Role of oxytocin in mate-choice copying of female rats

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**ROLE OF OXYTOCIN IN MATE-CHOICE COPYING
OF FEMALE RATS**

CHUA CHELSEA

SCHOOL OF BIOLOGICAL SCIENCES

2019

Role of Oxytocin in Mate-Choice Copying of Female Rats

Chua Chelsea

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Master of Science

2019

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Abstract

Mate-choice copying occurs when animals made decisions based on the mate choice of the conspecifics. Mate copying in rats is currently understudied. Past studies in oxytocin-knockout mice suggested the involvement of oxytocin, but the neurobiological mechanisms underlying mate choice have not been fully elucidated. Building on this, we investigated if female rats in oestrous exhibit mate-choice copying and whether oxytocin plays a role. The rats were injected with oxytocin (1 mg/kg), a cocktail of oxytocin (1 mg/kg) and its antagonist L-368,899 hydrochloride (5 mg/kg), or saline as control. We observed that both oxytocin and its antagonist reduced mate-choice copying instead of enhancing it. Furthermore, these treatments resulted in sedated behaviour up to 1h after injection. Locomotor activity recovered 24h post-injection. On the other hand, control rats showed mate-choice copying and no loss of locomotor activity. However, any learned mate choices did not seem to be strong enough to induce short- (30 min) and long-term (24h) memory. This might be due to a lack of information provided to the observer rat.

To fill the gap in the proximate mechanisms behind *Toxoplasma* infections in female rats, the behaviours of oxytocin-treated and *Toxoplasma*-infected rats were compared. *Toxoplasma* infection leaves mate-choice copying behaviours intact and unchanged in female rats. Strangely, cat odour aversion was not observed in all

tested rats (oxytocin-treated, *Toxoplasma*-infected, and their controls). The current findings suggest a possible role for oxytocin in mate-choice copying, but further investigation is required.

Brief backdrop

Mate-choice copying occurs when animals make adaptive decisions using social information obtained from their conspecifics' mate choices [1]. One of the neuropeptides involved is oxytocin (OT) [2]. The link between OT and mate-choice copying has been studied in mice but not in rats. In this study, we first investigated if rats display mate-choice copying, and whether supplementation of OT and its antagonist may enhance or disrupt mate-choice copying in rats (Chapter 1).

Next, we studied if mate-choice copying in *Toxoplasma gondii*-infected rats may differ from uninfected rats and whether their behaviours reflect those of either OT or OT antagonist treatment (Chapter 2), to determine if OT plays a role in *Toxoplasma gondii* infection. The infection has been well-studied in male rats, and a possible proximate mechanism involving testosterone hyperproduction and arginine vasopressin (AVP) promoter hypomethylation was found [3, 4]. Yet, this pathway appears to be sexually dimorphic as infection in female rats is not mediated by gonadal steroids nor AVP [5].

Another approach was taken in Chapter 3 using the rats' innate aversion to cat urine. *Toxoplasma gondii* is one of the most well-studied examples of host behavioural manipulation by parasites. Infected rodents exhibit a "fatal attraction" to feline scents, which is believed to enhance trophic transmission of the parasite

back into its feline definitive host [6, 7]. We investigated if OT treatment and *Toxoplasma* infection could lead to the loss of avoidance, to establish OT involvement in infection (Chapter 3).

Chapter 1

Oxytocin mediation of mate-choice copying behaviours in female rats

1.1 Introduction

1.1.1 Social learning

Social observation and learning are important skills that improve survivability and adaptability. Rozin and Kalat (1971) first theorised that learning abilities were naturally selected adaptations that enabled species to cope with specific ecological demands [8]. In later decades, this view was expanded to various types of learning, including social learning [9-11]. Behavioural patterns for foraging, finding refuge, sourcing for water, avoiding predators and selecting mates can be learnt from more experienced individuals [12]. Learning these behaviours are particularly important for young and inexperienced animals, allowing them to acquire essential survival skills without incurring the costs of individual trial-and-error learning [12]. Social learning can be observed in a myriad of species: chimpanzees learn to make and use tools to fish for ants [13]; blackbirds and fishes can socially learn to fear a previously neutral stimulus as though it were a predator [14]; guppies prefer a male they have previously seen mating [15]; cowbirds (which are brood parasitic birds) learn songs from conspecifics rather than their adoptive parents [16]; and bumblebees can even learn how to play soccer from each other [17].

Rats are no exception. For decades, rodent control efforts have been hindered by social transmission of food preferences. Norway rats can socially learn to avoid toxic foods they smell on their sick conspecific's breath [18]. Rats also use

observational learning to acquire an efficient spatial exploration strategy [19] and foraging methods [20]. Acquiring feeding techniques allows new feeding areas to be occupied. For instance, black rats (*Rattus rattus*) use a unique pinecone opening technique that allowed them to feed on the previously inhabitable pine forests of Israel [21]. The rats' adaptability and flexible feeding behaviour eventually led them to invade new territories worldwide [21]. In choosing a mate, female rats may also consider the mate choice of a more experienced female [22]. This copying behaviour is known as mate-choice copying and will be explored in greater depth in this thesis.

1.1.2 Mate-choice copying

Mate choice is vital for sexually reproducing organisms. Choosing a favourable partner increases offspring survivability and maximises fitness [23]. Benefits are apparent when resources such as nuptial gifts, parental care and territories are involved [24]. For instance, fifteen-spined stickleback males that court more intensely and are better at fanning nests (which aids hatching) are preferred. A resourceful feeding territory occupied by male dragonflies, frogs, and dunnocks attracts more females [24]. Other more indirect benefits involve inheritance, where specific indicators in a potential mate advertise 'good genes.' For example, starling males that sing more frequently have stronger immunity, which is believed to enhance offspring viability [24]. Having freedom of choice is an advantage as well.

When stripe-faced dunnart females were paired with their preferred male, pregnancies per cycle increased, and the time to conception was shortened [25]. Matings of mice where both the male and female preferred each other produced progeny that demonstrated higher fitness than in matings where they did not [26]. Conservation efforts have improved as a result of mate choice [27-29].

Mate choice is primarily determined by an individual's genetic framework [30], but the environment and personal experiences can also influence mate choice [31]. Animals can better increase their chances of obtaining a high-quality mate by gathering information from the environment. Mate-choice copying occurs when animals use social information of their conspecifics' mate choices to make their own adaptive decision [1]. It predicts that the probability of a female choosing a male as a mate increases if this same male was observed to be previously chosen by a conspecific female, and vice versa [32]. The earliest mate-choice copying behaviours were studied in lekking birds and mammals, as well as fish and crustacean [15, 33]. Copying is especially useful for young and inexperienced individuals who may avoid the costs of trial-and-error learning by observing a more experienced older individual [1, 33]. However, mate-choice copying can be disadvantageous when individuals choose mates of lower fitness or health [34].

1.1.3 Social odourant communication in rodents

It has been observed that mice [1] and rats [22] rely on urine odours to choose a mate in the absence of a demonstrator. Social odours are an important form of communication providing information to conspecific and allospecific recipients [35], including the owner's identity [36-38], territorial occupancy [39, 40], social status [41-44], and health and reproductive status [45, 46]. The informational content remains long after the animal has left its mark [46, 47].

Individual recognition in rodents is enabled by unique urine odours which are produced by differences in diet, commensal bacteria, major histocompatibility complex (MHC) genes, and major urinary proteins (MUPs) [48, 49]. Rats and mice are known to secrete MUPs in physiologically large amounts in their urines [50, 51]. The secretion of this mixture of small proteins (18 to 20 kDa) is sexually dimorphic. In the house mouse, MUP urinary output is 3- to 4-fold higher in males than females [52]. On the other hand, urinary MUP concentration of male Wistar rats have been found to be about 120-fold higher than their female counterpart [53]. Hepatic rat MUP expression is under the complex control of multiple hormones, including testosterone, growth hormones, and thyroid hormones [54], hinting at its role in mediating sociosexual interactions through chemosignalling [55, 56]. MUPs are critical in slowing the release of volatile compounds in urine, enabling scent marks to retain their informational content [57-59]. Mice MUPs in odourant communication has been widely studied, but the role of MUPs in rat

communication is more evasive. It is known that adult rats will urine mark to advertise their sexual availability. Male rats mark more when a receptive female is nearby [60]. Urine marking in males is mediated by testosterone; castrated males display reduced marking, but the behaviour is restored upon testosterone administration [60]. Rat MUP can stimulate neurotransmitter release in the female amygdala and activate vomeronasal organ (VNO) neurons [61]. Taken together with the rat MUP binding properties and their similarities to mouse MUP, it is widely accepted that MUPs play an important role in scent marking and social odourant communication in rats [62].

1.1.4 Neurobiological mechanisms behind mate-choice copying

The neurobiological mechanisms behind mate-choice copying remain evasive. Previous studies focused on the adaptive significance and social cues required for copying, but few have attempted to elucidate the molecular processes involved [1]. Social recognition, social learning, and social and sexual motivations underpin mate-choice copying. Thus, the hormones and neurochemicals involved were postulated to play vital roles in mate-choice copying as well [1]. A recent finding in fruit flies unveiled the importance of dopamine and serotonin in mate-copying [63]. Opioid peptides, sex steroids, oxytocin (OT) and arginine vasopressin (AVP) have also been implicated [1].

1.1.5 Oxytocin

OT is a nonapeptide that is synthesised in the hypothalamus and released into the periphery via the pituitary gland [64]. It is most commonly known for its roles in parturition and lactation. However, this discounts critical social functions of OT, such as its involvement in maternal care, affiliative behaviours, and social bonding [65-67]. OT facilitates pair-bonding without mating in prairie voles [68]. In mice, it enhances nest-building behaviour and is an essential modulator of social recognition memory and aggression [69-71]. It regulates social defeat posture [72] and context-dependent responses to female cues in male mice [73], and male-directed social behaviour in female mice [74]. Maternal behaviour [68, 75], olfactory exploration and social recognition in rats [76, 77] are mediated by OT as well. In humans, OT mediates generosity and forgiveness [68], reduces stress and anxiety [78], and increases trust [79].

Kavaliers et al. (2017) provided insight into how OT can impact mate choice. Wildtype mice that initially favoured uninfected over infected male urinary odours exhibited a reversal of mate choice when presented with infected male urinary scents paired with that of females in oestrous [2]. On the other hand, OT knockout (OTKO) mice seemed to make random mate choices [2]. These observations were attributed to the role of OT in the expression of sexual behaviours and trust-related decision making [1].

1.1.6 Specific Aims

The link between OT and mate-choice copying has yet to be established in rats. However, reports are conflicting. Mate-choice copying in rats was observed in one study [22], but appeared to be random in another [80]. Therefore, it is necessary first to establish if mate-choice copying occurs before further investigating the possibility of OT's role in rat mate-choice copying.

A myriad of methods have been used to study mate choice and copying. Direct behavioural tests include the observation of mate preference, mating frequency, and success in mounting and penetration [22, 80]. On the other hand, odour preference such as scent choice and time spent near an odour is used as an indirect measure of mate choice [1, 81, 82]. This study employs the odour preference method as it is a simple approach suitable for testing of mate-choice copying and its retention.

The questions we aim to address in this study are:

1. Can mate-choice copying be observed in female rats?
 - a. Is the effect strong enough to induce memory and preference for a neutral odour in the short- (30 min) and long-term (24h) period?
2. Does OT play a role in mate-choice copying of female rats?
 - a. Do OT-treated rats show a greater degree of mate-choice copying compared to vehicle-treated rats?
 - b. Do rats injected with a cocktail containing OT and its antagonist (L-368,899 hydrochloride) reverse mate-choice behaviours mediated by OT?

1.2 Materials and Methods

1.2.1 Animals

Adult female Wistar Han rats were procured from InVivos, Singapore. They were housed in groups of 2 to 3 in the NTU vivarium and maintained on 12/12 light/dark cycle (lights on at 0700h) with food and water *ad libitum*. All testing occurred during the light phase. The experiments were reviewed and approved by the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC). All animals were acclimatised in the vivarium for at least two days before experimentation.

1.2.2 Determination of oestrous

Vaginal lavage was collected with 100 µl 1X PBS under mild restraint. The wet, unstained smears were observed under the microscope using a 20x objective. Oestrous was defined by a high proportion of cornified cells relative to leukocytes and epithelial cells (Figure 1).

Figure 1 Cytological staining of vaginal lavage.

Proportions of leukocytes (L), nucleated epithelial cells (N) and cornified epithelial cells (C) in vaginal smears across the different stages of the rat oestrous cycle. This figure is copied from Pompili and colleagues' 2010 paper [83].

1.2.3 Treatment administration

All injections were done intraperitoneally. OT, OT antagonist L-368,899

hydrochloride (OTA) and PBS were administered 30 min before testing.

Experimenters were blinded to the injection solutions. The concentrations used were as follows:

Treatment	Concentration	Solvent
OT	1 mg/kg	1X PBS
OTA	5 mg/kg (excess)	1 mg/ml OT
PBS control	1 ml/kg	1X PBS

The concentration of OT was determined by considering the concentrations used in literature [84-87] and past experiments in our lab (Samira, unpublished). OT antagonist was supplied in excess to render the OT receptor ineffective regardless of its concentration.

1.2.4 Collection of male rat urine

Male Wistar Han rats (n=24) 10 to 12 weeks old, of which half were Toxoplasma-infected, were used. Infection status was not a concern since both infected and uninfected rats' urines could induce attraction in female rats [88]. Furthermore, urinary MUP levels are at a maximal level at this age, coinciding with sexual maturation of the male rats [51]. The rats were held over a falcon tube, while trans-abdominal pressure was applied to encourage micturition [89]. All collections were mixed in a stock solution. The urine was then aliquoted and stored at -20°C until required.

1.2.5 Mate-choice copying

The mate-choice copying experiment was adapted from Dass, S. A. (2011). Two identical runways consisting of opposing and identical arms with central start boxes and end goal boxes were used (Prime Bioscience, Singapore; Figure 2).

Animals were habituated in the arena for two consecutive days for 10 mins each day before any testing.

Figure 2 3D Model of the arena.

It consists of two runways connected along their longitudinal sides. Each runway is made up of a central start box, two opposing arms and two end goal boxes.

Only rats in oestrous were tested. Each rat underwent four trials to determine their preference for the odours presented (Table 1, Figure 3). In each trial, the rat was introduced into the centre of the runway and tracked for 10 min. Preference was quantified by its occupancy in each arm. The unfamiliar odours used were tested for neutrality prior to the experiment (Supplementary figure 1, Supplementary table 1). Nevertheless, Trial 1 served as a control trial to ensure no intrinsic preference

for the unfamiliar odours used. The two unfamiliar odours, almond and peppermint flavours (diluted 1:10 in water) were presented by five 10 µl drops along the respective arms of the runway. Male urine was similarly presented in trial 2. A demonstrator female undergoing oestrous was allowed to occupy and scent-mark an arm of the runway for 10 min before being confined to the end of the same arm for the duration of the trial. Peppermint or almond flavour was randomly tagged to the female scent (N*). Trials 3 (short-term memory trial) and 4 (long-term memory trial) were repeats of trial 1 but at 30 min and 24h after trial 2 respectively, to test for any maintenance of learnt mate choices through a preference for N*. All trials took place under low-light conditions and were videotaped from the top view (NC260 HD Day/Night Wi-Fi Camera, TP-Link, China). Position effect was controlled by counterbalancing the positions of the rats.

Trial	Odour 1	Odour 2
1	N*	N
2	N* + EF + M	N + M
3	N*	N
4	N*	N

Table 1 Odours presented to OT-treated and control rats.

N*: novel unfamiliar odour associated with an oestrous female; N: alternative unfamiliar odour; EF: oestrous female odour; M: male odour.

Figure 3 Timeline of experimental trials.

1.2.6 Statistical analysis

Occupancy in each arm of the runway was measured with AnyMaze software (Stoelting Co., USA). To ascertain if the different treatments affected the locomotor activity of the rats, distance and maximum speed obtained were also measured. Rats that did not pass through the centre of the runway at least twice were excluded from analyses. IBM SPSS Statistics 23 (IBM, USA) was used for data analysis. Collection and processing of all data were randomised and blinded to the experimenters.

1.3 Results

1.3.1 Test for intrinsic preferences and possible biases

1.3.1.1 Neutral odours

Trial 1 was conducted to determine if rats had an intrinsic preference for N*. Rats do not seem to show such preference for N* nor for either arm of the runway.

There was no significant deviation from chance level of 300s (one-sample t-test:

$t(29) = -0.344$, $p = 0.734$; Figure 4).

Figure 4 Female rats do not show an intrinsic preference for the neutral odours used. Time spent by female rats in arm containing N* (n=30). Bar chart depicts the mean and standard error of the mean (SEM). Y=300s is the chance level.

1.3.1.2 Social preference for demonstrator female

To ascertain that any preference effect seen in trial 2 of the mate-choice copying experiment was due to sexual preference rather than a social one, female rats undergoing oestrous were tested using trial 2's setup excluding the male odours. Both OT- and saline-injected rats did not appear to show significant preference for the demonstrator female (one-sample t-test: OT: $t(6) = 0.816$, $p = 0.446$; PBS: $t(9) = 1.109$, $p = 0.296$). The time spent near a demonstrator female did not differ between OT-treated and control rats (Independent t-test: $t(15) = 0.269$, $p = 0.792$; Figure 5).

Figure 5 Social preference for demonstrator female is not significant. Time spent by OT- (n=7) and saline-injected (n=10) rats in the arm containing N*. Bar chart depicts the mean and SEM. Y=300s is the chance level.

It was observed that some rats seemed to behave in a sedated manner after injection. Hence, locomotor activity was analysed as well. As compared to vehicle-injected rats, OT-treated rats displayed decreased activity, evident from lower distance travelled (Mann Whitney U-test: $U = 14.0$, $p = 0.040$; Figure 6) and lower maximum speeds attained (Mann Whitney U-test: $U = 13.0$, $p = 0.032$; Figure 7).

Figure 6 OT-treated rats travelled less than control rats. Total distances travelled by OT-treated (n=7) and vehicle-injected (n=10) female rats. The box plot shows median, upper and lower quartiles, and minimum and maximum values. An outlier is depicted by a circle (○).

Figure 7 OT-treated rats displayed lower maximum speeds than control rats. Maximum speeds attained by OT-treated (n=7) and vehicle-injected (n=10) female rats. The box plot shows median, upper and lower quartiles, and minimum and maximum values. An outlier is depicted by a circle (○).

1.3.2 Mate choice preference of OT-treated females

1.3.2.1 Preference for a female-associated male odour

Female rats seemed to prefer the male odour tagged to a female demonstrator.

Vehicle-injected rats exhibited greater time spent in the arm containing N* (one-sample t-test: $t(15) = 5.237$, $p < 0.001$; Figure 8). On the other hand, rats injected with OT did not show any significant preference in trial 2 (one-sample t-test: $t(13) = 1.018$, $p = 0.327$; Figure 8), suggesting the lack or loss of mate-choice

copying. Yet, the differences in time spent between the groups did not seem to be significant (independent t-test: $t(28) = -1.511$, $p = 0.142$; Figure 8).

Figure 8 Mate-choice copying is shown in control but not OT-treated rats. Time spent by OT-treated ($n=14$) and control ($n=16$) rats in the arm containing N* for trial 2. Bar charts depict mean and SEM. Chance level is depicted by the dotted horizontal line at 300s. One-sample t-test: *** $p < 0.001$.

1.3.2.2 Short- and long-term memory of preference

Of the 30 rats, half underwent trials 3 and 4 to test for short- and long-term memory of any learnt mate choice (Figure 9). Control rats did not exhibit preference for N* in both trials 3 and 4 (one-sample t-test: trial 3: $t(7) = -0.429$, $p = 0.681$, Figure 9A; trial 4: $t(7) = -0.081$, $p = 0.938$, Figure 9B). OT-treated rats did not display mate-choice copying in trial 2, hence there was naturally a lack of

preference in trials 3 and 4 as well (one-sample t-test: trial 3: $t(7) = 0.092$, $p = 0.930$, Figure 9A; trial 4: $t(7) = 0.063$, $p = 0.951$, Figure 9B). Between OT-treated and control rats, timings obtained in each trial did not differ significantly (Independent t-test: trial 3: $t(14) = 0.326$, $p = 0.749$, Figure 9A; trial 4: $t(14) = 0.100$, $p = 0.922$, Figure 9B).

Figure 9 Female rats do not display short- nor long-term memory of preference. Time spent by control (n=8) and OT-treated (n=8) rats in the arm containing N* for (A) trial 3 and (B) trial 4. Bar charts show means and SEMs. Chance level is depicted by the dotted horizontal line at 300s.

1.3.2.3 Changes in locomotion

Female rats had reduced total travelled distance 30 min after OT injection in trial 2, as compared to before the injection, i.e., trial 1 (Wilcoxon signed ranks test: $Z = -2.201$, $p = 0.028$; Figure 10A). Meanwhile, saline-injected rats did not exhibit any differences in distance travelled before and after injection (Wilcoxon signed ranks test: $Z = -0.840$, $p = 0.401$; Figure 10B).

Between the 2 groups, differences in distances travelled in each trial were not statistically significant (Mann-Whitney U test: trial 1, $U = 24.0$, $p = 1.000$; trial 2, $U = 9.0$, $p = 0.053$).

Figure 10 OT treatment reduces the total distance travelled by female rats. Figures illustrate total distances travelled by (A) OT-treated (n=6) and (B) vehicle-injected (n=8) female rats. The box plot shows median, upper and lower quartiles, and minimum and maximum values. An outlier is depicted by a circle ($\circ$).

There were no significant differences in maximum speed attained before and after OT injection (Wilcoxon signed ranks test: $Z = -0.105$, $p = 0.917$; Figure 11A). However, it is notable that the distribution of maximum speeds attained is much higher after OT injection (Figure 11A). Saline-injected rats did not show significant differences in maximum speeds as well (Wilcoxon signed ranks test: $Z = -0.700$, $p = 0.484$; Figure 11B).

There were no significant differences between the treatment groups (Mann-Whitney U test: trial 1: $U = 17.0$, $p = 0.366$; trial 2: $U = 20.0$, $p = 0.606$).

Figure 11 OT treatment does not seem to affect maximum speeds attained by female rats. Figures illustrate maximum speeds attained by female rats injected with (A) OT (n=6) and (B) vehicle (n=8). The box plot shows median, upper and lower quartiles, and minimum and maximum values. Outliers are depicted by circles (○).

Consistent with previous results (Figure 10A), total distance travelled is reduced from trial 1 to 2 in OT-treated rats (Wilcoxon signed ranks test: $Z = -2.240$, $p = 0.025$; Figure 12A). Distance travelled in trial 3 differed significantly from that of trial 1 (Wilcoxon signed ranks test: $Z = -2.100$, $p = 0.036$), but not 2 ($Z = -0.420$, $p = 0.674$). Locomotor activity seemed to revert to normal after 24h, as distance travelled in trial 4 was comparable to that of trial 1 (Wilcoxon signed ranks test: $Z = -0.840$, $p = 0.401$), but was significantly different from that of trials 2 ($Z = -2.100$, $p = 0.036$) and 3 ($Z = -2.380$, $p = 0.017$).

For saline-injected rats, total distance travelled remained similar across all trials (Wilcoxon signed ranks test: trials 1 vs 2: $Z = -1.540$, $p = 0.123$; 1 vs 3: $Z = -0.980$, $p = 0.327$; 1 vs 4: $Z = -0.980$, $p = 0.327$; 2 vs 3: $Z = -0.420$, $p = 0.674$; 2 vs 4: $Z = -0.140$, $p = 0.889$; 3 vs 4: $Z = -0.420$, $p = 0.674$; Figure 12B).

Before injection, both groups did not differ significantly in distance travelled (Mann Whitney U test: $U = 26.0$, $p = 0.529$). However, 30 min and 1h after injection, total distance travelled diverged markedly (Mann Whitney U test: trial 2: $U = 6.0$, $p = 0.006$; trial 3: $U = 2.0$, $p = 0.002$). Twenty-four hours after injection, distance travelled reverted to normal levels (Mann Whitney U test: $U = 25.0$, $p = 0.462$).

Figure 12 OT treatment affects distances travelled.

Graphs illustrate total distances travelled by female rats injected with (A) OT (n=8) and (B) saline (n=8) for the memory trials. The box plot shows median, upper and lower quartiles, and minimum and maximum values. Outliers are depicted by circles (○).

There are no significant differences in maximum speed across trials for OT-treated rats (Wilcoxon signed ranks test: trials 1 vs 2: $Z = -0.280$, $p = 0.779$; 1 vs 3: $Z = -1.260$, $p = 0.208$; 1 vs 4: $Z = -1.120$, $p = 0.263$; 2 vs 3: $Z = -1.680$, $p = 0.093$; 2 vs 4: $Z = -1.260$, $p = 0.208$; 3 vs 4: $Z = -1.680$, $p = 0.093$; Figure 13A). Similarly, there are no significant differences exhibited by vehicle-injected rats across all trials (Wilcoxon signed ranks test: trial 1 vs 2: $Z = -1.820$, $p = 0.069$; 1 vs 4: $Z = -1.920$, $p = 0.069$; 2 vs 3: $Z = -0.140$, $p = 0.889$; 2 vs 4: $Z = 0.000$, $p = 1.000$; 3 vs 4: $Z = -0.701$, $p = 0.483$), except between trials 1 and 3 (Wilcoxon signed ranks test: $Z = -2.100$, $p = 0.036$; Figure 13B). Treatment had no impact on maximum speed attained in all trials except trial 3 (Mann Whitney U test: trial 1, $U = 32.0$, $p =$

1.000; trial 2, $U = 19.0$, $p = 0.172$; trial 3, $U = 10.0$, $p = 0.021$; trial 4, $U = 29.0$, $p = 0.753$).

Figure 13 OT treatment does not affect maximum speeds attained. Graphs show maximum speeds attained by female rats injected with (A) OT (n=8) and (B) saline (n=8) for the memory trials. The box plot shows median, upper and lower quartiles, and minimum and maximum values. An outlier is depicted by a circle (○).

1.3.3 Mate-choice copying of OTA-treated female rats

Fifteen rats were treated with OT while the other 13 were treated with OTA to reverse OT's effects. Of which, two rats from each group failed to cross the centre line of the runway at least twice and hence were excluded from the following analyses.

1.3.3.1 Test for intrinsic preferences

Before treatments, the female rats did not appear to have any intrinsic preference for either arm of the runway, nor for peppermint and almond flavours (one-sample t-test: $t(23) = -0.424$, $p = 0.676$; Figure 14).

Figure 14 Female rats do not have an intrinsic preference for the neutral odours. The figure illustrates the time spent by female rats ($n=24$) in the arm containing N* in trial 1. Bar chart depicts mean and SEM. $Y=300s$ is the chance level.

1.3.3.2 Preference for female-associated male odour

In line with previous results, OT-treated rats do not seem to display mate-choice copying (one-sample t-test: $t(12) = -1.419$, $p = 0.181$; Figure 15). Rats treated with OTA did not display any preference for the female-associated male odour either (one-sample t-test: $t(10) = -0.673$, $p = 0.516$; Figure 15).

Figure 15 OT- and OTA-treated rats do not display mate-choice copying. The figure illustrates the time spent by rats injected with OT (n=13) and OTA (n=11) in the arm containing N* for trial 2. Bar chart depicts mean and SEM. Chance level is depicted by the dotted horizontal line at 300s.

1.3.3.3 Short-term memory of preference

There was an absence of short-term memory in both groups (one-sample t-test: OT: $t(12) = 0.515$, $p = 0.616$; OTA: $t(10) = 0.097$, $p = 0.925$; Figure 16) likely due to the lack of preference displayed in trial 2.

Figure 16 Absence of short-term memory of preference of OT- and OTA-treated rats. The figure illustrates the time spent by rats injected with OT (n=13) and OTA (n=11) in the arm containing N* for trial 3. Bar chart depicts mean and SEM. Chance level is depicted by the dotted horizontal line at 300s.

1.3.3.4 Changes in locomotion

For OT-treated rats, total distance travelled in trials 2 and 3 differed significantly from that of trial 1 (Wilcoxon signed ranks test: $Z = -3.110$, $p = 0.00187$ and $Z = -3.180$, $p = 0.00147$, respectively; Figure 17A). A similar result was obtained for OTA-treated rats (Figure 17B). Total distance travelled in trial 1 was significantly different from that of trials 2 and 3 (Wilcoxon signed ranks test: $Z = -2.934$, $p = 0.00335$ and $Z = -2.934$, $p = 0.00335$, respectively).

A comparison between OT- and OTA-treated rats yielded no significant differences in total distance travelled in each trial (Mann-Whitney U test: Trial 1, $U = 56.0$, $p = 0.369$; Trial 2, $U = 62.0$, $p = 0.582$; Trial 3, $U = 65.0$, $p = 0.706$).

Figure 17 OT and OTA treatments reduced the total distance travelled. Figures illustrate total distance travelled in each trial by (A) OT-treated ($n=13$) and (B) OTA-treated ($n=11$) female rats. The box plot shows median, upper and lower quartiles, and minimum and maximum values. Outliers are depicted by circles ($\circ$).

The maximum speed attained across all trials for both OT- and OTA-treated rats were similar (Wilcoxon signed ranks test: OT trial 1 vs 2: $Z = -1.922$, $p = 0.0546$; OT trial 1 vs 3: $Z = -1.642$, $p = 0.101$; OT trial 2 vs 3: $Z = -0.280$, $p = 0.780$; OTA trial 1 vs 2: $Z = -0.663$, $p = 0.508$; OTA trial 1 vs 3: $Z = -1.245$, $p = 0.213$; OTA trial 2 vs 3: $Z = -0.357$, $p = 0.721$; Figure 18).

Between treatments, the maximum speeds attained in each trial did not differ significantly (Mann-Whitney U test: Trial 1, $U = 52.0$, $p = 0.259$; Trial 2, $U = 50.5$, $p = 0.224$; Trial 3, $U = 69.0$, $p = 0.885$).

Figure 18 OT and OTA treatments do not have significant effects on maximum speeds attained.

Figures illustrate maximum speeds attained by rats treated with (A) OT (n=13) and (B) OTA (n=11). The box plot shows median, upper and lower quartiles, and minimum and maximum values. Outliers are depicted by circles (○).

1.4 Discussion

1.4.1 Evidence of mate-choice copying in rats

While mate-choice copying has been extensively studied in many animals such as fish and birds, knowledge of mate-choice copying in *R. norvegicus* remains rudimentary [22]. Hence, it was imperative first to establish if rats display mate-choice copying behaviours.

We first established that the rats had no intrinsic neutral odour nor preference for a particular arm of the runway in the absence of social odorant cues (Figure 4). After that, demonstrator females in oestrous were used to scent-mark because rats scent-mark more significantly when in oestrous [90], and urinary odours of oestrous female mixed with a male can induce a preference for the male [1]. Female rats may have an innate preference for the arm containing the demonstrator female due to their tendency to aggregate [32] and interact with conspecifics [91]. Yet, we found that the presence of a demonstrator female and her scent marks were also insufficient to induce an increased occupancy in the respective arm of the runway (Figure 5). Therefore, the stark increase in preference for the arm containing both male and female social cues over the arm lacking female cues could be attributed to mate-choice copying (Figure 8).

1.4.2 Lack of mate-choice copying in OT- and OTA-treated rats

Previous studies found that OTKO mice did not display mate-choice copying; hence, OT's roles in mediating trust and sexual behaviours were expected to increase mate-choice copying [1]. Surprisingly, when rats were treated with OT, they seemed to make random choices rather than have a clear preference for the female-tagged male odours (Figure 8 and 15). The same was observed with rats treated with OTA (Figure 15).

One plausible reason is the dose dependency of OT's effects. OT has been reported to exert bidirectional effects depending on its dose, such as in social recognition [92], social interaction [85], and short-term olfactory memory [93]. For instance, rats given 0.5 mg/kg OT exhibited increased adjacent lying, which was not observed in rats given 0.1 and 1.0 mg/kg OT [85]. It is possible that an OT concentration of 1 mg/kg exerted a suppressive effect while higher or lower doses might enhance mate-choice copying. Alternatively, acute and chronic OT administration may lead to different outcomes as well. Multiple doses of OT in neonatal or adolescent rodents can impact social behaviours in adulthood, with broad developmental consequences [94, 95].

Complex mechanisms involving multiple receptors underlie OT's effects. Since the OTA, L-368,899 hydrochloride, did not reverse the suppression of mate-choice copying, it is possible that OT acted via receptors other than OT receptors (OTRs).

Oxytocin belongs to the nonapeptide family and is highly similar in structure with another member of this family, AVP. They differ by only two amino acids and are essential neuromodulators of many social behaviours. Their similarities in structure and function have led to speculation of cross-talk between them [96].

Subsequently, they were found to be able to work through each other's receptors [97]. Specifically, other than OTRs, OT can also bind and signal through the vasopressin 1a receptor (V1aR) [98]. Taken together, the results suggest that mate-choice copying by OT may be mediated through V1aR other than OTR. Further studies using antagonists and agonists selective for each individual receptor will be useful in pinpointing the receptors involved in mate-choice copying.

The most feasible explanation could be that occupancy in the runway was affected by changes in the locomotor activity of the rats. This idea is further elaborated in the next section.

1.4.3 Decrease in locomotion

The total distance travelled and maximum speeds attained by both OT- and OTA-injected rats decreased, indicating suppressed locomotor activity (Figure 6, 7, 10A, 12A, 17). It is unlikely that treatment with OT nor the OTA severely impeded movement since rats were also found to attain similar maximum speeds both before and after treatment (Figure 11A and 18).

Intraperitoneal injection of 1 mg/kg of OT is commonly used for a myriad of behavioural studies in rats, including social behaviour [84, 85], hunger discrimination [86] and addiction [87, 99]. Yet, only some publications reported decreases in general motor activity with such OT treatment [100-103]. OT concentrations as low as 250 ug/kg was found to be sufficient in reducing locomotor activity in male rats [104]. Others report significantly reduced locomotor activity only at 2000 ug/kg and not at 0.5 ug/kg nor 50 ug/kg [105]. Besides, antagonists of OTR (L-368,899 hydrochloride) or V1aR (SR49059) alone did not reduce ambulation counts, but when given in a cocktail with OT, led to decreased counts, implying that the lowered mobility was not a result of OT acting via OTR nor V1aR [106]. OT is a hormone with complex effects that are not yet fully understood. The possibility of OT acting via interactions and feedback mechanisms in the peripheral endocrine system to affect locomotion could be considered. On the contrary, a few publications have reported increases in locomotor activity, but only when rats were subjected to stress and tested in an open field [107, 108]. Taken together, OT and OTA do not affect the ability of rats to move (i.e., maximum speeds attained may not be affected), but suppress the locomotor activity of the rats (i.e., reduced total distances travelled).

Due to the potent effect on locomotion, the preferences displayed in the mate-choice copying experiments should be taken with caution. OT- and OTA-treated rats tend to spend more time in a presumably random arm of the runway, which

could easily be mistaken for it making a “choice.” As evidenced in Figure 15, the distribution of time spent in N* arm varied widely in trial 2 for both OT- and OTA-injected rats, as compared to trial 1.

1.4.4 Learned mate choices were not maintained

In rats that showed mate-choice copying (i.e., preference for N* arm in trial 2), no short- and long-term maintenance of such learned mate choices were observed (Figure 9). Perhaps the lack of social cues in trials 3 and 4 removed the motivation to choose. Male urine could have been provided together with the neutral odours in these memory trials. Alternatively, the information provided in trial 2 was sufficient to induce mate-choice copying, but not memory of the mate choice. For example, female sailfin mollies displayed maintenance of learned mate choice when they were allowed to observe a demonstrator for 20 min, but not two demonstrators consecutively for 5 min each, despite showing copying behaviour in both cases [31].

1.4.5 Auto-critique

Mate choices here are quantified by the rats’ occupancy in an arm of the runway. While this is commonly accepted as a proxy for copulation, it is ultimately an indirect measure of resulting mating success [32]. Further investigation using a more direct method, by measuring time spent and the probability of copulation with male rats, could be employed.

It is also noteworthy that the mate-choice copying displayed by the 2 groups of OT-treated rats seemed to differ (Figure 8 and 15). It appears that the time spent in N* for OT rats in Figure 8 is above the chance level while that of Figure 5 is below chance level. Despite no significant deviations from chance, this observation could hint at possible carry-over or learning effects since the same animals were used for both experiments. However, a carry-over effect is unlikely as animals that have learnt to copy should instead show an increased preference for the arm containing N*, contrary to the results in Figure 15. Care was taken to ensure that demonstrator females were unfamiliar to the observer rats (i.e. demonstrator females for an observer rat in the first and second experiments were different). Hence, the disparity can not be explained by familiarity of demonstrator females either. The most plausible explanation for the difference would be an intrinsic variability in preference.

1.4.6 Future studies

In light of the unexpected results, performing follow-up experiments would be valuable in determining the reliability of the behaviours observed in this study.

The protocol may be modified to address the issues previously raised. Varying concentrations and doses of OT and OTA could be included to investigate any dose-dependent effects they may have on mate-choice copying behaviour. To determine if OT acted via receptors other than OTR, V1aR antagonists such as

SR49059 could be used as well. Mate-choice copying by rats injected with an OT-SR49059 mixture would be an indication of OT signalling via V1aR. Also, more information could be provided for the rats, such as increasing the duration of the trials, as well as providing social cues in trials 3 and 4 in the form of male urine.

Alternatively, one could approach the experiment from another angle. Optogenetics or Designer Receptors Exclusively Activated by Designer Drugs (DREADD) can be utilised to selectively activate neurons involved in social behaviours and not locomotor activity [109, 110]. The suppressive effect of OT is thought to stem from signalling in the substantia nigra [105], while social behaviour is mediated by OT signalling in the medial amygdala and medial prefrontal cortex [1, 111]. Thus, by selectively activating the medial amygdala and medial prefrontal cortex, both locomotor activity and mate-choice copying behaviours should be kept intact. This approach could also provide an insight into the neural pathways through which OT acts via to induce mate-choice copying.

Chapter 2

Mate-choice copying in female rats infected with

Toxoplasma gondii

2.1 Introduction

2.1.1 Behavioural manipulation hypothesis

Parasites and hosts share an intimate relationship; parasites can evolve to control some aspects of their hosts' behaviour to facilitate their transmission [112]. The resulting host behaviour is believed to be under a parasite's genetic control, thus representing a parasite's "extended phenotype" [113].

Host manipulation by parasites is not uncommon. Grasshoppers and crickets infected with hairworms display erratic water-seeking behaviour, then 'commit suicide' by jumping into an aquatic environment, enabling the worm to swim away to look for mates [114]. Carpenter ants under the control of the *Ophiocordyceps unilateralis* fungus climb down from their typical habitats up in rainforest trees to lock jaws on sapling leaf veins at prime locations with the ideal humidity, temperatures, and height required for fungal spore dispersal [115]. Plants, too, are subject to manipulation by fungi and plant pathogens to deceptively attract pollinators and herbivores to aid in their spread [116, 117].

Behavioural manipulation by parasites is often discussed in terms of survival and natural selection under the assumption that both sexes experience selection pressures equally. Yet, parasite prevalence and disease expression often differ between sexes. Males generally present a higher parasite prevalence and susceptibility than females [118, 119]. Such dimorphism has been reported in a

myriad of hosts from birds [120] to mammals [121, 122]. This phenomenon is mainly attributed to the immunosuppressive effects of testosterone [119]. Since testosterone is also vital in producing and maintaining secondary sexual characteristics, the production of testosterone represents a physiological trade-off between reproductive advertisement and immune competence. This dualistic effect was believed to be a form of honest and costly sexual advertisement and communication. The idea was first proposed by Zahavi (1975, 1977) who theorised that greater investment in the advertisement relates to greater reliability of communication since sexual advertisement is costly [123, 124]. Hamilton and Zuk (1982) furthered this concept and proposed that secondary sexual characteristics advertises a male's ability to resist detrimental parasitism, whereby females would select for higher parasite resistance such that their offspring may inherit the desired immune competency [125]. In addition, males are the signalling sex in most species, putting them at greater risk of parasitic infections through eavesdropping and therefore creating sexual dimorphism [126].

Since parasites preferentially exploit gender-specific physiologies, one would expect sexual dimorphism in host manipulation and behaviours. An example is the hantaviral infection of rodents, which is more prevalent in male than female rats [127]. The virus increases aggressive behaviour only in infected males [127, 128]. However, it remains unclear if such aggression increases the chances of transmission or if it is an effect of infection [128]. While the exact mechanisms

remain to be elucidated, neuroendocrine players such as testosterone, serotonin, corticosterone are believed to be involved [127, 128].

In this study, we are interested in a classical case of “fatal attraction” by host behavioural manipulation, the *Toxoplasma gondii*-*Rattus norvegicus* association.

2.1.2 *Toxoplasma gondii*

Toxoplasma gondii (henceforth *Toxoplasma*) is an obligate intracellular protozoan that represents one of the most well-known examples of host manipulation of vertebrates. *Toxoplasma* matures and reproduces sexually only in members of the cat family (*Felidae*), undergoing gametogenesis and mating within the gut epithelium before being excreted in the cat's faeces [129, 130]. Intermediate hosts, including small birds and mammals, may then be infected via these shed oocysts, or upon ingestion of tissue cysts such as through cannibalism [131-133]. Upon entry, *Toxoplasma* reproduces asexually via rapidly-dividing tachyzoites and slowly-dividing bradyzoites. These bradyzoites encyst in the brain, liver, and muscle tissues, establishing a life-long infection of the intermediate host. The life cycle of *Toxoplasma* is complete when the cyst re-enters its definitive feline host via the food chain [130, 134]. Rats have a strong innate aversion to feline urine odours. Laboratory rats that have not been exposed to cats for hundreds of generations still exhibit increased anxiogenic responses up to an hour after felid scent exposure [135]. Yet, rats infected with *Toxoplasma* seem to have a fatal attraction to these felid odours [136]. Such an attraction is feline-specific and is not observed for odours of non-predatory mammals such as rabbits [6, 136], nor predators that are not definitive hosts, including minks, dogs, and foxes [136]. More recently, *Toxoplasma*-infected chimpanzees showed specificity in their loss of fear to cat odours as well. Infected chimpanzees increased their approaches to

areas scent-marked by their natural predator, the leopard, but not to other big cats like lions and tigers [137]. Thus, it is widely believed that *Toxoplasma* had evolved mechanisms that alter behaviour to enhance parasite transmission back into its definitive feline host [6, 7].

Humans may also be infected with *Toxoplasma*. Infected undercooked meat, cat litter, contaminated water or soil, or congenital transmission can cause toxoplasmosis [138]. Extant humans represent a dead-end host, but our ancestors may once have been preyed upon by large felines and thus served as intermediate hosts for *Toxoplasma* [137]. Toxoplasmosis affects 30-50% of the world population [139]. The behavioural effects of a latent infection are dependent on sex [140]. Most cases of infection are asymptomatic, but toxoplasmosis can be life-threatening to immunocompromised patients and unborn children [139]. Infected children experience relapsing disease such as chorioretinitis, while patients deficient in T-cell mediated immunity face a high risk of encephalitis and disseminated infections [141]. *Toxoplasma* preferentially infects the central nervous system and has been associated with higher frequencies of psychiatric diseases, most notably, schizophrenia [142, 143]. These associations between infection and behavioural changes and disorders remain largely correlative rather than causative [139, 144]. More studies in animal models will help to strengthen these associations and possible causations. Uncovering the underlying proximate

mechanisms of infection would be a substantial step to understanding the observed behavioural changes as well.

2.1.3 Proximate mechanisms in *Toxoplasma* infection

The main argument against behavioural manipulation by *Toxoplasma* is that behavioural changes are merely side effects of infection [145]. Sick rodents generally exhibit weight loss, unkempt fur, and lethargy. They are unlikely to expend energy on costly behaviours such as aggression and mate choice. Yet, *Toxoplasma*-infected animals do not display these behaviours; they groom normally, put on weight (Supplementary figure 1), and compete for mates as their healthy counterparts do [146]. Some speculated that infection might disrupt olfaction in rodents, or that it could affect both innate and learned defensive behaviours, but these hypotheses have been refuted [146, 147]. Others are concerned if cat hosts are even required in *Toxoplasma*'s life cycle [148]. Could they be maintained via vertical transmission and carnivory without predation by their definitive host? These are all valid concerns that can be addressed with a deeper understanding of the mechanisms behind *Toxoplasma* infection. By elucidating these pathways, one can also probe further into the historical benefits brought about by behavioural manipulations. Three main hypotheses have been proposed to explain the mechanisms behind behavioural manipulation by *Toxoplasma* [149].

The first is the tropism model. It theorises that the presence of *Toxoplasma* in specific brain regions drives behavioural changes [150]. Specifically, *Toxoplasma* cysts were found in the nucleus accumbens, ventromedial hypothalamus, and amygdala [149, 151], areas which are involved in reward motivation, fear, and decision-making [152-154]. However, further studies revealed inconsistencies in the quantities and spread of the parasites in these brain regions. Cyst burdens were highly variable, leading to suggestions of a probabilistic distribution rather than selective tropism [155]. Rodents also exhibit fatal attraction to cat urine long after parasite clearance, suggesting a more indirect effect by the parasite [156]. Moreover, this theory fails to explain why other functions of the pinpointed brain regions, including neophobia to new foods, learned fear and social status, remain [157].

Shifting away from the tropism model, it has been proposed that dopamine is involved. Tissue cysts isolated from the brain are high in dopamine, and coculturing dopaminergic cells with *Toxoplasma* increases dopamine secretion [158]. Blocking dopamine binding to its receptors also improves aversion to predator scents [7]. Two genes (AAH1 and AAH2) which have similar sequences to tyrosine hydroxylase, a mammalian enzyme essential in the rate-limiting step in dopamine synthesis [159], may be responsible for the hyperdopaminergic drive in the brain. Yet, the link between *Toxoplasma* infection and dopamine levels has not been widely reproducible [160].

The latest hypothesis posits that *Toxoplasma* shifts the host away from defensive behaviours towards sexual behaviours [149]. In uninfected rats, exposure to cat odour increased c-fos activation in the posterior ventral medial amygdala (MePV), the brain region involved in regulating responses towards threats [161]. Infected rats, on the other hand, showed an atypical activation in the posterior dorsal medial amygdala (MePD), which is involved in mediating responses to sexual cues [161]. The “leakiness” between these adjoining regions in the amygdala enabled the parasite to exploit the sociosexual pathway to manipulate host behaviour. The proposed mechanism involves epigenetic modifications. *Toxoplasma* invades testes and upregulates testosterone synthesis in the Leydig cells [162]. This increase of testosterone in infected rodents is linked to hypomethylation of AVP receptors in the MePD, which in turn heightens sociosexual motivations even when presented with cat odours [3, 4]. Moreover, higher testosterone production also increases the production of major urinary proteins (MUPs) [88] which enhances a male’s attractiveness to potential mates and potentially increases the chances of sexual and vertical transmission of the parasite [81, 163]. Sexual transmission, however, remains a topic of debate due to conflicting findings [163-165].

Of the three hypotheses, the third appears to be the most parsimonious. Yet, a significant gap exists. The mechanism is non-analogous in female rats. Unlike male rats, the resultant change in behaviour in female rats is not attributed to gonadal

steroids nor AVP [5]. The exact mechanisms underlying the behavioural change in females remain hitherto unknown [5].

2.1.4 Significance of sexual dimorphism

Historically, women are seen as “hormonal” and treated as inferiors [166]. This unhealthy patriarchy has found its way into scientific studies and affected experimental designs, leading to a severe imbalance in the understanding of male and female behaviours [166]. It is becoming increasingly clear that biological sex differences can vastly affect brain and behaviour [167]. Synapses of the amygdala, an essential node in the mediation of social behaviour, are organised differently and vary in miniature excitatory postsynaptic potential frequencies between male and female rats [168]. In another critical region of the social behaviour network, the bed nucleus of stria terminalis, is sexually dimorphic in volume, neurochemistry, and function [169]. Sex influences on brain anatomy, chemistry, and functions are widespread and should be taken into consideration to progress our understanding of neuroscience and to improve neurological health [166, 170, 171].

2.1.5 Specific aims

This study is an attempt to bridge the gap in the proximate mechanisms of *Toxoplasma* infection in female rats. Previous studies in our lab revealed that infection increases OT and OTR mRNA transcript levels in the MePD, implying enhancement of OT/OTR activity by *Toxoplasma*. Ovariectomy and estradiol supplementation did not reverse fatal attraction to cat odour, suggesting that behavioural changes were independent of gonadal steroids [5]. Hence, we aimed to further investigate, as an extension of Chapter 1, whether:

1. Female rats infected with *Toxoplasma* display mate-choice copying behaviour.
2. The behaviours displayed by infected rats match those observed in OT-treated rats.

2.2 Materials and Methods

2.2.1 Animals

Adult female Wistar Han rats were procured from InVivos, Singapore. They were housed in groups of 2 to 3 in the NTU vivarium and maintained on 12/12 light/dark cycle (lights on at 0700h) with food and water *ad libitum*. All testing occurred during the light phase. The experiments were reviewed and approved by the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC). All animals were acclimatised in the vivarium for at least two days before experimentation.

2.2.2 Infection

This study uses the Type 2 Prugniald strain of *Toxoplasma*. Tachyzoites were maintained on human foreskin fibroblasts (HFF). Seven-week-old female rats were injected intraperitoneally with 5 million syringe-lysed tachyzoites suspended in 500 µl 1X phosphate-buffered saline (PBS) while control animals were injected with only PBS; experimenters were blinded to the injection solutions. Animals were monitored weekly for signs of illness. Seven weeks were given for the infection to reach the chronic stage before the start of any behavioural experiment.

Infection status was determined using the serological detection of anti-Toxoplasma IgG antibodies (Supplementary Figure 3). Blood serum was obtained by

centrifugation of clotted whole blood at 2000 g for 10 min in a refrigerated centrifuge. The supernatant was transferred to a clean tube and stored at 4°C for a few days, or in aliquots at -80°C for long-term storage. HFF cells were grown in 24-well plates and infected with *T. gondii* for 48h. When almost all HFF cells were infected after 2 days, the cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min at 37°C, incubated with 0.1% Triton X-100 for 5 min at 37°C, blocked with 3% bovine serum albumin (BSA) for 1h at room temperature and incubated in serum (1:1000) overnight at 4°C. Between each step, cells were carefully washed with 1X PBS (3 x 5 min). The sera of infected animals should contain anti-*Toxoplasma* IgG antibodies which can be detected using immunofluorescent Cy3-conjugated goat anti-rat IgG antibodies (Merck; 1:200, 2h at room temperature). Since the *Toxoplasma* strain expresses GFP, colocalisation of green and red fluorescence indicated a successful chronic infection of the animal.

2.2.3 Mate-choice copying

Two identical runways consisting of opposing and identical arms with central start boxes and end goal boxes were used (Figure 2). Animals were habituated in the arena for two consecutive days for 10 mins each day before testing. Oestrous status was determined using vaginal lavage (detailed in Section 1.2.2), and only rats in oestrous were tested. Rats underwent three trials to determine their preferences for the odours presented (Table 2, Figure 19). In light of the absence of both short- and

long-term memory of preference as shown by uninfected rats in Chapter 1, Section 1.3.2.2, trial 4 was excluded from this experiment. All trials took place under low-light conditions and were videotaped from the top view. Experimenters were blinded to infection status of the rats.

Trial	Odour 1	Odour 2
1	N*	N
2	N* + EF + M	N + M
3	N* + M	N + M

Table 2 Odours presented to infected and control rats.

N*: neutral odour associated with an oestrous female; N: alternative neutral odour; EF: oestrous female odour; M: male odour.

Figure 19 Timeline of experimental trials.

2.2.4 Statistical analysis

Occupancy in each arm of the runway was measured with AnyMaze software. Rats that did not pass through the centre of the runway at least twice were excluded from analyses. IBM SPSS Statistics 23 was used to analyse the data. Collection and processing of all data were randomised and blinded to the experimenters.

2.3 Results

2.3.1 Test for intrinsic preferences and possible biases

Trial 1 was conducted to determine if infected and uninfected rats displayed an intrinsic preference for N*. One-sample t-test on the time spent by infected ($t(10)=1.079$, $p=0.306$) and uninfected ($t(10)=-0.002$, $p=0.999$) rats in the arm containing N* showed no significant deviation from the chance level of 300s (Figure 20). There was no significant difference in time spent between the two groups either (Independent t-test: $t(20)=0.757$, $p=0.458$; Figure 20).

Figure 20 Female rats do not have an intrinsic preference for the neutral odours used. The figure illustrates the time spent in the arm containing N* by infected (n=11) and control (n=11) rats in trial 1. Bar chart depicts the mean $\pm$ SEM. Y=300s is the chance level.

To test for the presence of social or sexual preference for male odours, a setup similar to that of trial 1 was used, with the neutral odours replaced with equal volumes of male urine or water. Interestingly, female rats did not spend more time near the male urine, implying lack of mate choice (one-sample t-test: infected: $t(11) = 0.576$, $p = 0.576$; control: $t(11) = -1.283$, $p = 0.226$; Figure 21). Time spent did not differ between both groups (independent t-test: $t(22) = 1.381$, $p = 0.181$).

Figure 21 Female rats do not show a preference for male urine. The figure illustrates the time spent in the arm containing male urine by infected (n=11) and control (n=11) rats over 600s. Bar chart depicts the mean $\pm$ SEM. Y=300s is the chance level.

2.3.2 Mate-choice preference of *Toxoplasma*-infected females

The preference of the rats for a female-associated male odour was quantified by the time spent in the arm containing N* in trial 2. The time spent in trial 2 for both infected and uninfected rats differed significantly from chance level, suggesting mate-choice copying (one-sample t-test: infected: $t(10) = 7.706$, $p < 0.001$; uninfected: $t(10) = 2.553$, $p = 0.029$; Figure 22). Infection status did not significantly affect time spent in N* for trial 2 (Independent t-test: $t(20) = 0.627$, $p = 0.538$).

Figure 22 Infected and control rats displayed mate-choice copying.

The figure illustrates the time spent in the arm containing N* by infected (n=11) and control (n=11) rats in trial 2. Bar chart depicts the mean $\pm$ SEM. Y=300s is the chance level. One-sample t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3.3 Short-term memory of preference

Comparison of the time spent in the arm containing N* in trial 3 against chance level suggested that both infected and uninfected rats did not display short-term memory of preference (one-sample t-test: infected: $M= 290.291$, $SD= 55.356$, $t(10)= -0.582$, $p= 0.574$; uninfected: $M= 287.009$, $SD= 38.015$, $Z= -0.800$, $p= 0.424$; Figure 22). Both groups did not differ significantly in the time spent in trial 3 (independent t-test: $t(20)= 0.162$, $p= 0.873$; Figure 23).

Figure 23 Infected and control rats do not exhibit short-term memory of preference. The figure illustrates the time spent by infected (n=11) and uninfected rats (n=11) in the arm containing N* in trial 3. Bar chart depicts the mean $\pm$ SEM. Chance level is depicted by the dotted horizontal line at 300s.

2.4 Discussion

2.4.1 Evidence of mate-choice copying in *Toxoplasma*-infected rats

We investigated if *Toxoplasma*-infected female rats would display mate-choice copying behaviours and if these behaviours were similar to those observed in OT-treated rats. The results of trial 1 assured us that the rats did not have intrinsic preferences for the neutral odours nor for particular arms of the runway in the absence of social cues (Figure 20). The presence of a demonstrator was insufficient to increase occupancy in the respective arm of the runway (Chapter 1, Figure 5).

Rats did not seem to prefer male urine over water either (Figure 21). Taken together, both infected and control rats did not display a strong preference for female social cues nor for male odourant cues. Therefore, the increase in occupancy in the arm containing N* in trial 2 can be attributed to mate-choice copying (Figure 22). *Toxoplasma* infection left mate-choice copying behaviours intact. The strength of mate-choice copying in infected rats was greater than uninfected rats, though not significantly.

OT is said to mediate mate choices because OTKO mice make random mate decisions [2]. It was proposed that *Toxoplasma* infection atypically increases OT (and OT/OTR activity). However, whether infected rats would show greater mate-choice copying or display typical mate-choice copying behaviours due to a ceiling effect remains debatable. The results from Chapter 1 seem to suggest that OT

supplementation beyond normal levels reduces or eliminates mate-choice copying, but this could be the result of the dose-dependent effects of OT and the unexpected changes in locomotion post-treatment (Section 1.4.2). It would be useful to study the effects of chronic OT administration as well since the *Toxoplasma* infection manifests as a latent rather than acute infection.

2.4.2 Mate choice preference was not retained

In Chapter 1 (Section 1.4.4), we suggested that the lack of preference in the memory trials could be due to the insufficiency of social information provided. Hence, male odourant cues were provided in the short-term memory trial (trial 3). Yet, both infected and uninfected rats did not seem to retain the preference either (Figure 23). Rats have been known to sport impressive olfactory and episodic memory [172, 173] and should theoretically be able to remember their mate-choices both over short and long periods. It would be useful to determine if this was due to inadequate time given to commit mate choice preference to memory, such as in the case of the sailfin mollies [31].

2.4.3 Further studies

The current findings are insufficient for us to conclude if OT plays a role in *Toxoplasma* infection of female rats. Follow-up studies would be useful in providing a more definitive answer. A simple approach would be to compare the behaviours of OT-treated and *Toxoplasma*-infected rats towards cat odour (Chapter 3).

Chapter 3

Study of aversion to cat odours in *Toxoplasma gondii*-infected and oxytocin-treated female rats

3.1 Introduction

3.1.1 *Toxoplasma* infection in female rats

Behavioural manipulation by parasites is common. One of the most well-established examples of host manipulation is *Toxoplasma*. Felid urine odours are known to evoke a loss of avoidance response in rodents [136]. Rodents exhibit a “fatal attraction” specifically to these feline odours, and not urines of predators that are not definitive hosts, such as minks, dogs, and foxes [136]. Such behaviour is believed to be a parasite-evolved mechanism which allows *Toxoplasma* to be transmitted back into its definitive feline host by increasing predation rates on infected intermediate hosts [6, 7].

The proximate mechanisms behind such “fatal attraction” remain a mystery. The most parsimonious explanation is that testosterone hyperproduction in male testes results in hypomethylation of AVP promoters in the MePD and an atypical increase in sociosexual motivations even in the presence of cat odours [3, 4]. Evidently, this theory would not work in females due to sexual dimorphism. Gonadal steroids and AVP are not involved in behavioural manipulation of female hosts [5]. A gap in understanding of the exact mechanisms in females still exists [5].

3.1.2 Specific aims

We hypothesise that OT may play a fundamental role in behavioural manipulation in *Toxoplasma* infection. Previous findings point to the possibility of an atypical increase of OT/OTR activity by *Toxoplasma* in the MePD, the brain region involved in sociosexual behaviours, shifting rat behaviour from defensive to sexual when presented with felid urinary odours. In this chapter, we aim to:

1. Determine if *Toxoplasma*-infected rats display a loss of avoidance response to cat urine.
2. Determine if OT-treated rats display a fear response to cat urine.
3. Analyse using (1) and (2) whether OT might be involved in “fatal attraction.”

3.2 Materials and Methods

3.2.1 Animals

All animals used in this study are those that have undergone mate-choice copying assays, as discussed in Chapters 1 and 2 and were 22 and 18 weeks old, respectively.

3.2.2 Cat odour aversion assay

The same rectangular arena (Figure 2) was used in this experiment. Cat odour aversion was measured by the occupancy of the rat in each arm in 10 mins, with each goal box containing either 2 ml undiluted bobcat (Maine Outdoor Solutions) or an equal volume of horse (Bukit Timah Saddle Club) urine on a paper towel. Between each trial, the apparatus was cleaned thoroughly with 70% ethanol and urines were replenished.

3.3 Results

3.3.1 OT does not alter aversion to cat odour

Two of the five rats treated with OT failed to cross the centre line at least twice and were excluded from the analysis. PBS-injected rats did not show aversion to cat urine (one-sample t-test: $t(4) = -1.663$, $p = 0.172$; Figure 24). The same can be seen in OT-treated rats (one-sample t-test: $t(2) = 0.598$, $p = 0.610$; Figure 24). The difference in time spent between the two treatment groups was not significant (independent samples t-test: $t(6) = 1.117$, $p = 0.307$).

Figure 24 Aversion to cat odours by OT-treated and control rats at different bobcat urine volumes.

The figure illustrates the time spent by (A) OT-treated ($n=3$) and (B) control rats ($n=5$) in the arm containing cat urine at various urine volumes (1, 2 or 3 ml). Bar charts depict mean $\pm$ SEM. Chance level is depicted by the dotted horizontal line at 300s.

3.3.2 Infected rats did not show fatal attraction

The time spent by control and infected rats in the arm containing 2 ml cat urine was not significant (one-sample t-test: control: $t(11) = 0.541$, $p = 0.599$; infected: $t(11) = -0.935$, $p = 0.370$; Figure 25). Infection status had no effect on cat aversion (independent t-test: $t(22) = -1.074$, $p = 0.294$).

Figure 25 Infected and control rats do not differ in cat odour aversion. The figure illustrates the time spent by infected ($n=12$) and control ($n=12$) rats in the arm containing cat odour. Bar chart depicts the mean $\pm$ SEM. Chance level is depicted by the dotted horizontal line at 300s.

3.4 Discussion

3.4.1 Lack of aversion towards cat odour

All the tested rats did not display significant aversion towards bobcat urine (Figure 24 and 25). The lack of aversion in even the control rats is puzzling; either (1) the rats were not fearful towards cat odours, or (2) their aversion towards horse urine was equal to that of bobcat urine. It is highly unlikely that the rats were not averse to cat urine, as this innate fear has been widely studied and replicated, especially in the study of *Toxoplasma* [4, 5, 147, 161, 174]. Uninfected rats prefer rabbit urine over cat odour, while infected rats are known to be fatally attracted to cat urine [146].

In this study, rabbit urine was replaced with horse urine due to the unavailability of rabbit urine. Theoretically, horse urine should not induce an aversion in rats as horses are herbivorous and would not naturally prey on rats. A rat's fear of predator odours is attributed to enriched concentrations of 2-phenylethylamine (PEA) in carnivore urines [175]. Like many carnivores, bobcat urine contains high levels of PEA (20.3 μ M) [175]. On the contrary, the urines of horses and rabbits contain less than 0.1 μ M PEA [175]. When PEA was removed from lion urine, aversion responses by rats were eliminated, showing that urine-evoked avoidance was due to PEA [175]. Therefore, it is improbable that the horse urine evoked the same avoidance response rats show towards bobcat odours.

A possibility could be that the difference in urine collection methods of the bobcat and horse urines resulted in differing potencies. The horse urine was collected fresh by holding a container under a horse while it micturated, and frozen immediately. On the other hand, the bobcat urine was sourced from the Predator Pee Store, where urine is collected from floor collection drains in pens and cages, and then shipped from the USA to Singapore. It was frozen upon reception. Urine is full of odourant information, many of which are volatile compounds. For instance, the key component of carnivore odour that rodents avoid, PEA, is a moderately volatile compound with the vapour pressure of 35 Pa [176]. As such, it is likely to be ineffective over a long time [176]. The nature of bobcat urine collection suggests that the urine may have been left in the open for too long, leading to the dissipation of these crucial volatile compounds. Experimenters also noted that the smell of bobcat urine was not as strong as they had expected (as compared to previously used batches of bobcat urine). Therefore, the lack of aversion to the bobcat odours could be due to the insufficiency or absence of aversive volatile compounds in the bobcat urine.

It would be useful to repeat the experiment with a fresh batch of cat urine that is locally sourced or collected. Cat collars could also be used [146]. A follow-up test could also be done to determine if the bobcat urine used in this study does elicit an avoidance response in rats and whether the horse urine is aversive to them as well.

For instance, by having each rat undergo two trials with either cat or horse urine at one end of the runway and a plain paper towel on the other end.

3.4.2 Future studies

The sample sizes of the rats treated with OT and PBS control is too small for us to draw definite conclusions. Nevertheless, the results are useful in providing an indication of the aversive responses of the treated rats towards cat odours. Other than repeating the experiment with a larger sample size, I suggest a few other methods that could be employed for further study.

Immunohistochemistry would be useful in determining if OT is involved and in pinpointing the brain regions activated when female rats are presented with cat odour. If our hypothesis of atypical activation of OT/OTR system in infected rats is correct, antibody staining of brain sections would reveal more colocalisation between OT and c-fos in the MePD in infected rats as compared to uninfected rats. Novel imaging techniques such as electroencephalogram radiotelemetry could also offer a glimpse into the brain regions activated *in vivo* [177].

Conclusion

In recent years, it is becoming increasingly clear that sex is an important biological variable. The lack of female representation in preclinical studies could skew our understanding of neuroscience and might adversely affect neurological health [166, 170, 171]. There is growing evidence of sexual dimorphism in behavioural traits and brain anatomy, chemistry and functions [170]. Parasites may exploit sexually dimorphic mechanisms to their advantage. In *Toxoplasma gondii* infections, the parasite manipulates rodent behaviour towards felid odours. While *Toxoplasma* is believed to hijack testosterone production in male hosts to cause a shift of behaviours away from defensive to sociosexual [3, 4], the levels of these intrinsically “masculine” substrates remain unchanged in infected female rats [5]. Preliminary findings suggest that the OT/OTR system may be exploited in infected female rats. OT is a vital neuropeptide mediating a plethora of social behaviours, including maternal care, social bonding, and mate choice [1, 65-67]. Could *Toxoplasma* hijack this system to make innately aversive feline scents attractive to infected female rats? It is also likely that OT mediates mate-choice copying which is an important form of social learning in sexually reproducing species. Despite OT being a widely used drug (mainly in childbirth), the mechanisms behind its actions are not fully comprehended.

This thesis is an attempt to bridge the gap in understanding of the proximate mechanisms behind mate-choice copying and *Toxoplasma* infection in female rats with a focus on OT. However, we were met with many unexpected hurdles. The main issue was that OT and OTA treatment had an adverse sedative effect on the rats, which severely affected the accuracy of their preferences in the mate-choice copying and cat odour aversion tests. As a result, it was difficult to ascertain the role of OT in *Toxoplasma* infection using a behavioural approach as employed in this thesis.

Alternative approaches should be sought to avoid the problems surrounding pharmacological supplementation of OT and OTA. The necessity of OT expression in mediating behaviour could be studied using OTKO rats, similar to the study by Kavaliers et al. (2017). Conversely, the sufficiency of the OT/OTR system in behaviour change could be determined via OT overexpression in uninfected rats. Optogenetics and DREADD could be used to selectively activate neurons in the MePD to determine if this brain region is responsible for the change in the observed behaviour. The stimulation could be done in mice, rats or other transgenic animals. Finally, identifying the downstream regions and substrates involved would considerably deepen our understanding of the proximate mechanisms behind mate-choice copying and *Toxoplasma* infection in female rats.

We hope the findings of this study would add to the growing literature on OT. An increased understanding of OT's effects would open up possibilities for diagnoses

and treatments of social disorders such as autism [111]. From an evolutionary point of view, knowledge of OT could give insight into the co-evolution of parasites and their hosts. Further clarity of OT's effects would also shine light on the behaviours of social animals and how it may confer them a selective advantage or improved fitness.

Annexe

I Abbreviations

AVP	arginine vasopressin
BSA	bovine serum albumin
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
HFF	human foreskin fibroblasts
KO	knockout
MePD	posterior dorsal medial amygdala
MHC	major histocompatibility complex
MUPs	major urinary proteins
N*	neutral odour tagged to female scent
OT	oxytocin
OTA	oxytocin antagonist, L-368,899 hydrochloride
OTR	oxytocin receptor
PBS	phosphate buffered saline
PEA	2-phenylethylamine
PFA	paraformaldehyde

SEM standard error of the mean

V1aR vasopressin 1a receptor

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III Supplementary information

Supplementary figure 1 Preference of female rats for 4 unfamiliar non-social odours.

This figure illustrates the time spent by female rats in the arm containing an unfamiliar odour. For each scent, n=4. Bar charts depict mean $\pm$ SEM. Chance level is depicted by the dotted horizontal line at 300s.

Scent	N	Mean	Std. Deviation	Std. Error Mean
almond	4	305.25	46.176	23.088
banana	4	261.50	59.164	29.582
chocolate	4	320.25	59.033	29.517
peppermint	4	310.50	29.034	14.517

Supplementary table 1 Statistics of preferences for the unfamiliar scents.

The table shows the mean, standard deviations and standard error of mean of the time spent by female rats in the arm containing the respective unfamiliar odours.

Four unfamiliar scents were tested to determine the two scents that should be used for the mate-choice copying experiment. The 4 scents (almond, banana, chocolate and peppermint) were chosen as they are non-social and novel odours. Female rats were presented five 10ul drops of one of the four scents (diluted 1:10 in water) or water along the opposing arms of the runway. They were videoed for 10 min and the time spent in each arm was quantified. For all scents, there was no significant preference for the unfamiliar scent over that water, showing that the rats found them to be neutral (one-sample t-test: almond: $t(3) = 0.227$, $p = 0.835$; banana: $t(3) = -1.301$, $p = 0.284$; chocolate: $t(3) = 0.686$, $p = 0.542$; peppermint: $t(3) = 0.723$, $p = 0.522$; Supplementary figure 1). Almond and peppermint were chosen for subsequent experiments as their means were closest to 300s and they displayed the lowest variance in time spent (Supplementary table 1).

Supplementary figure 2 Infection does not affect weight gain in female rats.
Line chart depicts the mean weights of infected (n=12) and control (n=12) rats from 8 to 19 weeks of age.

Supplementary figure 3 Serology to determine infection status. *Toxoplasma* expresses GFP. Anti-*Toxoplasma* antibodies in rat serum were detected with Cy3-conjugated goat anti-rat IgG secondary antibodies. Colocalisation indicates a successful infection of the animal.

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