problem from another perspective: the brain. The loss of aversive behaviour observed post infection is by extension, an increase in affiliative or approach type behaviour. Extensive work by Choleris *et al* [243, 244], has shown that OT and OTR are important for social recognition and other approach and affiliative behaviours in females. Choleris and colleagues subjected estrogen receptor alpha knockout mice, estrogen receptor beta knockout mice and oxytocin knockout mice to social recognition behavioural paradigm and demonstrated from their results that estrogen receptor alpha and beta as well as oxytocin and the oxytocin receptor are important for social recognition in female mice. With that in mind, we decided to assay for the transcript levels of oxytocin (OT) and oxytocin receptor (OTR) levels in the PVN, MeApd, BNST and MPOA in control and infected females that were sham operated in the experiment above. We also assayed for the levels of OT mRNA transcript levels in the SON to serve as an internal control.

We observed an upregulation of OTR transcript levels in the MeApd of infected females relative to uninfected females (Figure 4.3). Previous reports have suggested that the

distribution and density of OTR is a measure of OT/OTR activity [240]. This is because very little OT is produced by extrahypothalamic neurons and most central OT is produced in the PVN and transported via volume control [210]. The marginal increase in PVN OT observed in our experiments supports this assertion (Figure 4.4). This suggests a role for the OT/OTR system in mediating the observed behaviour manipulation.

The OT/OTR system is a well-documented mediator of social behaviour in females, often promoting prosocial behaviour [236, 243, 245]. This is similar to the role of extrahypothalamic AVP in males [234, 246]. This suggests that *Toxoplasma* employs parallel gender dimorphic pathways in mediating behaviour manipulation of rodents. Another way to think about this is to consider that *Toxoplasma* induces increased approach behaviour to an otherwise aversive stimulus in both males and females. This suggests the underlying mechanism in both males and females may involve the pathway involved in sociosexual behaviours. House and colleagues have previously demonstrated that infected males perceive cat odour stimulus as a sociosexual cue [182]. Based on our data with OT/OTR and what is known from literature [243, 244], it is likely the same occurs in infected females.

Toxoplasma infection has been demonstrated to specifically alter male reproductive behaviour and “hijack” the sexual pathway. This raises the possibility that infection may “hijack” sociosexual behaviour in females in a similar manner. It is yet unclear what this behaviour may be. However, understanding this may provide several insights into downstream mediators of this behaviour.

Chapter 5

Experimental attempts to delineate necessity or sufficiency of oxytocin system for host behavioural change in female rats post-infection

Aim

I presented evidence in chapter 3 that *Toxoplasma* infection results in loss of aversion towards cat odor in female rats. In a subsequent chapter I explored the role of ovarian steroids, showing that loss of these steroids was insufficient to rescue parasitic behavioural change. At the same time, *Toxoplasma* infection increased abundance of mRNA transcripts for oxytocin (OT) and oxytocin receptor (OTR) in brain regions known to be involved in approach behaviors. These observations led me to a follow-up experiment that tested if the OT/OTR system was necessary or sufficient for behavioural change in female rats post-infection. I summarize results of these experiments in the present chapter.

Background

Prior work in males posits that “leakiness” between socio-sexual behavior and innate fear underlies behavioural change in male rats infected with *Toxoplasma* [182]. Control rats show a modular recruitment of neurons within the medial amygdala in response to cat odor exposure. Thus neurons in the postero-ventral aspect of amygdala show robust activation in response to cat odor exposure. These neurons are non-AVP reactive. In contrast AVP-containing neurons in the postero-dorsal medial amygdala remain relatively quiescent [182]. Infected animals on the other hand, show atypical activation of postero-dorsal AVP neurons: a population that is more typically recruited during exposure to sexual pheromones in male rats [182]. This atypical activation of a nucleus responsible for processing positively-valenced odours is thought to be largely responsible for approach behaviour observed in *Toxoplasma* infected males.

The parallel and modular nature of brain circuits for aversive and attractive pheromones plausibly reflect constant negotiations animals must make about reproductive opportunities and predatory challenge in their ecologies. I argue that the contours of this communication remain similar in males and females, although mediators vary depending on the molecular landscape of gendered brains. In males, *Toxoplasma* recruits a pre-existing socio-sexual circuit comprising of brain nonapeptides during the behavioral change.

Choleris [232] proposed a model of social recognition and social discrimination that involved the OT/ OTR system. She demonstrated that mice with OT knockout were deficient in social recognition. Social recognition and discrimination is important for animals to identify conspecifics from heterospecifics [231, 247]. The ability of animals to

correctly identify conspecifics allows them to avoid predators [231, 232]. Our data has previously showed that *Toxoplasma* infection results in increased OTR mRNA transcription in females (chapter 4). Coupled with the model proposed by Choleris, we hypothesized that *Toxoplasma gondii* manipulates the pathway for social recognition in females. We proposed that *Toxoplasma* caused female rats to perceive cat odour which is a heterospecific odour as a conspecific odour, and approach it. This model is a generalization of the effect observed in infected males. An infected male processes cat odour (defensive kairomone) in the same manner it will process odour from a sexually receptive female [95, 154, 182]. In other words, an infected male rat perceives a heterospecific odour as a conspecific odour as well.

In addition to exploring the ability of *Toxoplasma* to compromise the social recognition mechanism in females, we sought to determine the necessity of the OT/ OTR mechanism to mediate this behavior. We hypothesized that use of an OTR antagonist L-368899 would block the action of OT on OTR thus preventing the expression of the atypical behaviour post *Toxoplasma* infection.

This chapter will be presented a little differently to make it easier to understand. Data will be presented in a chronological manner that reflects the order in which tests were conducted. This is necessary because the chapter contains some negative data and an attempt at troubleshooting to understand what did not work.

Methods and Techniques

Animals and Infection

Adult female rats approximately 7-8 weeks (49-56days) old were used for all experiments. Animals were procured from InVivos animal rearing facility, Singapore and housed two per cage at the NTU vivarium. Animals were provided food and water *ad libitum* under controlled temperatures (24-26°C) and humidity (60-70%) in a 12 hour light on/ light off cycle (light on at 7am). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technology University. All animals were allowed to acclimatize to the facility for a minimum of two days before being subjected to any experiments. A Prugniald strain of *Toxoplasma gondii* with both Luciferase and GFP inserts (PA7) was used. This strain is similar to the strain used for prior work with male rats [95, 184, 185]. The parasites were maintained in vitro in human foreskin fibroblast (HFF) monolayers and harvested using syringe lysis. Female rats were randomly assigned in experimental groups and injected with either *Toxoplasma gondii* (5×10^6 tachyzoites suspended in 500 μ l of phosphate buffered saline (PBS); i.p.) or the vehicle (PBS) alone. All rats were weighed weekly. Absence or presence of overt sickness behavior was noted every week. Experiments were conducted from the 7th week of infection onwards.

Determination of estrus

Vaginal lavage was collected under mild restraint using 10 μ l of buffered saline. Stage of the estrus cycle was determined using previously published criteria under 200X (total) magnification. Specifically, vaginal estrus manifested as preponderance of cornified and epithelial cells.

Treatment with Antagonist

On cat odour avoidance day, 0.9% saline (vehicle) or 1mg/ kg of L-368899 (antagonist) was administered 30 minutes before testing to control and infected females depending on group.

Cat Odour Avoidance Assay

Aversion to cat odor was quantified in a rectangular arena with dimensions (76 x 9 cm). The entire length of this arena was used during testing. Cat odour was presented in one extreme of the arena (2ml bobcat urine soaked on a gauge of towel; Maine Outdoor Solutions, ME, USA; trial duration = 20 minutes). Animal was placed at the opposite end of the arena and allowed to move freely within the confines of the arena. Animals were habituated to the arena for 3 consecutive days, 10 minutes each day. Exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA). The arena was divided into 2 halves and the amount of time spent exploring the half containing the cat odour soaked towel was used as a measure of innate aversion to cat odour.

Social Recognition (Habituation/ Dishabituation Paradigm)

Individual female rats were tested 5 times (trial 1 to 5) in a rectangular apparatus (56cm x 39cm x 28cm). Stimulus juvenile rats were placed in a plastic holder (16cm x 10cm x 7cm) and covered with a wire mesh. The rectangular apparatus containing the stimulus rat was placed in one corner of the test apparatus. Each trial lasted for 5 minutes and conducted at 15 minute intervals. The same stimulus juvenile rat was presented to the test female in trial 1-4. In test 5, a novel stimulus rat was presented to the test female. In the

interval between trials, rats were left undisturbed in the test apparatus. Prior to testing, each experimental female was habituated to the testing apparatus for 10 minutes. Stimulus animals were also habituated to the holding apparatus for 10 minutes before testing began.

Effect of Isoflavone levels of feed on cat odour avoidance assay

20 female rats were ovariectomised and randomly placed into two groups. One group was fed normal animal house feed with isoflavone levels ≥ 300 ppm. The other group was maintained on a low isoflavone (Labdiet 5v5r, 50ppm) diet. Females were tested in the cat odour avoidance assay after 10 days.

Luciferase Assay

2×10^5 of MCF7 cells were cultured into a 6 well plate overnight. Once cells are approximately 60% confluent they were transfected with luciferase plasmid construct and incubated for approximately 24 hours. Cells were then treated with serum from ovariectomised females for 24 hours. Treated cells were treated with reporter lysis buffer to lyse the cells and dislodge them from the dish. The solution was collected and centrifuged at 14000rpm at 4°C for 15 min. 20µl of supernatant from each sample was added to each well in a 96 well plate. 40µl of LARII reagent was added to each well containing a sample. BSA standards were added to the plate. 200µl BCA working reagent was added to every well including standards. Plate was incubated at 37°C for 15 to 20mins. Absorbance was read at 562nm.

Statistics

Student's t-test was used to determine statistical significance between control and infected animals. Analysis of variance (ANOVA) was used to determine main effects and interactions of the infection and the surgical status. In view of non-normal distribution, Mann-Whitney test was used to determine statistical significance.

*Please refer to the Annexe for a more detailed methodology.

Results

Infection did not improve social recognition in females.

Repeated measures ANOVA revealed no effect of infection on social recognition in the habituation-dishabituation paradigm (Figure 5.1A; $F_{(1,26)} = 0.865$, $p = 0.361$; Effect size = 0.032; Power (β) = 0.146). A planned comparison of individual trials revealed significantly different interaction for trial 2 (control = 15.07 ± 2.2 , infected = 28 ± 5.42 ; $F_{(1,28)} = 4.402$, $p = 0.045$), trial 4 (control = 10 ± 1.79 , infected = 22.31 ± 4.81 ; $F_{(1,28)} = 5.157$, $p = 0.031$) and trial 5 (control = 36.27 ± 5.96 , infected = 20.57 ± 3.46 ; $F_{(1,27)} = 5$, $p = 0.034$). An analysis of area under the curve generated for each animal showed no difference between control and infected females (Figure 5.1B; Mann-Whitney test, $Z = -1.103$, $p = 0.27$; Cohen's $d = 0.579$; Power (β) = 0.334)

Infection did not result in increased approach behaviour to cat odour during this cohort

32 female rats were randomly assigned to groups and infected with five million *Toxoplasma gondii* tachyzoites ($n=16$) or injected with physiological saline ($n=16$). Innate aversion to cat urine was quantified during the proestrus/estrus phase (>6 weeks post-infection). A two-way ANOVA revealed no effect of infection $F_{(1,27)} = 0.06$, $p = 0.35$. There was also no effect of antagonist $F_{(1,27)} = 0.904$, $p = 0.809$. The interaction effect between infection and antagonist was however marginally significant (Infection * antagonist $F_{(1,27)} = 4.069$, $p = 0.054$; Figure 5.2A). A comparison of saline treated control and infected animals showed infected animals displayed an atypical decrease in aversion in response to cat odour when they spent 89.14 ± 22.25 seconds in the vicinity of cat odour. This was less than the 205.25 ± 57.49 seconds spent by control saline treated animals in the vicinity of cat odour. This difference was however not statistically significant ($|t|_{13} =$

1.783; $p = 0.098$, paired comparison: independent t-test; Cohen's $d = 0.949$; Power (β) = 0.47). Figure 5.2B: A repeat cat odour experiment without antagonist was run. Females infected with *Toxoplasma gondii* atypically spent significantly less time near cat odor compared to rabbit odor ($|t|_{17} = 2.703$; $p = 0.015$; Cohen's $d = 1.239$).

Figure 5.1: Habituation/ Dishabituation. Panel A shows mean time spent by both control and infected animals interacting with the stimulus animal. A repeated measure ANOVA revealed no statistical difference between the two groups in the time spent interacting with stimulus animals. Panel B represents the mean area under the curve for each group of animals. There was no statistical difference between control and infected females in this measurement.

*Please see legend on next page

Figure 5.2: Effect of antagonist on response to cat odour. Panel A shows mean time spent by both control and infected animals with the cat odour stimulus. There was no statistically significant difference in test females for the main or interacting effects of infection or antagonist. A planned comparison of control and infected females with saline was not significant. Similarly, a planned comparison of control and infected females with antagonist was also not significant. Panel B represents a repeat infection experiment with normal cycling control and infected females. Control females spent significantly more time exploring the area with cat odour compared to infected females. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution.

Isoflavone levels of animal feed affect response to cat odour stimuli

Data presented in the previous section created uncertainties about the basic model of infection-induced behavioral change. This period of experimentation unfortunately coincided with massive upheaval in institutional animal house. Since experimental treatments were coded until analysis was completed, I did not become aware of the atypicality of results until four months from start of experiment when codes were broken. I then started to carefully control for environmental factors that could have influenced experimental endpoints. As part of these efforts, I tested if variation in food chow influenced endpoints and baselines being measured. My main concern was the inclusion of soybean in the feed and the possible unintended supply of phytoestrogens from this source. I compared performance of female rats on low-isoflavone diet and the diet being provided to animals as norm. Female rats maintained on normal animal house feed spent more time on average exploring the vicinity of cat odour (45.2586 ± 3.45) compared to females on low isoflavone diets (31.28 ± 6.20) ($|t|_{18} = 1.969$; $p = 0.065$; Cohen's $d = 0.880$; Power (β) = 0.504; Figure 5.3A).

Isoflavone levels of animal feed did not affect ER alpha activity in blood

Female rats maintained on normal animal house feed had a lower ER alpha activity (700.46 ± 41.97) compared to females on low isoflavone diets (859.39 ± 72.54) ($|t|_{10} = -1.896$; $p = 0.087$; Cohen's $d = 1.095$; Power (β) = 0.475; Figure 5.3B).

A **B**

*Please see legend on next page

Figure 5.3: Effect of Phytoestrogens on ER activity. Panel A represents mean time spent by ovariectomised females on normal animal facility feed and those on Labdiet 5v5r with the cat odour stimulus after 10 days of treatment. Females maintained on normal animal house feed spent more time exploring the vicinity of cat odour compared to females maintained on Labdiet 5v5r. The difference was marginally significant. Panel B represents a luciferase assay to measure the activity of estradiol receptor alpha. There was no statistical difference between the two groups in the activity of estradiol receptor. Box plots represent the distribution between the 25th percentile, and 75th percentile. The middle line represents the median. The Y error bars represent the mean and standard error of mean (SEM) of the distribution.

Interpretation

The aim of this experiment was initially to ascertain if *Toxoplasma* was hijacking the social recognition or social discrimination pathway in female rats. In other words, did *Toxoplasma*-infected females lose their ability to differentiate conspecifics from heterospecifics? We observed from previous experiments that infected females showed an increase in OTR mRNA expression (Chapter 4). Congruently, previous work carried out by Choleris [232, 247] demonstrated that OTKO female mice were incapable of recognizing conspecifics. Based on this, our control and infected animals were subjected to the habituation-dishabituation paradigm. This was to determine if *Toxoplasma* infection improved social recognition. Furthermore, test females were to be used to determine OT/OTR necessity in a 2 x 2 experiment; half of the females from each group were to be intraperitoneally injected with L-368899, an OTR antagonist, thirty minutes before testing. The rest of the animals were treated with physiological saline thirty minutes prior to testing.

A repeated measures ANOVA analysis of our data showed no difference between control and infected females in the habituation-dishabituation paradigm. Similarly, an analysis that compared the mean area under the curve (AUC) generated for each female based on time spent with stimulus animals after all five trials also did not show any significant difference between control and infected females. However, the general trend of the data suggested that control females demonstrated better social recognition compared to infected females. An independent t-test of trial 5 comparing control and infected females showed control females spent significantly more time with stimulus animals compared to

infected females. This essentially confirmed that control females were demonstrating better social recognition (Figure 7.1).

Among saline treated females, infected females spent much less time exploring the vicinity with cat odour compared to control females. Among females treated with the OTR antagonist, infected females spent more time exploring the vicinity of cat odour compared to control females. This data was very surprising and completely opposite to what we were expecting. This is especially true of the saline group. To ensure that the animals had not been mistakenly assigned to the wrong groups, *Toxoplasma* conversion was confirmed using serology to determine seroconversion. To further confirm the data, the cat odour avoidance assay was repeated using another cohort of control and infected females. Again, control females spent significantly more time exploring the vicinity with cat odour compared to infected females (Figure 7.2). This data was completely opposite to other cat odour experiments where infected females spent significantly more time exploring the vicinity of cat odour compared to controls. This raised an important question of what could be causing this change.

One likely possibility was that the animals were being extremely stressed by construction that was going on next door. Around the same period of time several other groups reported atypical results in well-established behavioural and biochemical experiments. Another likely possibility involved the animal house feed supplied to the animals. The feed supplied to the animals had been changed around the same time making them a second variable in the equation. There was not much to be done concerning the construction except to wait for its completion and then re-establish baselines. Even after construction was over, data from the experiments still did not revert back to what it was

pre construction. After some research we realized the new feed supplied to the animals had a greater amount of isoflavones compared to the previous feed lots.

Isoflavones are also known as phytoestrogens and are derived from soy foods. Isoflavones are structurally similar to the major female hormone 17β estradiol. This allows phytoestrogens to bind estradiol receptors and assert an estrogenic or anti estrogenic effect [248]. Several behavioural effects of high phytoestrogen diets have been reported. These include effects on learning, memory and anxiety-related behaviours [249-251]. It has also been shown to affect aggressive behaviour in male rats [252] and affect food intake in female rats [253]. There have also been reports that phytoestrogens affect the size of certain sexually dimorphic nuclei in the rat brain including the anteroventral periventricular nucleus [248] and preoptic area [254]

To ascertain the effects of phytoestrogen on my experiments, ovariectomised uninfected females were fed on food with high (>300ppm) and low (50 ppm) phytoestrogen levels. Ovariectomy was done to remove the major source of endogenous phytoestrogens. After two weeks on the different diets, females were exposed to the cat odour avoidance assay. Females on the high phytoestrogen diets spent a marginally significant time ($p = 0.065$) in the vicinity of cat odour. This is similar to the effect an anxiolytic compound such as estradiol would produce. It remains unclear what the interacting effects a diet high in phytoestrogen coupled with a highly stressful environment were on the experiments. However once construction ended and females were placed on a low phytoestrogen diet, we have been able to replicate infection effects once again in normally cycling females (Figure 3.1C).

We were unable to demonstrate the ability of *Toxoplasma* to hijack the social recognition/discrimination pathway. This was mainly due to the stressful effects of ongoing construction works possibly interacting with high phytoestrogen levels in the diet of experimental animals. The experiment however afforded us the ability to identify the effect of phytoestrogen in the animal diet and account for this.

The main experimental goal of this chapter still remains unfulfilled. I was able to confirm the presence of behavioral manipulation in females by using a separate cohort when the uncontrollable and interacting influences of construction and diet were removed. I decided against re-attempting OTR antagonist experiments mainly because the animal house in my institute is slated to undergo another round of transition and movement in 2017.

Chapter 6

Discussion

I have placed discussion about each set of observations at the end of relevant chapters. I will not duplicate that discussion here for the sake of brevity. Instead I will try to provide a broad discussion of this thesis in the current chapter.

Gender as a biological variable

The role of gender in designing and interpreting experimental data has in recent years become a topic of debate. This is especially true in preclinical research where there is a noticeable absence of female subjects in research that is not female-specific. This has prompted the NIH to direct that all grant applications must consider gender (sex) as a biological variable in the design and analysis of research proposals [197, 202]. In neurobiology, there is growing evidence of sexual dimorphism in rodent behavioural traits [189, 198]. This suggests that individuals of the different sexes may respond differently to the same stimuli. It also allows for the possibility that a common behavioural trait may be mediated by different substrates within the two genders [199, 200].

This thesis argues for the need to consider gender in the design of experiments and the interpretation of results especially in preclinical experiments. We have shown that a similar phenotypic presentation of behaviour can be mediated by different substrates in the brain. Without access to this data, our knowledge of the underlying mechanism of *Toxoplasma* mediated behaviour manipulation is myopic at best. Similarly, any research that seeks to understand social behaviour must take into consideration the gender dimorphic nature of what is considered the social brain. We must understand how the

different genders respond to stimuli in order to understand how to develop therapies that effectively treat disorders of social behaviour.

Gender and Parasitic Behavioral Change.

Several groups including our own has sought to understand the mechanisms underlying *Toxoplasma* induced behavioural manipulation in rodents. To this end, three different hypotheses have been proposed [163]. The most parsimonious of these models proposes an interaction between peripheral testosterone and AVP in the MeApd to explain both the increase in attractiveness of infected males to uninfected females and the increased approach to cat odour observed [95, 134, 184]. Both of these substrates are expressed in a gender dimorphic manner in rodents with greater expression in males compared to females. The general assumption is that *Toxoplasma gondii* is able to manipulate expression of these substrates resulting in the observed behavioural changes. If this holds true, then without these specific substrates, *Toxoplasma* should not be able to induce the host to exhibit approach behaviour to cat odour. The “maleness” of both substrates is very striking and begs the question of specificity in relation to *Toxoplasma*-induced behavioural manipulation. In other words, is the ability of *Toxoplasma* to manipulate host behaviour exclusive to males?

Another way to think about this is that manipulation occurs at the level of approach-avoidance negotiation. Negotiation of the approach-avoidance conflict holds true irrespective of gender. In this case *Toxoplasma* manipulates host behaviour at the level of the approach-avoidance conflict and not at the level of specific substrates. In this scenario, it is possible for female rats to display atypical approach to cat odour post *Toxoplasma*

infection. Data from experiments outlined in this thesis supports this theory. I have demonstrated that female rats infected with *Toxoplasma gondii* showed increased approach behaviour to cat odour compared to uninfected controls (Chapter 3). This is similar to what has been observed in the case of male rats [94, 136]. Some researchers have been skeptical of the behaviour manipulation hypothesis. They have argued that observed behaviour changes are most likely a result of general sickness behaviour rather than a specific effect on a particular behaviour [148]. In male rats, *Toxoplasma* infection does not affect olfactory behaviour, weight gain, normal grooming behaviour or hippocampal dependent non-aversive learning [94, 138, 149]. Similarly in females, general anxiety levels do not appear to differ between control and infected female rats as measured in the EPM and OFT. This argues against the notion that observed atypical attraction to cat odour post infection is a symptom of general sickness behaviour or a general disruption in adaptive or survival-related behaviours. It points to specificity in the actions of *Toxoplasma* where it alters only those behaviours that would improve its transmission efficiency. This is the central idea of the behavioural manipulation hypothesis.

The demonstration that *Toxoplasma gondii* is able to alter behaviour in female rats raises the question of how the parasite achieves this manipulation. What are the substrates involved? One possibility will be that *Toxoplasma* is masculinizing infected female rats causing them to increase production of testosterone and AVP. The ability of parasites to alter the gender of the host post-infection has been previously reported in the sand crab *Portunus pelagicus* [57]. To determine if *Toxoplasma* was making females more masculine, we determined the levels of plasma testosterone as well as mRNA levels of

AVP in the MeApd. We observed no difference in the levels of these substrates between control and infected female rats. This suggests that mechanism underlying *Toxoplasma* mediated behaviour manipulation does not rely on masculinizing female rats. We also did not observe any difference between control and infected female rats in the levels of estradiol, progesterone and corticosterone.

The testosterone-AVP hypothesis in its most basic form describes an interaction between the periphery and central systems to drive a behaviour change. We were unable to identify a peripheral hormone that could be a substrate in this behaviour manipulation and so we turned our attention to the brain. Reports from work conducted in male rats served as a template in deciding which areas of the brain to focus on. Work on affiliative behaviours has implicated the oxytocin system to be an important substrate in mediating approach behaviour in female rats [232, 236, 247]. Based on the proposed model by Choleris, we assayed for the mRNA levels of OT and OTR in the MeApd, PVN, BNST and MPOA. We also assayed for OT levels in SON as an internal control. We observed a significant increase in OTR transcript level and a marginal but statistically insignificant increase in OT transcript levels in the MeApd. OTR density and distribution are widely accepted to be a more reliable indicator of oxytocin activity since oxytocin is mainly produced in the PVN and transported to other brain regions via volume transfer [240]. The marginally significant increase in PNV OT levels observed in our experiment supports this assertion. This is in line with the observation in males that MeApd is atypically activated post *Toxoplasma* infection. Transcript levels of OT and OTR were not statistically different in the MPOA and BNST of control and infected females.

In male rats, infection is hypothesized to “hijack” the male reproductive pathway. The MeApd which is innately responsible for processing positively-valenced odours such as reproductive pheromones is atypically activated in *Toxoplasma* infected male rats when they are exposed to cat odour [182]. This raises the question of which innate behaviour is being manipulated by *Toxoplasma gondii* in the case of female rats. We hypothesized that the social recognition/ social discrimination pathway in was being manipulated in the case of females. This was based on the model proposed by Choleris and her colleagues. Unfortunately, we were unable to determine if this truly was the case due to challenges arising in the animal facility that were beyond our control. We were however able to prove that phytoestrogen-containing animal feed played an important role in behavioural response in female rats. This is especially true for anxiety or fear-related behaviours or behaviours mediated by estradiol.

In the preceding chapters we have established that *Toxoplasma*-mediated behaviour manipulation exists in females. We have further demonstrated that this effect is not a symptom of general sickness behaviour or an indication of breakdown in general defensive behaviours in infected rats. We have gone ahead to show that infection does not masculinize female rats and have proposed a parallel pathway that involves the oxytocin system acting within the MeApd as being a mediator for the atypical approach behaviour observed. *Toxoplasma* is able to alter the host brain in such a manner that a stimulus that is innately aversive suddenly becomes affiliative. This has immense implications in social behaviours and interactions. This is particularly true for the study into neuropsychological disorders such as schizophrenia where correlative evidence exists that *Toxoplasma* infection increases prevalence and intensity of disease presentation. It also opens several

possibilities for neuropsychological disorders where OT/ OTR and AVP are thought to be important.

Auto-critique and Future Possibilities

I have tried in preceding chapters to elucidate the mechanism underlying *Toxoplasma* mediated behaviour manipulation. In spite of my best efforts, I am aware of several shortcomings in this document. I have demonstrated an increase in OTR expression in the MeApd and implicated the oxytocin system as a substrate important in driving this behaviour. I have, however, been unable to prove the necessity and sufficiency of this substrate to drive the atypical behaviour observed. This is important to validate the data presented in this thesis. One way to do this would be to knockdown the OTR gene in the MeApd of infected female rats using the CRISPR/ Cas9 system and observe their response to cat odour. This would help to determine the necessity of OTR expression in the MeApd in mediating the behaviour. On the other hand, an overexpression of OTR in the MeApd of uninfected female rats could be used to determine the sufficiency of the OT/ OTR system to drive the behaviour change in the absence of infection. I have also been unable to implicate any hormone in the periphery as a mediator in the mechanism underlying this behaviour. It will be important in the future to determine what hormone is important here and understand how it interacts with the brain. Finally, it still remains unclear which innate affiliative behaviour is co-opted by *Toxoplasma* in females. Having an idea of which behaviour is manipulated will help identify downstream brain regions important in mediating this behavioural manipulation. It will also allow for a better holistic understanding of the mechanism involved. An in-depth understanding of what exactly is happening in both genders will make it easier to develop an inclusive model.

This will help conceptualize the information and hopefully increase our knowledge of disorders of social behaviour.

7. Annexe

Materials and Methods

1. Animal Work

a. Animals

Adult female rats approximately 7-8 weeks (49-56days) old were used for all experiments. Animals were procured from InVivos animal rearing facility, Singapore and housed two per cage at the NTU vivarium. Animals were provided food and water *ad libitum* under controlled temperatures (24-26°C) and humidity (60-70%) in a 12 hour light on/ light off cycle (light on at 7am). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technology University. All animals were allowed to acclimatize to the facility for a minimum of two days before being subjected to any experiments.

b. *Toxoplasma* Parasite and Infection

A Prugniaud strain of *Toxoplasma gondii* with both Luciferase and GFP inserts (PA7) was used. This strain is similar to the strain used for prior work with male rats [95]. The parasites were maintained in vitro in human foreskin fibroblast monolayers, harvested using syringe lysis. Female were randomly assigned in experimental groups and injected with either *Toxoplasma gondii* (5×10^6 tachyzoites suspended in 500 μ l phosphate buffered saline (PBS); i.p.) or the vehicle (PBS) alone. All rats were weighed weekly. Absence or presence of overt sickness behavior was noted every week. Experiments were conducted from the 7th week of infection onwards.

c. Confirmation of Infection Status

Infection was confirmed using serological detection of anti-*Toxoplasma* IgG antibodies in rat serum. Human foreskin fibroblasts (HFF) cultured in 24 well plates were infected with *Toxoplasma*. 24 hours post infection, media was aspirated out, wells were washed with PBS and fixed with 4% PFA. Infected wells were incubated with 1ml volume of 1:1000 dilution of serum and kept overnight at 4°C. Animals infected with *Toxoplasma* would express anti *Toxoplasma* antibodies which would bind to the *Toxoplasma* in culture. Bound antibodies were visualized using anti Rat IgG Cy3 (1:200 Millipore; red fluorescence).

d. Determination of estrus

Vaginal lavage was collected under mild restraint using 10µl of buffered saline. Stage of the estrus cycle was determined using previously published criteria under 200X (total) magnification. Relative ratios of leucocytes, cornified and epithelial cells were observed under the microscope. Specifically, vaginal estrus manifested as preponderance of cornified and epithelial cells.

e. Ovariectomy and estradiol supplementation

Surgery was performed using aseptic techniques under isoflurane anesthesia (2.5% gaseous isoflurane with pure O₂). After placing animals in ventral recumbency, ovaries were approached bilaterally using incisions caudal to ribcage. Ovaries were excised after suturing fallopian tubes. The wound was subsequently sutured. Animals assigned to sham surgery group were operated in a similar manner, except that fallopian tubes were not tied and ovaries were not excised.

Silastic capsules containing estradiol were prepared by packing 5mm of tubing with pure estradiol (total length of capsule = 15 mm). This length of silastic capsules resulted in constant delivery of estradiol for 60 days, creating constant estrus as determined by the presence of cornified and epithelial cells in vaginal lavage. Animals undergoing estradiol supplementation received one silastic capsule subcutaneously immediately after ovariectomy. Animals receiving placebo treatment received an empty silastic capsule of similar length.

Animals were given pre-operative prophylactic antibiotic (Baytril 10mg/kg, sc; Bayer) and pain relief (Carprofen 5 mg/kg, sc; Pfizer). After surgery, animals were housed singly for >3 days with supplemental pain relief daily (Carprofen 5 mg/kg, sc). Animals were re-housed with prior cage-mates once wound healing was visually confirmed. At least one week elapsed between surgery and the start of further experiments.

2. Behavioural Assays

a. Cat odor Avoidance Assay

Aversion to cat odor was quantified in a rectangular arena with two opposite and identical arms (76 x 9 cm each), separated by a central part (9 x 9 cm in size; white Perspex). Three different protocols were used. In the case of the intact animals (Figure 3.1), the entire length of the straight maze was used during testing. Cat odour was presented in one extremis of the arena and rabbit odour was presented in the other extremis (2ml bobcat urine soaked on a gauge of towel; Maine Outdoor Solutions, ME, USA; 2ml rabbit urine soaked on a gauge of towel; Biopolis Vivarium, Singapore; trial duration = 20 minute). Animal was placed in the centre of the maze

and allowed to move freely between the two odours. In the second protocol (Figure 4.2 and 5.2), cat odor was presented in one extremis of the arena (2ml bobcat urine soaked on a gauge of towel; Maine Outdoor Solutions, ME, USA; trial duration = 20 minute). In both protocols, animals were habituated to the arena for 3 consecutive days, 10 minutes each day. The exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA) in both protocols.

Ovary-intact cycling females were always tested in this assay during estrus phase of their cycle.

Figure 7.1: Cat odour avoidance assay apparatus. (A) represents apparatus for protocol 1. (B) represents apparatus for protocol 2.

b. Elevated plus-maze

Intact estrus female rats (control, infected) from were tested on the elevated plus maze (EPM) as a measure of anxiety between the hours of 10am and 3pm each day for 300s per animal. The lux conditions used were 508 at the edge of the open arm and 390-398 towards the centre of the apparatus. The closed arm was kept as dark as possible. The

open arm is 75cm x 11cm, the closed arm is 75cm x 11cm x 26cm. The arms are raised 60cm off the ground.

Figure 7.2: Elevated Plus maze

c. Open Field Test

A square Perspex open field apparatus with dimensions 100 cm x 100 cm x 30 cm was used. Control and infected estrus female rats were tested for anxiety and locomotion during the dark phase of their light cycle. Test females were placed in the centre of the apparatus and allowed to explore the apparatus. The exploration was video recorded and automatically analyzed using AnyMaze (Stoelting, IL, USA). Quantification of time spent in the centre, walls and corners was used as a measure of anxiety. Total distance travelled served as a proxy for locomotion in test animals.

Figure 7.3: Open Field apparatus

d. Habituation/ Dishabituation Assay

Individual female rats were tested 5 times (trial 1 to 5) in a rectangular apparatus (56cm x 39cm x 28cm). Stimulus juvenile rats were placed in a plastic holder (16cm x 10cm x 7cm) and covered with a wire mesh. The rectangular apparatus containing the stimulus mouse was placed in one corner of the test apparatus. Each trial lasted for 5 minutes and conducted at 15 minute intervals. The same stimulus juvenile rat was presented to the test female in trial 1-4. In test 5, a novel stimulus rat was presented to the test female. In the interval between trials, rats were left undisturbed in the test apparatus. Prior to testing, each experimental female was habituated to the testing apparatus for 10 minutes. Stimulus animals were also habituated to the holding apparatus for 10 minutes before testing began.

3. mRNA extraction and cDNA synthesis

Preparation of lysis buffer (components from Sigma USA)

i. Tris 1M (10X stock) Set pH to 8.0 after autoclaving (working solution 100mM)

- ii. EDTA 0.5M (25X stock) (working solution 20mM)
- iii. Sodium deoxycholate 10% (10X stock) (working solution 1%)
- iv. SDS 10% (10X stock) (working solution 1%)

Note: all solutions were prepared using autoclaved DEPC water.

RNA extraction was performed using the Trizol method. Briefly, tissue was microdissected and, placed in lysis buffer and homogenized in ice cold Trizol (~double the volume of extraction buffer). Ribolock was added to prevent RNA from degrading. Choloform (~1/5 volume of Trisol) was added and samples were shaken for 15-30 sec followed by incubation at room temperature for 3min. The samples were centrifuged at 4°C and 12000g for 15 min. The aqueous phase was pipette into a new eppendorf tube and ice cold isopropanol (~ same volume as aqueous phase) added. This was incubated at room temperature for 15-25 min and centrifuged again for 15 min. The supernatant was discarded carefully and pellet washed three times with 1 ml of 75% ethanol prepared using DEPC water and allowed to air dry at RT for ~15-30 min. It was resuspended in 30 µL DEPC water with 1 µL Ribolock. Samples were immediately converted to cDNA. cDNA synthesis was performed using the RevertAid first strand cDNA synthesis kit from Fermentas (Catalogue number #K1621, #K1622). 20 µL reactions were prepared (described below). cDNA was synthesized at 42°C for 1 hour (Eppendorf Mastercycler personal, Singapore). Samples were stored at -20°C for short time or -80°C for longer periods.

Reagent	Volume (μl)
5X reaction buffer	4
Ribolock RNase inhibitor	1
10mM dNTP	2
Revertaid M-MuLV Reverse Transcriptase	1
Oligo dT Primer	1

Table 7.1: cDNA synthesis master mix composition

4. Quantitative PCR

Brain tissue was harvested post-decapitation and flash-frozen in liquid nitrogen. The tissue was stored at -80°C till further analysis. Subsequently, mRNA abundance was quantified for the nonapeptide oxytocin (OT) in the paraventricular nucleus of hypothalamus (PVN) and oxytocin receptor (OTR) in posterodorsal medial amygdala (MePD). The standard method based on SYBR green dye was used as described before. PCR cycles needed to reach a pre-determined fluorescence threshold at early linear phase of the amplification were determined (Ct, threshold cycle number). Greater mRNA abundance in this assay results in lower Ct values. Ct values for OT and OTR were normalized by subtracting corresponding Ct values of 3 housekeeping genes (HPRT, β -actin and 18s rRNA). Three technical replicates were used for each determination. The median coefficient of variation between technical replicates was 15%. Please refer to Table 10.3 for primer sequences and their sequences.

Reagent	Volume (μl)
ddH ₂ O	9
Forward Primer (10mM)	0.5
Forward Primer (10mM)	0.5
Rox Low	0.5
Sybr Green (Kapa Biosystems)	12.5
cDNA (100 ng/ μl)	2

Table 7.2: qPCR master mix composition

Figure 7.4: Diagram of cycling conditions for qPCR

5. Primers Used

All primers were synthesized by Integrated DNA Technologies, Singapore.

Primer	Primer sequence 5' - 3'
OT	TCTGCTGTAGCCCGGATGG
OT	GGAATGAAGGAAGCGCCCTA
OTR	AAATCCGCACGGTGAAGATGA
OTR	CATGTAGATCCACGGGTTGCAG
AVP	TGCCTGCTACTTCCAGAACTGC
AVP	AGGGGAGACACTGTCTCAGCTC
HPRT	AGGCCAGACTTTGTTGGATT
HPRT	GCTTTTCCACTTTCGCTGAT
18s	ACGGACCAGAGCGAAAGCAT
18s	TGTCAATCCTGTCCGTGTCC
β -actin	TGTCACCAACTGGGACGATA
β -actin	GGGGTGTTGAAGGTCTCAAA

Table 7.3: Primers used and their sequences

6. Quantification of Estradiol, Progesterone, Corticosterone and Testosterone levels

Trunk blood was collected during sacrifice. Plasma estradiol, progesterone, testosterone and corticosterone were determined using commercially available enzyme-linked immunoassay kits (Enzo Life Sciences, NY, USA). Protocol recommended by the manufacturer was used in all cases. With the exception of corticosterone, serum displacement reagent was used to make steroids in plasma available for assay. Diethyl ether was used to extract steroids for corticosterone assay.

7. Statistical Analysis

Statistical analysis was performed using SPSS software (version 23, IBM). Mann-Whitney U test and independent sample t test were used to calculate statistical significance samples, as appropriate. When using non-parametric statistics, exact statistics was used to ensure freedom from normality and distributional assumptions. Two way ANOVA with LSD pot hoc was used for experiments with 2x3 design. Box plots in this thesis depict 25th percentile, median and 75th percentile. Central dot and whiskers depict mean and SEM respectively. In bar charts, the bar height depicts mean and whiskers the SEM. PCR efficiency for each primer set was quantified using a series of sequentially diluted pooled samples that were quantified using qPCR in similar conditions. Primer efficiency was calculated using an online calculator (<http://www.thermoscientificbio.com/webtools/qpcrreffiency/>).

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