Parasitic manipulation of male sexual advertisement in *Toxoplasma gondii*- *Rattus norvegicus* association

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2016

Vasudevan, A. (2016). Parasitic manipulation of male sexual advertisement in *Toxoplasma gondii*- *Rattus norvegicus* association. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/68901>

<https://doi.org/10.32657/10356/68901>

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Jan 2016

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A thesis submitted to the Nanyang Technological
University in partial fulfillment for the degree of Doctor
of Philosophy

Acknowledgments

I would like to thank my wife, Prabha for her constant support during my PhD. I never thought that when I began my PhD that I would also find my life partner.

This study was carried out with the financial aid of the School Of Biological Sciences, NTU and the Ministry of Education under the supervision of Assoc Prof Ajai Vyas. He was an excellent mentor always ready to listen without ever being too imposing, allowing me to think on my own. I would also like to thank my lab mates with whom I have worked in close collaboration. First, Shantala- we need did many of the initial behavioral experiments and Vineet who produced the recombinant protein for the behavior experiments and sequenced MUPs clones. I would also like to thank Donna for her revamp of the behavior room into a more professional setup that made experiments easier to conduct. Also I would like to thank Samira for help with castration surgery. And Linda, who was really good at improvising and coming up with novel ways to design animal behavior setups. I also would like to thank John Tayki Williams for his help with LCMS and Prof Joanne Yew for conducting the DART-MS experiment.

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Abbreviations

Arginine vasopressin	AVP
Complex life cycles	CLC
Diethylpyrocarbonate	DEPC
Direct analysis in real time mass spectrometry	DART-MS
Fast protein liquid chromatography	FPLC
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH
Green fluorescent protein	GFP
High molecular weight fraction	HMW
Liquid chromatography mass spectrometry	LCMS
Low molecular weight fraction	LMW
Major Urinary Proteins	MUPs
Paraformaldehyde	PFA
Phosphate buffered saline	PBS
Reverse-Transcription Polymerase Chain Reaction	RT-PCR
Simple life cycles	SLC

Abstract

Parasitic manipulation of male sexual advertisement in *Toxoplasma gondii*- *Rattus norvegicus* association

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Parasitic manipulation of host behavior is often exploitative in nature as they seek to derive benefits in terms of food, shelter or as a conduit to another host. An example is *Toxoplasma gondii* that has shown to alter the innate fear in rats toward cat odors, increasing its chances of transmission. This is critical for the parasite to complete its two-stage life cycle. Host-parasite interactions are a constant battle in which each player tries to outfox the other. So how has *T. gondii* evolved and sustained such a detrimental (for the rat) behavioral manipulation? This could be possible if *T. gondii* offers some benefits/ trade-offs to the rat. To this end, I have shown that *T. gondii* manipulates mate choice, causing females to prefer infected male rats and is also sexually transmissible. Thus, a parasite that can enhance reproductive opportunities of a host will benefit from blunted selection pressure and gain advantage of transmission. This is unique as females detect and avoid parasitized males based on phenotypic traits to reduce chances of spreading infections and choosing for heritable resistance. In rodents, such social information can be relayed by male odors, often via urinary cues. A putative candidate for a rat sexual signal is Major Urinary Proteins (MUPs) - it is secreted in copious amounts in the urine, a common communication means for rats and hence a good way to advertise suitability as a mate. I show that *T. gondii* increased levels of MUPs in the liver and urine of infected rats. Moreover, I demonstrated the sufficiency and necessity of MUPs to attract females and that preference is dose dependent manner. Subsequently, a potential cost of greater MUPs expression is easier detection by possible predators or prey. I

showed that mice prey can detect and avoid infected male rat urine. Moreover, they were able to discriminate differing concentrations of rat urine (and MUPs) in a dose dependent manner. One of the main themes of sexual selection puts forward that sexual signals reflect condition of the individual. Expression of traits is dynamic in nature and can accurately reflect condition as signaling often depends on the same internal and external factors, hence the phrase condition dependence. As such, expression would vary in different contexts and be restricted when the cost of signaling becomes too high. I studied three such condition altering factors- age, health and social environment. MUPs expression decreases with age and due to sickness induced by lipopolysaccharides (LPS). Moreover, presence of a challenger (animal supplemented with testosterone) caused the biggest increase in MUPs levels, however, when exposure females (while co-housed with challengers) led to sharp decline in MUPs. This fits into the idea of an individual increasing signaling only in conditions or contexts where the benefits outweigh the costs. In this way, the dynamism of signaling can accurately reflect condition or status of the individual and ability to manage the cost-benefit associated.

1. Introduction

Parasitism refers to the exploitative relationship between two species, where the parasite benefits at a cost for the host. In contrast to predators, parasites do not kill the host immediately and can cohabitate in their hosts for as long as years (1). Parasitism is widespread across kingdoms (with most animals serving as a host) and evolved convergently across phylogenies (2-4). Parasites often have a detrimental effect on fitness of the host either by a generalized effect of illness/sickness behavior or specific pathology like parasitic castration (5). This refers to the ability of a parasite to inhibit reproduction of the host for example, the crustacean *Hemioniscus balani* castrates barnacles by feeding on the host's ovarian fluid, causing a loss of reproductive ability (5). The parasite can tap into the energy and resources spared by restricting the host's reproduction (energy that would be otherwise invested in development of sexual signals, courting, competition and parental care) (2). In this way, parasites improve their own fitness by exploiting hosts to derive benefits in terms of food, shelter and as a conduit to another host (from prey to predator) (4). However, parasitism is not always harmful to the host- a form of commensalism or mutualism may develop in co-evolving partners, as it is beneficial for the parasite if the host survives (6). In humans, our gut is full of microflora that serves useful functions such as digesting unused energy substrates (7), aid cell growth and stunt dangerous microorganisms (8). Gut microbiota can also teach immune system to recognize pathogens, develop tolerance to helpful bacteria (9, 10) and protect against certain disease (11).

From the parasite's perspective, often a host is a temporary stop in its quest to complete its multi-host complex life cycle (CLC) (12). Such life cycles have evolved in parallel in numerous parasites from single simple life cycles (SLC) (13-15). In CLC, parasites have distinct morphological phases, which utilize different habitats or hosts (1, 16). Trophic

transmission is a common means of transfer between hosts where a definitive host predates upon the intermediate host (12). As such, CLC depends on predator-prey (predation) interactions. Some suggest that under certain conditions the addition of a host to SLC possibly enhances transmission and thereby fitness benefits (i.e. adaptive), while others claim CLC are caused by changes to ancient events or simply accidental (17-20). This evolution would depend on whether the current intermediate or definitive host served as the ancestral host in the SLC, as ecology and selection pressures to add hosts to life cycle would vary in each case (17). In the case of an ancestral intermediate host, predation by a new species would lead to evolution of trophic transmission under pressure to bypass predation of their hosts and parasitize the predator to avoid dead end hosts (17, 21). Moreover, the addition of a predator to the life cycle increases the probability for parasites to find a sexual partner (12). Parasites (even those that are hermaphrodites) prefer sexual reproduction due to fitness benefits such as quantity and/or viability of offspring (15, 22-24). However, locating a mate is a problem for parasites if they are in low density in the host and their inability to move freely hinders mate finding. Thus, finding a mate in an intermediate host is often random for the parasite. Predators can help solve this problem as they consume bigger quantities of prey, possibly bringing together and concentrating isolated parasites in one location (predators). This would increase the chances of the parasite finding a conspecific for sexual reproduction (12).

However, these theories of predation survival and finding mates cannot explain evolution of CLC if an ancestral host is the definitive host to begin. Instead, addition of intermediate prey hosts in trophic transmission of parasites could be an adaptive means of greater dispersal or transmission (25). Similar to plants using animals as a mode of dispersal, parasites can potentially reach greater number of definitive hosts (predators) by using intermediate preys as carriers (like malaria and mosquitoes). For instance, parasites in the predator would release

its propagules into the environment. If the chance of direct transmission to other definitive hosts is lower than the probability of infecting prey and then being eaten by predators, the latter mode would be chosen (25). Such a model would work as predators would find prey more effectively than the parasites locating predators directly. Moreover, as prey population tend to be greater in numbers, it is also more likely for parasites to infect them than predators. Interestingly, death of a carrier that normally would be disadvantageous to a parasite is actually required in cases of trophic transmission. Thus, the pressures of predation can lead to the evolution of multi-host life cycles.

In the above scenarios, the critical step is the predation of the intermediate host by the correct predator. Consequently, the parasite has an incentive to manipulate host behavior in ways that would increase chances of predation and transmission and reinforce the evolution of CLC. This would affect the development and dynamics of co-evolution cycles between the host and parasite. As such, it is vital to study how the parasite and host influence each other especially as co-evolution over evolutionary time scales can lead to surprising outcomes.

Extended Phenotype

Animal behavior is under the influence of proximate factors in its environment, for example, availability of food, onset of breeding season and predation (26). These factors will interact with the internal state (e.g. hormones) of an animal to determine behavioral outcomes. For example, the onset of breeding season often causes a spike in sex steroid hormones leading to activation of sexual and territorial behaviors. In a lizard species, *Sceloporus jarrovi*, territorial aggressive behavior is associated with elevated testosterone levels even during nonbreeding-season territoriality (27). This opens up the possibility of parasites hijacking and manipulating this pre-existing machinery, altering hormonal \ or neurotransmitter levels and thereby behavior, to its own end (28-32). The wasp, *Ampulex compressa*, targets the

Chapter 1

cockroach, *Periplaneta americana*, by injecting a toxin into the brain paralyzing the central nervous system and inducing hypokinesia. This results in a loss of “normal escape behavior and motivation to walk” in the host. It allows the wasp to bury the cockroach and use it as a larvae incubator (33). Though the underlying mechanism is yet to be determined, disrupted neural communication (depletion of biogenic amines (33)) responsible for escape and motor activity is most likely to be involved.

The above example shows a phenomenon where one organism's genes (genotype) exerts influence on another organism's behavior (phenotype). This falls into the paradigm that views the gene as the basic unit of selection, passed on between generations and organisms and their phenotypes are the means of transfer of genetic material (34). Another pertinent example is seen in the suicide like behavior of grasshoppers parasitized by hairworms where a genotype exerts influence in interesting ways (35). The grasshoppers jump into water bodies so that the adult worm can escape its host to continue its life cycle. The hairworm genes control this behavior (31, 36). Clearly, the genotype of the parasite (worm) is extending its reach and expressed in the phenotype of another individual (grasshopper), that is, the phenotype is not physically constrained to the organism whose genotype is responsible. Richard Dawkins first put forward this phenomenon and gave the term ‘extended phenotype’ (37). The various ways that extended phenotype manifests are (summarized in Figure 1): firstly, animal architecture as seen in beaver dams that are physical demonstrations of behavior, which increases fitness of genes encoding the behavior (34). Even more impressive is the termite mound, which serves varied functions like nursery, waste management and egg incubation (38) and humans have become masterful at manipulating their environment to meet their needs. The second variation is action at a distance, such as pheromonal communication between insects and rodent conspecifics. In such cases, pheromones are mode of communication which does not require the signaler to be physically present when a

conspecific encounters it (39). Finally, parasitic manipulation of host behavior and its underlying physiological mechanisms as described in the above grasshopper example fit well into this paradigm. In this thesis, I studied an extended phenotype that is a combination of action at a distance and parasitic manipulation.

Figure 1. Flow chart depicting the different phenotype levels extending from the gene: within and organism phenotype and extending to other individuals.

Behavioral manipulation hypothesis

The behavioral manipulation hypothesis states that parasites will seek to manipulate the behavior of the intermediate hosts to increase chances of trophic transmission to a predator that serves as a definitive host for the parasite (21, 40). In this way, the parasite will be able to complete its life cycle. Three major evolutionary hypotheses attempt to explain the development of host manipulation. First, “manipulation *sensu stricto*”, in which parasite’s genes are responsible for modifications in host behavior enhancing parasitic transmission, i.e. extended phenotype (41). In this case, there is a clear link between parasite genes and manipulation of behavior and are selected for this ability. The grasshopper and hairworm would be such an example. Second, “mafia-like strategy”, puts forward that parasites could influence host behavior to benefit itself by imposing extra fitness costs when the host fails to co-operate. In this view, parasites influence the host to co-operate as it would be less costly than not doing so. The great spotted cuckoo and the magpie share such a relationship (42).

Cuckoos lay their eggs in magpie's nest, where the latter raises its own and the cuckoo's. Magpies that fail to do so come under higher rate of nest predation than those who raise the "parasite eggs" (43). Third, "compensatory responses", suggests that parasites could manipulate other fitness traits in hosts (growth, reproduction) to "induce host compensatory responses"(41) when such traits can also serve as potential transmission routes (44). This is in contrast to the mafia-like strategy where there is no fitness compensation to hosts. For instance, parasites that transmit directly between conspecifics could benefit from increased host reproductive output or sexual faculties (45). Greater reproductive opportunities for the host would concomitantly increase social interactions (46) with conspecifics and thereby aid parasite transfer (44, 47). For example, in sexually transmitted *Chrysomelobia labidomerae* and its host, *Labidomera clivicolis*, infected males have lower survival rate but possibly compensated with enhanced sexual behavior (48, 49). This would increase social interactions with males (competition) and females (reproductive). Thus, parasites that can achieve transmission through compensatory responses would be favored as it does not need a direct manipulative effort (*sensu stricto*) but is rather piggybacking on normal host behaviors (41). In addition, there would be less resistance from the host as it is also benefiting from parasitism-induced behaviors. Both hypotheses are not mutually exclusive but are more likely to lie on a progressive continuum without clear-cut distinctions (44).

Behavioral manipulations reflect a dynamic balance between selection pressures on co-evolving hosts and parasites, observed in many trophically transmissible parasites. *Diplostomum spathaceum*, a trematoda, affects cryptic coloration and cryptic behavior of its host, *Oncorhynchus mykiss*, making it more easily detected by birds (50, 51). The parasite causes cataract formation affecting its crypsis behavior (ability to avoid detection from predators) and leaving it open to greater chance of predation (51, 52). Similarly, another trematoda, *Euhaplorchis californiensis*, has a three-host life cycle of horn snails, killifish and

shorebirds as final hosts. Feces of shorebirds that contain parasite eggs infect horn snails. The snails release parasite larvae into marshes, where they infect the killifish and lodge in the brain cavity (53). The parasite reduces the fish's ability to avoid predators as infected killifish are four times more likely to "shimmy, jerk, flash, and surface" increasing their chances to be predated by 30-fold and completing the parasite's life cycle (21, 53). Such manipulation has been termed as "parasite increased trophic transmission" where increased transmission would correspond to increased fitness for the parasite (21).

Multidimensionality of behavioral manipulations

Manipulations of host behavior as described above seem straightforward; however, parasites rarely affect just one host phenotypic trait (41, 54). It is more likely that a wide range of traits are affected, for example, *Microphallus papillorobustus*, parasitizes on *Gammarus insensibilis*, which cause phenotypic manipulation in photophilic behavior, negative geotaxis and abnormal escape behavior (41). However, often it is unclear whether all manipulations of phenotypes are truly adaptive, that is, whether each alteration translates into increased trophic transmission or is a byproduct of infection pathology. The occurrence of multiple manipulations, either simultaneous or successively, has led to the study of multidimensionality of host manipulation by parasites (55-57). For a manipulation to be multidimensional, it must have at least two alterations in different phenotypic traits (behavior, physiology, morphology) or in the same trait (58). In addition, parasitic manipulations are often studied in context of its ability to increase parasite's fitness, specifically transmission. As such, only phenotype alterations that contribute to this end can fall under the multidimensionality category (59). In the above example of *G. insensibilis*, the parasite causes a greater inter-moult duration in addition to the other changes mentioned. However, as it is unlikely to be useful for trophic transmission, it does not fall under the multidimensionality model as proposed by some. Thus, this view looks at manipulations that

increase transmission as specific and adaptive, moulded by natural selection (panselectionist perspective) (54, 60, 61).

The contrarian view states that all parasite induced phenotype manipulations are part of the ‘infection syndrome’ (similar to clinical syndrome) and should be considered under the multidimensionality manipulation (54). This also includes manipulations that have no apparent purpose for parasite transmission. This is important, as it is always not possible to determine clearly the specific effects of all phenotype alterations in relation to trophic transmission (41). Moreover, even in hosts that display phenotype manipulation leading to increased predation susceptibility, there is not always a direct causal link between the two (54) even in tightly calibrated experiments (62). There are also might be scenarios where manipulations increase parasite fitness independent of trophic transmission. For example, there could be phenotype manipulations in the host that enhance the parasite’s survival, growth or reproduction without enhancing transmission (54). Thus, the definition of multidimensionality becomes too restrictive to only its ability to increase fitness in context of transmission. Given the lack of clarity behind the mechanisms of parasite based phenotype manipulations, it may be early to define multidimensionality explicitly (56). The different manipulations observed might arise from a single physiological process either simultaneously or sequentially. Alternatively, it may be due to distinct processes behind each manipulation. In addition, the various phenotypes could be the result of imbalance in important molecular pathways as side effects of the tussle between parasites and host immune system (32, 41, 63). There has been extensive work done in arthropods that show the role of neuromodulator dysregulation by parasites. In many organisms, neural networks are usually multifunctional (a set of neurons that respond to different stimuli for varying functions) which when affected (by parasites) would naturally cause various phenotype alterations. This could be because parasites are linked with manipulation of neuromodulators (biogenic amines- dopamine (64),

serotonin (65, 66)). In turn, this can have an effect on neurohormones that regulate physiology and behavior of arthropods (67). Detailed studies on crustaceans with acanthocephalan parasites (*G. pulex* and *P. laevis* (68-70)), have characterized many phenotype alterations and effect on neuromodulator expression, though mechanisms linking them are yet to be resolved. Thus, it is difficult to distinguish between manipulations that could be due to general dysregulation of key pathways or adaptive means to enhance trophic transmission.

Evolution of multidimensional manipulations

Natural selection would favor multidimensionality manipulation as influencing multiple traits could increase chances of transmission (41). For trophic transmission, prey showing both color and behavioral manipulations are at greater risk of predation (cestod parasites and *Artemia* shrimps (71)). However, the cost of each alteration would have to fit in context of overall effect of all manipulations on parasite fitness. In the adaptive scenario (58), multidimensionality initially involved a single manipulation to which other alterations that provided fitness benefits were added successively. The added transmission benefit would have to cover the extra costs of the new manipulation. As mentioned above (color and behavior), a new manipulation could work synergistically or decrease costs of initial manipulation to increase efficiency (for example, in *G. sensibilis* with phototaxis and abnormal escape behavior, see Box 1) (41). On the other hand, in line with the ‘infection syndrome’ hypothesis (54) another scenario is put forward on how multidimensionality manipulations evolved. In this parsimonious scenario, it is more likely that parasites produce a single compound that causes a series of effects, which include manipulations that increase transmission. The example of neuromodulators and myriad of behavioral effects in crustaceans fits in this paradigm. In this view, only the genes of the parasite that are needed to produce the compound are under selection. It will evolve in balance with the holistic

effects of all manipulations on parasite fitness versus the cost of compound production. Similar to the role of both ‘manipulation *sensu stricto*’ and ‘compensatory responses’ in evolution of host manipulation, multidimensional manipulation can be due to both panselectionism and infection syndrome as they are not mutually exclusive hypotheses.

Box 1: Possible scenarios in evolution of multiple manipulations

The example of *G. insensibilis* parasitized by *M. papillorobustus* can help us understand how multiple manipulations could have evolved. The abnormal escape behavior of *G. insensibilis* features swimming to the surface and turning at the air–water edge. This makes it vulnerable to predation though would be energetically costly. Thus, positive phototaxis might have developed as it reduces energy costs in relation to the first manipulation (abnormal escape behavior) by ensuring that gammarids stay at the water surface. However, a different evolutionary history would reveal a different sequence of events. In this case, the first change to increase transmission would be positive phototaxis. Subsequently, natural selection would favor those who can produce abnormal escape behavior in the host. In the first hypothesis, phototaxis would have evolved as a secondary manipulation without direct effect on transmission while in the second view both alterations have positive influence on transmission.

Now I will be discussing another trophically transmitted parasite that will be the focus of the thesis.

***Toxoplasma gondii*-rat model**

The protozoan parasite *Toxoplasma gondii* found commonly in rats and other warm-blooded organisms including humans (16). Post a short acute phase, the fast replicating tachyzoites infects the immune-privileged organs like brain and testes of rats, forming cysts for long periods of latent infection (16). The latent phase is characterized by slowly replicating bradyzoites (tissue cysts) which are thought to be asymptomatic. The most interesting *T. gondii* manipulation or effect (see Table 1 for summary of *T. gondii* effects) is the apparent reduction in innate fear of rats toward cat odors (measured by time spent near stimuli- urine and towel worn by cat) and a subset instead develop a fatal attraction to such stimuli (72-74).

Various behaviors that could cause increased vulnerability to predation include increased activity level (75), greater time spent near cat odor (73), impaired motor performance/reaction time (76), lowered neophobia (77, 78) and anxiety (79). This loss of fear manipulation appears to be specific as it did not affect memory, fear conditioning, spatial learning (73) and it did not cause a similar loss or gain of attraction for non-predator odors (rabbit or mink) (72, 73, 80, 81). However, there are reports of negative effects on spatial memory and learning (82, 83) that might not directly relate to increased predation (Table 1). The reasoning behind such a behavioral manipulation is to allow the parasite to increase its transmission efficiency, in this case via predation of rats by cats (trophic transmission). This is critical for the parasite as a means to complete its two stage life cycle that involves cats as the definitive hosts (can only sexually reproduce in cat intestines) and rats as the intermediate hosts (84). After growing in cats, highly stable oocysts are excreted in the feces. The intermediate hosts (grazing animals) which include rats then ingest these oocysts and complete the life cycle when predated by felines (85).

However, there are arguments that the *T. gondii*-rat behavioral manipulations seen might not be adaptive for increased transmission but rather a byproduct of infection pathology or host defense. Firstly, the varied nature of experimental methodology (strain & mode of infection, gender & strain of animals) and conflicting data undercut evidence of adaptive manipulation. As described in Table 1 and in (86), there are contradictory results in various behavior tests making clear conclusions difficult. Moreover, behaviors that might not have an adaptive role in transmission also are affected. This relates to the idea of multidimensionality where not all behavioral manipulations are adaptive but instead are a part of the infection syndrome.

Secondly, the fundamental assumption that *T. gondii* induced manipulations increase transmission to cats to complete its sexual stage of its life cycle is questionable (86). Another parasite, *Eimeria vermiformis*, causes similar decrease in avoidance of cat odor in mice but

does not need predation to complete its life cycle (87). Thus, in this case, predation is not to the benefit of the parasite and it could be a byproduct of a generalized decrease in anxiety and fearfulness. Moreover, the decrease in fear towards cat urine does not directly ensure predation. Though urine could be a source of territorial marking, it does not accurately convey the exact location of the cat in real time as the urine marking might be old. More indicators that are reliable would be cat fur, sound and movement (86). Therefore, there must be evidence of increased predation for a behavioral change to be an adaptive means to increase transmission.

Finally, the idea that the sexual stage of the parasites in cats increases its fitness and thus drove evolution of *T. gondii* induced manipulation to complete life cycle is debatable (88, 89). Fitness benefits could be in terms of transmission to new hosts and sexual reproduction to create genetic variation. *T. gondii* is mainly clonal with three major lineages (at least in North America and Europe (88, 90)) suggesting that trophic transmission routes and sexual reproduction are rare. This is due to its ability to directly transmit (via sexual, vertical transmission (91) and carnivory (89)) between its numerous intermediate host range. Such a scenario would result in a clonal population of *T. gondii* and render the need for a definitive host (cat) unnecessary (88). As such, the need for sexual stage may not put enough selective pressure to evolve adaptive manipulations. Later in the chapter, I discuss examples of sexual and vertical transmission of *T. gondii* in intermediate hosts. It is possible that sexual and vertical routes are equally sufficient and beneficial for infecting new hosts. Moreover, these routes would increase prevalence among intermediate hosts and when predated by cats, increase chances of completing life cycle of *T. gondii*.

Chapter 1

Table 1. Summary of *T. gondii* induced behavioral changes in rodents

Host	Observations		PMID#
Sprague Dawley rats (male)	Spatial reference learning Spatial memory recall	Neutral Decreased	25220582
Balb/c mice	Elevated plus maze Learning & short-term memory	Decreased anxiety Decreased	25076438
Long Evans rats (female)	Time spent near cat urine Light-dark box Social investigation	Increased Increased in lit zone Increased	24907696
Long Evans rats (male)	Time spent near cat urine	Increased	24269877
Balb/c & Swiss Albino mice (male)	Time spent near cat urine	Neutral	23907633
Wistar rats (female)	Elevated plus maze	Decreased anxiety	23328726
Balb/c mice (female)	Time spent near cat urine	Increased	22530040
AD mice	Water & Y maze	Increased learning and memory	22470449
CD-1 mice	Motor performance	Neutral	22468990
C57BL/6J mice (female)	Elevated plus maze Locomotion	Decreased anxiety Increased distance and speed	22431975
Balb/c (male & female)	Time spent near cat urine Locomotion	Increased (female) Increased (female)	22240252
Mice	Open field test	Decreased exploration	22223035
Wistar rats (males)	Mate choice	Increased preference by females	22073295
Mice	Time spent near cat urine Locomotion Spatial memory	Increased Increased Decreased	20608478
C57BL/6J mice (male)	Elevated plus maze	Neutral	20348009
Lister hooded rats	Time spent near cat urine	Increased	18620624
Balb/c (male & female)	Learning (static rod test)	Decreased	17445326
Long-Evans rats (male) BALB/c mice (female)	Time spent near cat urine	Increased	17404235
Wistar rats	Elevated plus maze	Decreased	17169442
Mice	Open field test Hot-plate test *Transient effects	Decreased exploration Increased reaction times	10958436
Rat	Novelty exploration	Increased	11023404
Mice	Elevated plus maze	Increased anxiety	7436599

A model of *T. gondii* transmission routes would have to incorporate all these routes to understand the value of each. Thus, trophic transmission may not be as critical for *T. gondii* to infect new hosts, undermining its status as an adaptive manipulation. However, trophic transmission is still important for sexual reproduction and sustaining genetic variation of *T. gondii* (86).

It is interesting to note the dynamic nature of *T. gondii* manipulation (loss of fear) is not an all or nothing effect. The parasite has to weigh if the energy and resources needed to induce behavioral manipulation come at a cost of reduced fitness, which would drive the parasite to optimize rather than cause absolute changes (92, 93). To this end, *T. gondii* infection causes a dose dependent and size dependent effect on aversion to urine stimuli and cat towel respectively (93). Another sign of this dynamic manipulation is the reversibility of the loss of fear or gain of attraction towards cat odor. The use of routine *T. gondii* treatment (pyrimethamine with dapson, which has shown to inhibit latent toxoplasmosis in rodents and humans) caused reduction in gain of attraction (80, 94).

So far, the examples of trophic transmissible parasites require the death of the intermediate host to reach the definite host. This would cause such parasites to evolve to be more virulent and manipulate behavior as seen in previous examples. On the other hand, a parasite that is able to transmit sexually and vertically (to offspring) will evolve to be less virulent to allow the host to have progeny. What would happen if a parasite could transmit trophically and sexually?

Evidence of sexual transmission of *Toxoplasma gondii*

A literature survey (Table 2) shows that *T. gondii* tachyzoites have been detected in semen and epididymis in numerous animals. Moreover, artificial insemination of females with semen of infected males and natural mating led to infection of females and their progeny (92).

Thus, it is clear that *T. gondii* is a sexually (horizontal & vertical) transmissible infection and a trophically transmissible parasite too. Why would a parasite put the effort to invest energy and resources in another mode of transmission when trophic transmission serves its goal?

Table 2. Evidence of sexual transmission of *T. gondii*

Animal	Infection	Mode of Transmission	PMID #
Goat	Oocysts	Sexual (presence in semen)	7406300
Ram	Tissue cysts	Sexual (presence in semen)	7123822
Dog	Oocysts	Vertical (in female & fetal tissue)	10496698
Dog	Oocysts	Vertical (miscarriage & in fetal tissue)	19137327
Rabbit	tachyzoites	Sexual (presence in semen and female blood)	17094613
Deer	Natural infection	Vertical (in female blood & fetal brain)	18187136
Dog	Oocysts & tachyzoites	Sexual and Vertical (presence in semen, female blood & in fetal brain)	19622353
Sheep	Oocysts	Vertical (presence in fetal brain & lungs)	20510517
Rat	tachyzoites	Sexual and Vertical (presence in epididymis, vaginal lavage & in fetal brain)	22073295
Swine	Oocysts & tachyzoites	Sexual (presence in semen)	
Sheep	Oocysts & tachyzoites	Sexual and Vertical (presence in semen, female blood & in fetal tissue)	23384578

Host-parasite interactions are a constant battle where one partner develops a novel invasive mechanism and the other responds with a defense mechanism, starting a co-evolutionary arms race between the parasite's virulence and the host's resistance (95). Thus, if a parasite alters behavior such that it leads to greater chances of predation, there ought to be an immense selection pressure from the host to defend against the manipulation. So how has *T. gondii* evolved and sustained such a detrimental (for the rat) behavioral manipulation? A clue can be found in the fact that *T. gondii* is able to infect reproductive tissues and transmit sexually (96). Parasites that manipulate behavior usually encyst and localize in the brain, which could have initially been to avoid the host immune system, but subsequently served as a good place to alter behavior of the host (97, 98). Similarly, *T. gondii* invades the testes (like the brain, an immune privileged organ) and its presence in semen, raises the possibility of manipulation in the reproductive domain, for example, increased sexual opportunities. This goes back to the "compensatory responses" hypothesis where a parasite could exploit compensatory behaviors to benefit itself and the host (possibly to compensate for decreased fitness) (41). Therefore, to blunt the selection pressure of the host, *T. gondii* could manipulate sexual or reproductive behaviors that increase its transmission (possibly sexually and vertically) rather than only decrease fitness of the host. Thus, *T. gondii* could offer potential benefits/ trade off to the host, rat, in lieu of the fatal attraction.

There are other reasons why a parasite could have evolved more than one transmission route. Adaptive manipulations for increased transmission is associated with greater fitness costs (56, 99, 100), such as physiological limitations of mechanisms behind the manipulation (101, 102). In addition, there is possibility that manipulated host becomes more vulnerable to all predators. This increases chances of death due to predation by wrong (non-definitive host) predators (103-105) where parasites would not be able to reproduce. Interestingly, some parasites could instead manipulate intermediate host to reduce predation by non-definitive

host. The trematode parasite *Microphallus*, changes foraging behavior of snails (intermediate host) in a time specific way to increase predation by waterfowls (definitive host). Infected snails forage on top surface of rocks during the day (mornings) when waterfowls forage the most. Infected snails then move to bottom of rock during rest of the day to avoid being predated by fish, which are dead end hosts for the parasite (106).

Therefore, multiple transmission routes would arise to maximize chances of infecting new hosts (107). This could affect evolution of host manipulation, as the different routes might be in conflict with each other and place different selection pressures. For instance, sexual & vertical transmission needs the survival of the host, while trophic transmission by definition needs the death of the intermediate hosts (107). Some studies have shown that magnitude of manipulation can decrease when there is a conflict between parasites in the same host (68, 108, 109). For example, two parasite species *Dictyocoela roeselum* and *Polymorphus minutes* use dissimilar transmission routes in the same host- *Gammarus roeseli* (110). The vertically transmitted *D. roeselum* inhibits the manipulation induced by the trophically transmitted *P. minutes*. This protection from the vertically transmitted parasite could be due to conflict between the routes of transmission (111). However, the case of *T. gondii* is unique as it transmits via both routes in the same host, which could lead to interesting outcomes or manipulations. Likewise, multiple transmission routes or manipulations might be due to differing selection pressures depending on the type of strain and host used in different experiments (107). This could explain the inconsistencies in expression and magnitude of *T. gondii* induced manipulations (Table 1). Moreover, as vertical transmission can only occur in females this can influence the sex dependent evolution and magnitude of manipulations. Males ought to show greater behavioral manipulation (loss of fear) as compared to females, though possibility of sexual transmission (male to female) may clash with this (107).

Hence, through either “compensatory responses” or effects of multiple transmission routes, a parasite can alter the evolution of host manipulation. Given that *T. gondii* uses sexual & vertical transmission route, there is the possibility of manipulation in the sexual or reproductive domain to enhance its transmission via this mode.

Parasitic Manipulation of Sexual signals

An avenue by which a parasite can increase its transmission without a detriment to the host fitness is by exploiting secondary sex features or sexual signals. Some hypothesis put forward that male sexual signals are a means to display parasite infection and resistance to females (112, 113). As such, most literature indicates that females normally detect and avoid parasitized males (Table 3). The discrimination benefits the hosts by reducing chances of spread of infections and choosing for heritable resistance. In other words, the preference for a sexual signal could act as a proxy for a genetic legacy of parasitic resistance that can be passed on to offspring (114, 115). Thus, co-evolving parasites will look to increase transmission by co-opting and exploiting sexual signals (116, 117).

Sexual signals like an elaborate peacock’s tail or song of a bird are conspicuous in nature such that they can aid to signal to mates from a distance but it also makes them vulnerable to predators. In this way, a parasite exploiting a sexual signal would also increase trophic transmission. For example, male fiddler crabs signal to females by claw waving which its predators, shorebirds, would also detect (21). These crabs are intermediate hosts for microphallid metacercariae (118), which would try to increase conspicuous claw waving to attract shorebirds (definitive hosts). To counterbalance the possibility of increased predation of the crabs, increased claw waving could improve their mating success to partly offset fitness costs. Similarly, flatworms (cestodes) that use copepods as first intermediate hosts increase their chances of predation by sticklebacks (second intermediate hosts) (119). The copepods are a source of carotenoids for sticklebacks necessary for their red coloration

around the throat (sexual display) (120). Hence, infected copepods will result in redder sticklebacks, which will benefit in courting mates. However, it would also increase susceptibility to predation by definitive host, birds, completing the parasite's life cycle (121). Thus, a parasite that can enhance sexual signals of a host will benefit from blunted selection pressure from the host and augment its transmission efficiency (21). This would resemble a trade-off between the host and the parasite that seeks to increase its trophic transmission. In return, the host would obtain advantage from possible fitness benefit (mate choice) to offset some of the costs of parasitism.

In this thesis, I aim to study if co-evolution between *T. gondii* and rats has led to similar trade-offs. Specifically, I tried to determine whether *T. gondii* caused other behavioral manipulations in the reproductive domain by exploiting sexual signaling in rats. Furthermore, the identity of sexual signaling in rats is not been fully known, so my work also focuses on a putative candidate- major urinary protein (MUPs) - for sexual signaling in rats. I have summarized my work into four specific aims around which the thesis is organized.

Specific Aim 1. To characterize Toxoplasma induced behavioral manipulation in mate choice

The Toxoplasma induced gain of attraction to cat stimulus has been extensively studied. In this specific aim, I characterized behavioral manipulations in mate choice that could act as a trade off to blunt selection pressure from the host. In addition, I characterized the effect of Toxoplasma on expression of a putative sexual signal in rats- MUPs. Finally, I sought to link changes in MUPs expression to the mate choice manipulation observed.

Specific Aim 2. Necessity and sufficiency of MUPs

In this aim, I moved away from a Toxoplasma infection paradigm to study how MUPs are capable to elicit female attraction. First, I demonstrated the necessity and sufficiency of

MUPs in mate choice. Second, I addressed the question of whether MUPs signaling is a quantitative or qualitative phenomenon.

Specific Aim 3. Kairomonal communication in mice

Often a cost of greater expression of a sexual signal is the increased chances of predation due to open nature of communication with sexual signals (body coloration, bird song or pheromonal cues). At the same time it is possible that even prey could eavesdrop on this system to detect its predators and avoid detection. In this aim, I tested if like female rats, mice displayed any difference in behavior (in this case, avoidance) to infected and control rat urine stimulus. In addition, I tested for dose dependency of kairomonal communication.

Specific Aim 4. Condition dependence of MUPs

Sexual signals are dynamic in nature responding to internal and external factors that affect condition dependence of an individual. The intensity of the signals varies with different contexts or challenges as it not possible to constitutively express due to cost in terms of resources. In this aim, I carried out a longitudinal study on MUPs expression to see if aging of a rat affected MUPs expression. In addition, I induced sickness behavior with LPS injections to see if this had an effect on MUPs. Finally, I tested how social interactions affect MUPs- for this rats were housed with individuals of varying dominance status and exposed to females to assess how the male rat responded to these challenges.

Table 3. Previous reports that females detect and detest parasitized males

Parasite	Host	References
<i>Heligmosomoides polygyrus</i>	Mouse	Kavaliers, M. and Colwell, D.D. (1995). <i>Anim. Behav.</i> 50:1161–1169 Kavaliers, M. et al. (1998). <i>Proc. R. Soc. Lond. B. Biol. Sci.</i> 265:1111–1118 Kavaliers, M. et al. (2003). <i>Anim. Behav.</i> 65:59–68 Ehman, K.D. and Scott, M.E. (2001). <i>Anim. Behav.</i> 62:781–789 Ehman, K.D. and Scott, M.E. (2002). <i>Parasitology.</i> 125:461–466
<i>Eimeria vermiciformis</i>	Mouse	Kavaliers, M. and Colwell, D.D. (1995). <i>Proc. R. Soc. Lond. B. Biol. Sci.</i> 261:31–35 Kavaliers, M. et al. (1997). <i>Behav. Ecol. Sociobiol.</i> 40:373–384 Kavaliers, M. and Colwell, D.D. (2003). <i>Ethology.</i> 95:202–212
<i>Polyplax serrata</i>	Mouse	Kavaliers, M. et al. (2003). <i>Behav. Ecol. Sociobiol.</i> 54:423–430 Kavaliers, M. et al. (2003). <i>Genes Brain Behav.</i> 2:220–230
<i>Salmonella enterica</i>	Mouse	Zala, S.M. et al. (2004). <i>Behav. Ecol.</i> 14:338–344
<i>Hymenolepis diminuta</i>	Rat	Willis, C. and Poulin, R. (2002). <i>Folia Parasitol.(Praha).</i> 47: 6–10
<i>M. pennsylvanicus</i>	Prairie voles	Klein, S.L. et al. (1999). <i>Behav. Ecol. Sociobiol.</i> 45:323–329
<i>M. orchogaster</i>	Prairie voles	Klein, S.L. et al. (1999). <i>Behav. Ecol. Sociobiol.</i> 45:323–329
<i>Influenza</i>	Mouse	Penn, D. et al. (1998). <i>Ethology.</i> 104:685–694

2. Materials & Methods

Animals

Rats

Adult Wistar rats (44-50 days) were obtained from NUS animal Facility. They were housed in pairs at NTU animal house facility. Males and females were housed in same room. Animals were provided with food and water *ad libitum* and maintained in a 12:12 hour light-dark cycle. The Nanyang Technological University institutional animal care and use committee reviewed and approved all procedures (IACUC number: ARF SBS/NIE-A-0106AZ). Experiments were carried out during the light phase.

Mice

Male Balb/c mice (7–8 weeks old, housed five/cage; (369 x 156 x 132 mm; 1145T, Tecniplast, UK)) were obtained from the vivarium of National University of Singapore. Standard corncob cage bedding was changed twice a week. Animals were placed on a 12 hours light-dark cycle, with temperature between 20–25 °C and relative humidity ranging around 70–80%, respectively. Experiments were carried out during the light phase. Food and water was available *ad libitum*. The diet was made up of standard laboratory chow (PicoLab Rodent Diet 20, 5053) with 20% protein content.

Parasite and Infection

A Prugniaud strain of *Toxoplasma* containing GFP and Luciferase inserts was used. They were cultured in human foreskin fibroblast monolayers. Infected fibroblasts were syringelysed (twice) using an 18 gauge followed by 25 gauge needle. Male rats were injected intraperitoneally with 5 million parasites resuspended in 0.5 mL of PBS. Control males were sham injected with the same volume of PBS. Infected rats were weighed once a week for 4

weeks and checked for any outward symptoms of illness. Behavioral experiments were done post 6 weeks of infection. (*Used in chapter 3*)

Confirmation of infection Status

Infection was confirmed by serological detection of anti-Toxoplasma IgG antibodies. Toxoplasma was cultured inside human foreskin fibroblasts in 24-well plates. 24 hours post infection; the wells were aspirated, washed with PBS and fixed with 4% PFA. They were incubated with 1mL of the serum (1:1000) overnight at 4 °C. If the animal had been infected with Toxoplasma the serum would contain anti Toxoplasma antibodies that would bind to Toxoplasma in culture. The bound antibodies were visualized using anti Rat IgG-Cy3 (1:200, Millipore; red fluorescence). (*Used in chapter 3*)

Castration

Male Wistar rats (6-8 weeks old) were used. Animals were anaesthetized using a ketamine (90 mg/kg) and xylazine (10 mg/kg) cocktail and maintained using 2-3% isoflurane. Additionally animals were injected subcutaneously with the analgesic combination of Meloxicam (0.2 mg/kg) and Lidocain (0.5 mL). A medial incision was made in the scrotum of heavily anaesthetized animal. Testes and vas deferens of each side was sequentially pulled out of the incision. Blood vessels supplying to the testes were sutured. Testes, vas deferens and associated fatty pads were severed just below the point of suture, followed by closure of the scrotum incision.

For the social environment experiment (Chapter 6) castration was followed by testosterone supplementation. One group (n=8) was implanted with empty silastic tubing (2mm length, 1.51 mm internal diameter., 3.12 mm outer diameter, Dow Corning Corp, Midland, MI modified from Serra, 2012) at its neck; the other group (n=8) was implanted with silastic tubing (as above) filled with testosterone propionate (Tp 86541-5G Fluka, 18 mg, Sigma-Aldrich, Singapore) at the nape of neck. A third group (n=8) of intact rats (not castrated,

testicle region cut and sewed back) were implanted with empty silastic tubing at the nape of the neck. *(Used in chapter 4 & 6)*

Determination of Estrus

Female rats were acclimatized in the vivarium for 2-3 days upon arrival. They were lightly restrained and vaginal lavages (~10 μ L) using PBS was collected at 11AM. Relative ratios of cornified, epithelial and leuckocytes in lavage was examined under 20X magnification. Estrus was characterized by majority presence of cornified cells. Only estrus females were used in behavioral experiments. Females used were uninfected and sexually inexperienced. *(Used in chapter 3, 4 & 6)*

Collection of urine

Urine was collected 6 weeks post infection. The rats were placed individually or in pairs in a metabolic cage (Harvard Apparatus, Holliston, USA) for a period of 5-6 hours. The urine samples were stored in 2 mL aliquots in -20 °C for upto a month. The rats had complete access to water and food throughout the collection period. For an experiment in Chapter 4, urine of Long Evans rats from collaborators was obtained. The urine was lyophilized and shipped and then were reconstituted with water upon arrival and stored in -20 °C. *(Used in chapter 3, 4, 5 & 6)*

Separation of low- and high- molecular weight fractions

Four mL of rat urine was placed in Amicon centrifugal devices (Millipore, Billerica, USA) with a 3 kDa cut-off. It was centrifuged at 3500 g for 10 minutes. This yielded about 2 mL of both the filtrate (LMW) and retentate (HMW). Displacement of volatiles was done by adding 10 μ L of 4 mg/mL of menadione (M5625 Sigma-Aldrich, Singapore; dissolved in absolute ethanol) to every 500 μ L of HMW fraction. The solution was incubated in an open eppendorf tube for 30 minutes at room temperature with slight shaking. The different aliquots of 500 μ L

were pooled and then centrifuged at 3500g for 5 minutes. For the grafting experiment, the control and infected LMW and HMW fractions were mixed in a 1:1 ratio before presenting the stimuli. This technique was adapted from (122, 123), where they fractionated and displaced rat urine in similar manner to study kairomonal responses in mice towards rat MUPs. (*Used in chapter 3, 4, 5 & 6*)

Mate choice assay

Experiment was conducted in a rectangular arena that had two opposing and identical arms (76 X 9 cm each) separated by a central compartment (9 X 9 cm size). Please figure below- this same apparatus was used in other behavioral tests too. At the start of experiment, the central gate was closed. One control and infected male was introduced and allowed to explore (and urine mark) their respective arms for 2 hour. They were then confined in extremities of the arena by closing the perforated gate. This allowed for olfactory cues to be emitted without possibility of physical contact. Naturally cycling females were used in all mate choice assays. Female mate choice was quantified by comparing time spent by estrus females in two opposing arms of an arena (46 X 9 cm each; 15 cm high, separated by a central chamber) during a 20 minute trial, containing two contrasting stimuli. Data was collected with an automated behavioral tracking software- ANY-maze, version 4.3, Stoelting. (*Used in chapter 3, 4 & 6*)

Paced Mating Assay

i. Non-competitive Assay

A rectangular arena was used. This was divided into two chambers (30 X 45 cm) by Plexiglas dividers. The dividers had cuts outs (50 mm diameter). These were big enough for the female to pass through but too small for the males. All experiments were conducted during the dark phase of the circadian cycle. Females were habituated in the arena for three consecutive days (15 minutes each). On the day of the experiment males and estrous females were brought to the behavior room and acclimatized for 10 minutes. Males were introduced in one arm and allowed to urine mark for 15 minutes prior to beginning of experiment. Females were then introduced into the other arm and the trials were recorded for 2 hours. Trials where the females did not show lordosis within the first 15 minutes were aborted. Trials were recorded using camcorder (model Samsung Schneider) under dim light conditions. Trials were timed manually and timeline of sexual events reconstructed.

ii. Competitive Assay

This experiment is similar to the above mentioned. The only difference being that the arena is divided into three chambers with two Plexiglas dividers with cutouts. The central small zone is the female chamber. (*Used in chapter 3*)

Cat odor avoidance assay

The response of male rats to cat odor (Predatorpee) was studied in 20 minute trials in same maze used in mate choice assay. Data was quantified (with ANY-maze) by comparing time spent by the rat within 15% of the stimuli. Subjects were habituated in a single arm arena, the stimuli was presented at the end of one arm. Animals were habituated 10 times (twice a day, over 5 days) prior to remove novelty effect of the arena. One arm was a home arm and the other had cat urine (2 mL) which was replaced with every new trial. (*Used in chapter 6*)

Visualization of Toxoplasma cysts

Presence of Toxoplasma in testes was visualized by histological staining of Toxoplasma tissue cysts in epididymis (7 weeks post infection). Epididymis tissue was homogenized in PBS. This was smeared on a suprafrost glass slide and air-dried overnight. Toxoplasma was detected using an anti-GFP antibody (1:1000; Millipore) as the Toxoplasma strain used in our study has a GFP insert. This signal was further amplified using a combination of avidin-biotin complex (Vector Labs) and tyramide signal amplification coupled with Cy3 dye (1:75, red color; Perkin Elmer). Cyst wall was visualized by staining with dolichos biflorus agglutinin (20 µg/mL; Vector Labs) coupled with fluorescein (green color). The DBA binds to polysaccharides found in the Toxoplasma cyst wall. Slides were counter-stained with DAPI (blue) (Vector Labs) and visualized in a fluorescent microscope using 40X objective (Carl Zeiss; LSM 710). *(Used in chapter 3)*

Fast protein liquid chromatography (FPLC)

FPLC-gel filtration was carried out using a Superdex 75 column (GE Healthcare, Piscataway, USA) that had been calibrated with molecular mass standards. 7 mL urine from each control, infected and castrated animals were lyophilized, suspended in phosphate buffered saline (PBS; 0.5 mL) and dialyzed against PBS overnight. These were further concentrated using Amicon Centrifugal Devices (molecular weight cut-off of 10kDa; Millipore, Billerica, USA). The concentrated protein solutions were run through a Superdex 75 10/300 GL column (300 X10 mm and bed volume of 25 mL) on an AKTA FPLC system (GE Healthcare). The column was equilibrated and eluted with PBS (12 mM phosphate salt, 127 mM sodium chloride and 2.7 mM potassium chloride, pH 7.4) at a flow rate of 0.5mL/min. Fractions (2 mL size) were collected and concentrated using Amicon centrifugal devices. For all samples, SDS-PAGE analysis was performed with 12% polyacrylamide gels (self-cast gels; composition for 2 gels

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shown below). Samples were boiled for 5 minutes at 95 °C with 10 mM β -mercaptoethanol. Gel running conditions were 100 V for 2 hours after which proteins were stained with Coomassie blue. The running buffer was composed of (for 1 L) 3.03 g Tris, 14.4 g glycine and 1 g SDS.

(Used in chapter 3 & 4)

	Stacking gel (4%)	Resolving gel (12%)
MilliQ Water	3.9 mL	4.3 mL
Tris buffer	0.5 mL (0.5 M, pH6.8)	2.5 mL [*] (1.5 M, pH8.8)
40% acrylamide (37.5:1)	0.5 mL	3 mL
10%SDS	50 μ L	100 μ L
10% APS	50 μ L	100 μ L
TEMED	5 μ L	10 μ L

^{*}For 15% resolving gel, 3.75 mL of Tris buffer was used.

Dialysis of denatured MUPs

Urine samples were lyophilized, suspended in phosphate buffered saline (PBS; 0.5mL) and dialyzed against PBS overnight. These were further concentrated using Amicon centrifugal devices with molecular weight cut-off of 3 kDa (Millipore, Billerica, USA). The concentrated protein solutions were rapidly run through a Superdex 75 10/300 GL column (300 X 10mm with a bed volume of 25mL) on an AKTA FPLC system (GE Healthcare, Piscataway, USA). The column was equilibrated and eluted with 50 mM phosphate buffered saline at a flow rate of 0.5 mL/min. Fractions (2mL size) were collected and further concentrated. FPLC-purified MUPs were denatured with 6M guanidinium hydrochloride. Denatured MUPs were dialyzed against PBS for 72 hours at 4 °C (Thermo Scientific, Slide-A-Lyzer Dialysis Cassettes; 3 kDa cut-off) to allow for refolding of proteins. Protein concentration after dialysis was renormalized to physiological range of 1.6 mg/mL and used for mate choice assay.

Renaturation was confirmed by running the denatured and renatured protein on a 12% Native PAGE. The gel composition was the same as mentioned previously but no SDS was added in both stacking and resolving gel. Moreover, there was no SDS in the loading dye and running buffer too and samples were not heated prior to loading. The loading dye also did not have β -mercaptoethanol. The gel was run at 100 V for 2 hours after which proteins were stained with Coomassie blue. In addition, the renaturation was further confirmed with circular dichroism (done in a collaborator's lab- Prof Surajit Bhattacharyya's lab in SBS-NTU). This showed that the renatured sample had the same spectra as the original native MUPs sample.

Mass spectrometry

Protein bands were excised from the SDS-PAGE gel and sent for analysis. The identity of bands was determined by MALDI-TOF/TOF and peptide fingerprinting after tryptic digestion.

(Used in chapter 4)

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 0.5 M (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 by the Trizol (Invitrogen, Carlsbad, CA, USA) method. Briefly, tissue was dissected 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.

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Chloroform (~1/5 volume of Trizol) was added and samples were shaken for 15-30 sec followed by incubation at room temperature for 3 minutes. The samples were centrifuged at 4 °C and 12000 g for 15 min. The aqueous phase was pipette into a new tube and ice-cold isopropanol (~same volume as aqueous phase) added. This was incubated at room temperature for 15-25 minutes and centrifuged again for 15 minutes. The supernatant was discarded carefully and the pellet washed three times with 1 mL of 75% ethanol prepared using DEPC water and allowed to air dry at RT for ~15-30 minutes. 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, Glen Burnie, MD, USA). 20 µL reactions were used (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. (*Used in chapter 3 &4*)

Reagent (Thermo Scientific, Singapore)	Volume
5X reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl ₂ , 50 mM DTT)	4 µL
Ribolock RNAase inhibitor (20 u/µL)	1 µL
Revertaid M-MuLV Reverse Transcriptase (200U/µg)	1 µL
Oligo (dT)18 primer (0.5 µg/µL)	1 µL
DNTP mix 10 mM	2 µL

Quantitative RT-PCR

Abundance of MUPs cDNA was quantified using the standard SYBR green based real-time quantitative PCR. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as

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internal control. For each sample, threshold cycle numbers required to reach a pre-determined fluorescence value was measured. Threshold values of GAPDH were deducted from that for MUPs to calculate delta Ct values. (*Used in chapter 3 &4*)

MUP	Forward primer	GCCTGACCCTGGTCTGTGGC
	Reverse primer	ACCTCGGGCCTGGAGACAGC
GAPDH	Forward primer	GGGCAAGGTCATCCCTGAGCTGAA
	Reverse primer	GAGGTCCACCACCCTGTTGCTGTA

Quantitative PCR reactions were run using an ABI 7500 machine. The reaction composition (volume: 25 μ L) and run method are described below.

Reagent	Volume
Sybrgreen 2X buffer (AppliedBiosystems, Singapore)	12.5 μ L
DEPC	10.5 μ L
Primers (Forward & Reverse- 100 mM)	0.5 μ L
DNA(100 ng/ μ L)	1 μ L

Production of recombinant major urinary proteins

Total RNA from Wistar rat liver was reverse transcribed to cDNA as described previously. Pairs of degenerate primers for MUPs were used to amplify MUP cDNA using 25 cycles of PCR. Following forward primers were used (3'-5'): ATGAAGCTGTTGCTGCTGCTGCTG; ATGAAGCTGTTGCTCCTGCTGCTG; and, ATGAAGCTGTTCTGCTGCTGCTG. Following reverse primers (5'-3') were used: TCAGGCCTGGAGACAGCGATC; TCAACCTTGGGCCTGGAGACA; TCAACCTCGGGCCTTGAGACA; TCATCCTCGGGCCTGGAGACA; and, TCAACCTCGGGCCTGGAGACA. Resultant cDNA were cloned into *Escherichia coli* TA vector and sequenced to identify MUP isoforms. All complete isolates contained only two MUP isoforms (amongst 30 colonies screened). Both of these isoforms were sub-cloned into an *E. coli* expression vector to allow protein expression in copious amounts (*E. coli* strain TB1, coupled with an expression vector pMALc2x). Expressed MUPs were isolated using combination of sonication and affinity chromatography. This procedure resulted in isolation of MUPs fused with maltose-binding protein, which was used in behavioral experiments. (Used in chapter 4)

Western blotting

All urine samples were standardized using a Creatinine Kinase detection kit (Enzo Life Sciences, 25-0740) following manufacturer protocol. The colorimetric detection was visualized at 520nm (Tecan Infinite M200 Pro Quadruple Monochrome Microplate Reader, Magellan Version 7 software). This acts as a method to normalize or equalize the dilution of the samples and serve as a loading control in western blots. With the creatinine levels, the samples were diluted to the lowest creatinine level in that batch of samples to be tested. Then protein was extracted via methanol and chloroform protocol¹ and resuspended in PBS. The samples were then used for western blotting. A 12% SDS-PAGE gel (same composition and running conditions as described on p. 28) was used and wet transfer with nitrocellulose membrane was run at 100V for 2 hours at 4 °C. Transfer buffer was composed (for 1 L) of 3.03 g Tris, 14.4 g glycine and 200 mL methanol.

The MUPs protein levels were quantified with Western blots using anti- MUPs antibody (1:2000 dilution, sc-66976, Santa Cruz Biotechnology, Santa Cruz, USA) and detected using a horse-radish peroxidase based (secondary antibody: goat-anti rabbit HRP, 1:5000 sc-2004) luminescence signal. A pooled sample (it composed of equal volumes from each sample in the whole assay after protein was extracted) was run with each gel and was used as standard against which all densitometry readings were normalized. The antibody used in this assay identifies all MUPs protein variants found in rats (supplementary Figure 1). (*Used in chapter 3, 4 & 6*)

Direct analysis in real time mass spectrometry (DART-MS)²

Samples (approx. 1 mL in volume) were extracted three consecutive times with an equivalent volume of hexane. Pooled extracts were evaporated under nitrogen gas and reconstituted in

¹ Adapted from <http://rtsf.msu.edu/MethanolChloroform.pdf>

² DART-MS was done by Prof Joanne Yew in Temasek Life Sciences Laboratory, NUS

100 μ L of hexane. The atmospheric pressure ionization time-of-flight mass spectrometer (AccuTOF-DART; JEOL USA, Inc.) was equipped with a DART interface and operated in positive-ion mode at a resolving power of 6000 (FWHM definition). Calibration was accomplished by acquiring a mass spectrum of polyethylene glycol (average molecular weight 600) as an external reference standard in every data file. A clean glass capillary was dipped into each sample and placed between the gas inlet and outlet of the DART ion source. 3-5 replicate measurements were obtained for each sample. The DART source was operated in positive ion mode with helium gas with the gas heater set to 200 °C. The glow discharge needle potential is set to 3.5 kV. Electrode 1 is set to 150 V, and electrode 2 (grid) was set to 250 V. (*Used in chapter 4*)

Iso-electric focusing

Iso-electric focusing was achieved using the Immobiline DryStrip pH4-7 Linear (GE Healthcare). The strips were rehydrated with protein solution (including DeStreak solution and 0.5% IPG buffer) for 2 hours. The strips were run on an Ettan IPGphor 3 Isoelectric Focusing Unit (GE Healthcare), subjected to current of 50 μ A per strip and running conditions in accordance with manufacturer's directions. The strips were then equilibrated first with buffer (6M urea, 75 mM Tris pH8.8, 30% glycerol, 2% SDS) containing DTT (0.1 g/10 mL) and then iodoacetamide (0.25g/10mL). Finally the strip was run on a 12% SDS-PAGE gel (same composition and running conditions as described on *p.* 27) and stained with Coomassie blue for visualization of spots. (*Used in chapter 3*)

Testosterone extraction & quantification

The method was modified from reference (124). Two microliters of 10 ng/mL internal standard (Testosterone-2,3,4- $^{13}\text{C}_3$ in acetonitrile) was added to 200 μ L of serum and vortex-mixed for 30s. Extraction was carried out by the addition of 1 mL hexane: ethyl acetate (90:10, v/v) mixture to each tube, vortex-mixed for 2 minutes and then further subjected to

automatic vortexing for 10 minutes at room temperature. The samples were then centrifuged at 3000 rpm for 10 min at 4 °C. The tubes were placed on dry ice until the aqueous bottom layer froze and the organic top layer was transferred into a fresh tube. The tubes were placed in a concentrator to evaporate the solvent to dryness and the sample reconstituted in 150 µL of 20 % acetonitrile as obtained during method development to give higher peak areas for testosterone while retaining good peak shape. After reconstitution, the extracted samples were centrifuged at 13,200 rpm for 10 minutes at 4 and 120 µL of the supernatant was taken into the HPLC vials for LCMS/MS analysis. Quantification was done with standard curve of internal standard. (*Used in chapter 6*)

LCMS/MS conditions³ (*Used in chapter 6*)

Pump: Thermo scientific accela 1250

Autosampler: Thermo scientific accela

Analytical column: Phenomenex Luna C18 (50 × 2.00 mm)

Mobile phase A: 0.1 % formic acid in water

Mobile phase B: 0.1 % formic acid in acetonitrile

Gradient: 10 % to 100 % B in 3.5 min

Flow rate: 0.2 mL/min

Injection volume: 20 µL

Mass spectrometry

MS system: LTQ XL (Thermo Finnigan)

Condition: LC/(+) ESI-MS/MS

MRM transition:

Testosterone: 289 → 97

Internal standard (Testosterone-2,3,4-13C3): 292 → 100

³ LCMS was done with help from John Tayki Williams.

Dose-responsivity to kairomones

Rat urine was collected using metabolic cages (Harvard Apparatus). A single pool of rat urine was used for all subsequent experiments. Displacement of volatiles was done as described previously. Only the high molecular-weight fraction containing MUPs and devoid of volatiles was used. The response of mice ($n = 10$) to increasing doses of MUP fraction of rat urine ((henceforth referred to as rat urine) was studied (trial duration = 600s). Avoidance was quantified by comparing time spent by mice in two opposing bisects of an arena (76×9 cm; 15 cm high). The bisects were defined by a virtual division of the arena in two equal halves (38×9 cm), with exploration in either half being considered as time spent near the stimulus presented in that bisect. The stimuli were presented at the terminal end of the bisects. Data on time spent in each bisect was collected by automated behavioral tracking software (ANY-maze, version 4.3, Stoelting). Opposing arms contained either rat urine or phosphate-buffered saline. The amount of rat urine was systematically varied from 1X to 16X (3.125, 6.25, 12.5, 25 and 50 μ L). X was arbitrarily defined as 3.125 μ L of rat urine. The different stimuli were made by a twofold serial dilution to ensure that a constant volume was presented (50 μ L) while the concentration varied. The stimuli was dotted (5 drops of 10 μ L) on filter paper and positioned at the terminal ends of the two bisect. The animals had direct access to the stimuli. The same set of mice was used in successive testing for all doses (starting from lower to higher doses) with 24 hours elapsing between two successive trials. (*Used in chapter 5*)

Discrimination threshold

Both arms of the arena contained rat urine in this condition. The amount of rat urine in one arm was varied in five discrete doses (6.25–25 μ L), equidistant on a Log_2 scale. The opposing arm contained volume that was greater by ratio of either 1.2 or 1.3. The percentage of time that mice ($n = 15$, the same mice that were used in the previous experiment) spent in the arm with the greater volume of urine, was quantified. The same set of mice were used in

successive testing for all doses (starting from lower to higher doses), with two successive trials (24 hours apart). (*Used in chapter 5*)

Kairomonal valence of control and infected male rat urine

For testing response to fresh urine, rat urine was collected using metabolic cages (Harvard Apparatus). The urine was collected on the same day of testing. Rat urine contains both volatile and non-volatile substances. Urinary volatiles tend to dissipate quickly with passage of time, while non-volatiles can remain stable for weeks. In order to ascertain the contribution of non-volatiles (i.e. aged urine), a plastic Petri plate was placed in a rat cage for twenty-four hours on which the rats would urine mark. Three days (stored at RT) after removal of the Petri plate, it was used as the stimulus in the avoidance-avoidance test with mice.

Response of male mice to fresh urine obtained from control or infected rats (pooled from two rats) was studied using an avoidance-avoidance conflict paradigm. Avoidance was quantified by comparing time spent by mice in two opposing bisects of arena (76×9 cm; 15 cm high) during a 20 minute trial. Data on time spent was collected by automated behavioral tracking software (ANY-maze, version 4.3, Stoelting). Opposing bisects either had urine from control and infected rats (5 drops of 20 μ L each, placed equidistant on absorbent paper of 5×7 cm). For testing response to aged urine, the three day old Petri plates from the control and infected rats were placed at terminal ends of opposing bisects. Again avoidance was quantified as described above with ANY-maze. The same set of mice was used for both behavioral assays. Experiments involving aged urine preceded the experiments with fresh urine. (*Used in chapter 5*)

Statistical Analysis

Statistical analysis was performed using SPSS software (version 20, IBM). Wilcoxon signed-rank test, 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. Repeated measure ANOVA was used to compare MUPs expression in the longitudinal study. In text, mean and SEM are mostly reported unless it is stated that 95% confidence interval (CI) is shown. All box plots in this thesis depict 25th percentile, median and 75th percentile. Central dot and whiskers depict mean and SEM respectively. In some figures instead of SEM, 95% confidence intervals are showed.

Effect size (Cohen's D) was calculated using an online tool (<http://www.uccs.edu/~lbecker/>) and sample size determination was done with a statistical power calculator (<https://www.dssresearch.com/Home.aspx>). An example is provided below for an initial pilot study testing female preference (time spent) for infected vs. control urine stimuli.

*Average (std dev) for infected urine: 420 s (65); control urine: 250s (32). Sample size= 10
 α -error level= 1% (Probability of incorrectly rejecting the null hypothesis that there is no difference in the average values)*

After inputting the above values in the online calculator mentioned, a statistical power of 100% was obtained. Thus, I tried to ensure that in most behavior experiments in this thesis, a sample size of 10 was used. This was not possible in Chapter 6 due to the fact that some experiments had more than 2 experimental groups, for example the LPS and social environment studies.

Outliers were determined and removed by plotting box plots. Data points which were greater than 1.5 times the inter quartile range were removed from analysis ($1.5 \times (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$).

3. *Toxoplasma gondii* infection induces behavioral manipulation in mate choice

This chapter includes work from two papers I published ([±] Equal contribution)

1. Dass, S.A[±], Vasudevan, A[±], Dutta, D., Soh, LJT., Sapolsky, R.M., Vyas, A. (2011) Protozoan parasite *Toxoplasma gondii* manipulates mate choice in rats by enhancing attractiveness of males. *PLoS One*. 6(11):e27229. **Figure 2 & 4 are from this paper under the terms of Creative Commons Attribution License.**
2. Vasudevan, A[±], Kumar, V[±], Chiang, Y.N., Yew, J.Y., Cheemadan, S., Vyas, A. (2015) Alpha2u-globulins mediate manipulation of host attractiveness in *Toxoplasma gondii*-*Rattus norvegicus* association. *Isme J*. 9(9):2112-2115. **Figure 5 has been adapted from this publication in accordance with journal guidelines.**

There have been numerous studies on *Toxoplasma gondii* behavioral manipulation in rats (72-74, 93). This is an example of the use of perturbation models to introduce experimental manipulations in biological systems. The *T. gondii*-rat model is a naturally occurring perturbation system that is characterized by the loss of fear and gain of attraction to cat odor or stimuli. This defensive behavior is part of the innate behavior of a rat that does not need learning or prior experience. In such behavior, a stimulus evokes responses selected for over an evolutionary time frame. Those who failed to display an innate aversion were more likely to be predated and failed to reproduce, leading to fixation of innate fear responses. In addition to defensive behavior, another facet of innate behavior is the reproductive or sexual behaviors. In this chapter, I am interested if there are similar manipulations in this domain.

As described in the introduction, the *T. gondii* fear manipulation in rats could be a means for the parasite to increase its transmission efficiency and thereby completing its two- stage life cycle. The basis of this manipulation is still unknown but certain hypotheses attempt to understand this phenomenon. One point of view looks at the major site of chronic *Toxoplasma* cyst localization- the brain. Some think that the cysts localize in a non-stochastic manner to specific brain regions (125). Reports have shown a slight but significant tropism to the amygdala, nucleus accumbens and ventromedial hypothalamus (73, 79). These areas are part of the brain circuitry involved in processing fear and decision-making. As such, tropism

could be the key to the fatal attraction behavior displayed by disrupting fear responses and decision-making abilities. Evidence for this hypothesis is contradictory with reports of a lack of tropism and maintenance of behavior manipulation even after clearance of cysts from the brain (126). Thus, it is critical to look outside the brain and into the periphery of the host for possible mechanisms behind this fear manipulation.

An alternative hypothesis for the fear manipulation looks at how the parasite could co-opt the hormonal cross talk between the brain and the reproductive organs. Testes are another immune-privileged site where *Toxoplasma* invades which has shown to up regulate rate-limiting enzymes in steroidogenesis in Leydig cells (exact mechanisms is still unclear) (28) and increase testosterone levels in the testes and plasma. As such, it is possible that greater testosterone levels is behind the fear manipulation. This is reflected in the fact that castration prior to infection prevents the loss of fear seen after *Toxoplasma* infection (28). However, the lack of testosterone during infection could influence the immune response to *Toxoplasma*, affecting nature of infection and subsequent behavioral manipulation. Nevertheless, testosterone could explain the loss of fear post infection but how about the gain of attraction observed in a subset of infected rats? For this, we have to see how the increased testosterone affects the brain. This hypothesis puts forward that higher levels causes leakiness at the molecular level between the sexual and defensive behavior circuitry in the brain (127). A focal point of this leakiness could be the medial amygdala, which expresses arginine vasopressin (AVP), a neuropeptide controlling reproductive behaviors (128). Expression of AVP is testosterone sensitive (the medial amygdala is rich in androgen receptors) through mediation of methylation levels of its promoter (129). Exogenous testosterone causes hypomethylation at the promoter and an increase in AVP expression that *Toxoplasma* infection mimics in rats (130). Activation of these AVP neurons normally occurs during reproductive opportunities in male rats (131). However, in infection these very neurons are

recruited when rats are exposed to cat odors, possibly causing the leakiness and atypical behavior response (130). In light of these observations, I was interested in other possible manipulations in behavior and given testosterone's key role in sexual investment and behavior, the reproductive domain was considered a great place to start the investigation.

Mate choice manipulation

To begin with, we investigated if *Toxoplasma* infection enhances attraction from females and sexual investment of males⁴. Urinary olfactory cues are a major means of communication in rodents (132, 133). Rat urine contain a range of volatiles bound to Major Urinary Proteins (MUPs) that are known to be chemical attractants to females (134, 135). We initially tested with this complete urine signal. We used estrous females to assess attractiveness of control and infected male urine stimuli. Attraction or preference was quantified based on amount of time they spent near the stimulus (approach-approach conflict assay) during 20 (1200s) minute trials. Males' urine marked the testing arena (straight maze) for 2 hours before females were introduced. During the trial, the males were enclosed to chambers at the terminal ends of the arena that did not allow for direct contact but they could smell each other. This mate choice assay was conducted with 12 different pairs of control and infected males and each pair was tested with 5-7 females (total- 72 trials). In 74% of trials, females preferred the infected male (Figure 2A, median preference score = 1.54; $\chi^2 = 16.1$; $p < 0.0001$; infected > control = 53/72 trials). In addition, results showed that in all 12 male pairs, females preferred the infected male to the control males (supplementary figure 2 & Figure 2B, 506 ± 30 s in infected bisect compared to 308 ± 17 s in control bisect; exact Wilcoxon signed ranks test: $Z = -3.059$; $p < 0.0001$). Analysis of variance suggested statistically significant variation because of infection status (bisect occupancy in control and infected as repeated measures; $F(1,11) = 48.5$; $p < 0.0001$) and between individual male pairs (between pairs; $p < 0.0001$).

⁴ Done with Shantala Hari Dass

Figure 2. Females preferred *Toxoplasma* infected males. (A) Preference was quantified by dividing time spent by an estrus female in infected bisect divided by control bisect ($n=72$ trials). Each bisect was urine marked by either control or infected males (6 weeks post infection) (trial duration = 1200 s, ratio >5 assigned arbitrary value of 5). Each dot represents raw data from one female. Box plots depict median, 25th percentile and 75th percentile. (B) Preference of females for each males in the 12 unique pairs was calculated by taking median of all females tested for that particular male pair (ordinate and abscissa depicts time spent in infected and control bisect, respectively). Each dot represents the median preference for an infected: control males pair over six females. Mean and SEM are depicted in grey color and the chance bars represent equal preference for both groups.

Figure 3. Increase in sexual activity in *Toxoplasma* infected Wistar males. Sexual episodes is defined as a mount followed by genital grooming (% sexual episodes= # sexual episode/# mount * 100). (A) Non Competitive Paced Mating Assay: The infected males showed more sexual episodes than their control counterparts (mean $\pm$ SEM: Infected mean=97.04% $\pm$ 1.2, Control mean= 92.05% $\pm$ 0.9, paired t-test: $p = 0.021$, $n = 7$ infected, 6 control males). (B) Competitive Paced Mating Assay: Infected males received more mating opportunities. 6 out of 7 females bestowed greater mating opportunities on infected males ($n = 7$ infected: control pairs; exact Wilcoxon signed ranks test: $Z = -2.21$, $p = 0.011$; one-sample t-test: $t = 4.36$, $p < 0.01$). The chance bars represent equal preference for both groups.

This showed that females were showing greater preference to some components in the infected male urine stimuli. However, this does not completely rule out visual or acoustic signals playing a role in female preference.

Next, we studied if the preference or attraction for infected males led to more sexual opportunities. The arena used had 3 compartments; two compartments at the ends either had an infected or control male. The middle compartment was for the female. The design of the dividers was such that the female could access the compartments with the male but not vice versa. This experiment would provide information on possible differences in consummatory behavior of infected and control rats and if scent based preference correlates in a mating set up. Female rats set the pace of control mating behavior in rats, which allows her to enter and exit the male compartment multiple times. This allows the females to choose their preferred mates and set the pace of the mating episodes. Mating behavior begins with the female entering the male compartment where she displays solicitation behavior by hopping, ear wiggling and arching her back (lordosis behavior) indicating receptiveness for copulation. Then the male mounts and penetrates the female. A sexual episode was defined as mount followed by genital grooming. However, as not every mount was a sexual episode (i.e. no genital grooming), a percentage (percentage sexual episodes) was derived by dividing number of sexual episodes with number of total mounts. Two variations of the paced mating was tested- in the first scenario, the female was mated with either a control or infected male (i.e. non-competitive paced mating- one chamber of the three compartments was empty). Infected males demonstrated greater number of sexual episodes (Figure 3A; paired t-test: $p = 0.021$, $n = 7$ infected and 6 control males; Effect size: Cohen's $D = 2.35$; statistical power = 95.6%). In the second scenario, the female had choice of mating with both control and infected male (competitive paced mating) allowing for a direct comparison. Seven pairs of males were tested, out of which in six trials, females had more number of sexual episodes

with infected males (Figure 3B; one-sample t-test: $t = 4.36$, $p < 0.01$). Thus, infected males obtained more sexual opportunities indicating that preference assay was a reliable indicator of female mate choice and that urinary cues could play a role in this behavior.

Sexual transmission of *Toxoplasma gondii*

This mate choice manipulation is quite a unique phenomenon as females normally detect and avoid parasitized males. The increase in preference and sexual episodes of infected males raises an interesting possibility that in addition to blunting selection pressure on *Toxoplasma* from the host, this manipulation could be a means to increase transmission to females and offspring. As described before, *Toxoplasma* transmits both sexually and vertically in livestock and dogs (Table 2) so rats were tested by dissecting the epididymis from infected males. The epididymis stores sperm where they mature prior to ejaculation. From this tissue mixture, *Toxoplasma* cysts were visualized with fluorescence staining (Figure 4A⁵). Females mated with infected males showed cysts in their vaginal lavage ($n=4$ Figure 4B). After these females gave birth, the brain homogenates of the pups also showed presence of cysts (Figure 4C). Thus, *Toxoplasma* transmits both horizontally and vertically transmission in rats and this sexual transmission could be a result of the increased attractiveness of infected males.

It is possible that the fatal attraction and mate choice manipulation are two sides of the same coin, by which I mean that could be a common physiological mechanism behind both- an increase in testosterone levels post infection. I previously described a possible role of testosterone in reduced fear/fatal attraction. Its effect on mate choice could be through its role in increasing male sexual investment or signaling, a widespread effect documented across species. The preference assays indicated that a component in rat urine could explain the enhanced attractiveness of infected males. Thus, I propose a role for MUPs as a putative sexual signal candidate in rats.

⁵ Done by Shantala Hari Dass and Deborah Dutta

Figure 4. *Toxoplasma gondii* is transmitted sexually and vertically. *Toxoplasma gondii* cysts were visualized in epididymis of infected males (A), in vaginal lavage of naïve females mated with infected males (B) and in brain smears of pups from these mating (C). Scale bar = 10 μ m. Parasites are stained red (anti-GFP antibody couples with fluorogenic detection using Cy3), cyst wall green (dolichos biflorus agglutinin coupled with fluorescein) and sperm nuclei in blue (DAPI).

Figure 5. Infection enhanced expression of MUPs (A) seen by greater abundance of MUPs mRNA levels in infected rat liver (n=8). Infected animals required fewer number of amplification cycles to reach a threshold for abundance. The y-axis depicts PCR cycles needed for MUP gene minus GAPDH gene (a housekeeping gene). The solid black circle with whiskers is the mean $\pm$ SEM; control= 3.71 $\pm$ 2.12; infected= -1.59 $\pm$ 1.56; (Mann-Whitney U test: $p = 0.041$). (B) Liver and urine from infected animals contained greater amount of MUP (n=8). The y-axis shows densitometric intensity, normalized to intensity of a pooled sample run in the same gel. The mean $\pm$ SEM in liver are control= 86.8 $\pm$ 4.1; infected= 158.7 $\pm$ 24.3 (Mann-Whitney U test: $p=0.007$) and urine are control= 68.5 $\pm$ 5.3; infected= 164.4 $\pm$ 41.1 (Mann-Whitney U test: $p=0.011$).

Figure 5 (C). Western blot images of liver urine and preputial samples for GAPDH and MUP protein. The red box denotes the area of the gel analyzed. GAPDH was used as internal control. No inter-group differences were observed in GAPDH levels. The urine samples do not have GAPDH protein and instead were normalized using creatinine levels.

Toxoplasma gondii affects MUPs expression

So first, I wanted to test if *Toxoplasma* infection had an effect on sexual investment, in other words, on the level of MUPs expression (see pg.103-7 for background). The liver is the major production site for MUPs in the male rat (136-140). Transcript levels were measured for MUPs mRNA abundance using qRT-PCR and infected male rat had greater levels compared to control males ($n=8$ each). Expression was assessed by number of amplification cycles required to reach a threshold as compared to reference gene (GAPDH) (Figure 5A control= 3.71 ± 2.12 ; infected= -1.59 ± 1.56 , Fold change= ~ 39 ; Mann-Whitney U test: $p= 0.041$; Effect size: Cohen's $D= 1.96$). This shows that infected animals had higher levels of MUPs mRNA. Next, protein levels were measured using western blots in liver and urine ($n=8$ each). In the liver, infected rats had 83% more MUPs protein than control rats (Figure 5B control= 86.8 ± 4.1 ; infected= 158.7 ± 24.3 ; Mann-Whitney U test: $p=0.007$) similarly in urine, infected rats had 138% more MUPs protein than control rats (Figure 5B control= 68.5 ± 5.3 ; infected= 164.4 ± 41.1 ; Mann-Whitney U test: $p=0.011$). In both liver and urine, 25th percentile of MUPs quantity in infected animals surpassed 75th percentile of respective controls. Preputial glands (they act as modified sweat glands located near the testes) also synthesize MUPs (141-144). However, MUP protein levels did not significantly differ between control and infected rats (Mann-Whitney U test: $p = 0.37$). Clearly, infected rats synthesized and excreted greater amounts of MUPs. Figure 5C is a representative figure of

the raw data used in this experiment to visualize changes in MUPs expression levels in liver, preputial glands and urine samples. For the urine western blots, the samples were creatinine normalized. This served two purposes- it acts as an internal loading control for western blots and provides information on infection effects on renal function. Both groups had comparable filtration rates of urine from kidney (creatinine content; control = 90.4 ± 13.3 mg/dl; infected = 87.1 ± 13.6 mg/dl; Mann-Whitney test $p > 0.9$). Thus, infection does not appear to have any adverse effect on renal function that could skew MUPs secretion into the urine. I have presented data that females prefer infected males possibly via urinary cues and greater MUPs expression. However, a pertinent question is whether MUPs is mediating the behavioral manipulation (i.e. female attraction)?

MUPs can recapitulate mate choice manipulation

The same setup was used as the initial female preference assay, the difference being that the stimuli used was only urine in this case. This setup allows us to rule out the role of acoustic or visual signals and assess role of urine stimuli on its own. First, we presented urine of infected and control males to estrous females to determine whether male urine is sufficient to recreate the *Toxoplasma* effect seen previously. In all preference assays mentioned henceforth, estrous females were used. The stimuli was dotted on filter paper and presented at terminal ends of the straight maze and I quantified time spent near stimuli. Females showed a clear preference for infected male urine. A preference score was calculated by dividing the time spent in the respective stimulus arms for each female. In this case, time spent in the infected arm divided by the control arm. A score greater than 1 would indicate a preference for infected arm. The average preference score is 2.12 ± 0.10 (one sample t-test: $p=0.019$) and 8 out of 11 females preferred infected stimulus (score>1).

Then urine from infected and control rats was competitively displaced for bound volatiles using a displacement agent, menadione (122, 123). This technique has been validated with

GC-MS (122) and they showed that this fraction retained behavioral activity. Using a centrifugal filter, the urine was fractionated and segregated into low (<3kDa containing volatiles and small peptides) and high (>3kDa containing mainly MUPs) molecular weight fractions (LMW and HMW respectively; more details in methods). First, the LMW fraction was tested. Females (n=16) did not show preference between the infected and control LMW fraction (50 μ L of stimulus). They spent on average $387s \pm 47s$ near the infected LMW stimulus compared to $398s \pm 35s$ near the control LMW stimulus (Wilcoxon signed ranks test: $p=0.96$; Effect size: Cohen's $d=0.26$). The average preference score is 0.95 ± 0.10 (one sample t-test: $p=0.68$) and 8 out of 16 females preferred infected LMW stimulus (score>1) (Figure 6A).

In contrast, females (n=16) showed a clear preference for infected HMW or MUPs fraction (50 μ L of stimulus). They spent on average $457s \pm 26s$ near the infected HMW stimulus compared to $282s \pm 20s$ near the control HMW stimulus (Wilcoxon signed ranks test: $p=0.001$; Effect size: Cohen's $d=3.46$). The average preference score is 1.59 ± 0.11 (one sample t-test: $p<0.0001$) and 15 out of 16 females preferred infected HMW stimulus (score>1) (Figure 6B). This supports that MUPs fraction is sufficient (after menadione displacement to remove bound volatiles) to recapitulate effects of *Toxoplasma* infection on rat mate choice. I went one step further and conducted a grafting experiment where I paired the infected MUPs with control LMW fraction and control MUPs with infected LMW fraction. If MUPs is the active attractive component, than preference for infected MUPs stimulus should remain. As such, females (n=12) still maintained preference for the infected MUPs (50 μ L of stimulus). They spent on average $493s \pm 34s$ near the infected HMW stimulus compared to $317s \pm 25s$ near the control HMW stimulus (Wilcoxon signed ranks test: $p=0.019$; Effect size: Cohen's $d=2.94$). The average preference score is 1.73 ± 0.21 (one sample t-test: $p=0.005$) and 9 out of 12 females preferred infected HMW stimulus (score>1) (Figure 7).

Figure 6. MUPs fraction is sufficient to elicit female attraction and recapitulate the mate choice manipulation by *T. gondii* infection. The y-axis depicts the preference score, which was calculated by dividing the time spent in the respective stimulus arms for each female. The filled black circles represent data obtained from individual females. (A) Females show no preference towards either control or infected LMW fraction (n=16). 8 out of 16 females showed preference for infected LMW fraction (score>1). The solid black circle with whiskers is the mean $\pm$ SEM; 0.95 ± 0.10 (one sample t-test: $p = 0.68$). (B) Females show preference towards infected HMW or MUPs fraction (n=16). 15 out of 16 females showed preference for MUP fraction (score>1). The mean $\pm$ SEM is 1.59 ± 0.11 (one sample t-test: $p < 0.0001$).

Figure 7. ‘Grafting’ experiment reinforces that MUPs is the active attractive component and attraction is dose dependent (n=12). I paired the infected MUPs with control LMW fraction and control MUPs with infected LMW. The y-axis depicts the preference score that was calculated by dividing the time spent in infected HMW stimulus arm by the control HMW stimulus arm for each female. The filled black circles represent data obtained from individual females. 9 out of 12 females showed preference for infected HMW fraction (score>1). The solid black circle with whiskers is the mean $\pm$ SEM; 1.73 ± 0.21 (one sample t-test: $p = 0.005$).

Toxoplasma infection increased expression of MUPs but as described in the discussion (pg. 103-107) there are multiple isoforms of MUPs, so could it possibly be that mate choice effect is due to alteration of MUPs expression pattern? 2D gels were run for urine of four infected and control animals to create a sort of MUPs “fingerprint” (Figure 8; see figure S7 for original gel picture with molecular weight marker). From the gels, there does not appear to be any consistent difference between infected and control rats in the various isoforms expressed. In other words, infection does not lead to expression of novel isoforms. From this, one can tentatively concluded that infection does not alter MUPs expression pattern. A possible reason for the complex pattern observed in the 2D gels is that in rats, there are 9 MUP genes (145) (so at least 9 MUPs could be expressed) and also some of the spots could be due to post translational modifications. Going forward, future research could focus on detecting possible alterations in expression of particular isoforms and identifying co-migrating spots.

We further looked into the HMW or MUPs fraction from four infected and control rats with FPLC analysis. FPLC chromatograms of both groups of animals were comparable (representative traces in Figure 9) with no novel peaks appearing in infected samples. Mass spectroscopic analysis of samples showed that the majority of urinary proteins consisted of two isoforms, MUP13 and MUP 10 (more details about identification in next chapter). In addition, numerous trials to clone MUPs from liver of control and infected rats only yielded MUP-13 and MUP-10 (from screening of 30 colonies). In other words, infected rats do not show expression of new MUPs isoforms.

In this chapter, I demonstrated that Toxoplasma creates a shift in female preference or mate choice towards infected males. The manipulation could serve to further increase parasite transmission through sexual and congenital modes. This phenomenon could be a result of the arms race between host and parasite with each trying to outwit the other resulting in constant

Figure 8. Representative 2D gels (Coomassie blue stained gel) of male urinary MUPs (n=8; 4 control and 4 infected). Urinary proteins were first separated using isoelectric focusing across a gradient from pH4-7 and then run on a SDS-PAGE for visualization. Infection with *T. gondii* does not seem to alter MUPs expression pattern.

Figure 9. Representative FPLC traces of control and infected urine samples. For each sample, urine was lyophilized and concentrated in PBS and 250 μ L was loaded. Elution buffer was PBS. Shaded region of chromatogram contains MUPs and peaks eluted before were characterized with mass spectrometry (details in next chapter). Both traces are comparable with no novel peaks in MUPs fraction in infected animals. Red arrow indicates the peak that was characterized as rat serum albumin.

Chapter 3

co-evolution. Such a dynamic has been termed the Red Queen hypothesis (24, 95) in which parasites that provide the hosts with reproductive advantage are favored. The trade-off between increased preference of infected males and cost of reduced fear/gain of attraction serves to blunt the selection pressure from hosts. To understand this manipulation, I demonstrated that *Toxoplasma* infected rats had greater expression of a possible sexual signal- MUPs, though no change was seen to its expression pattern. Moreover, I showed that MUPs fraction of urine is sufficient to recreate the mate choice manipulation. Taken together, this points towards female attraction being influenced by MUPs probably in a dose dependent manner rather than a qualitative or presence/absence of certain MUPs. The next chapter will continue along this theme to understand role of MUPs to elicit female attraction.

4. Necessity and sufficiency of MUPs

This chapter includes work published from ([±] Equal contribution) Kumar, V[±], Vasudevan, A[±], Soh, LTJ., Le Min, C., Vyas, A., Zewail-Foote, M., Guarraci, F. (2014) Sexual attractiveness in male rats is associated with greater concentration of major urinary proteins. *Biol Reprod.* **91**(6):150. **Figure 18 and 20 are reproduced from this paper in accordance with journal guidelines (<http://www.biolreprod.org/site/misc/permissions.xhtml>).**

Studies on rat mating strategies have described behaviors such as sperm competition, intrasex aggression and mate guarding that fall under the male domain (146, 147). In the following chapters, I turn the focus towards female mate choice and how male sexual signals could guide who they find attractive. Sexual signals have evolved with the ability to attract potential mates and provide conspecifics information on quality or suitability of an individual as a reproductive partner. As female rats are the limiting gender in mate choice (146), they are the ones exerting the choice while males display sexual signals. Interestingly, there is a clear and consistent aspect to female rat mate choice (148, 149). In mate choice studies using multiple males, females indicate preference for one male. They choose to spend greater time, more frequent visits, greater number of proceptive behaviors and shorter latency of revisiting the preferred male after sexual stimulation (150-152). In addition, female preference stays consistent for a specific male in a dyad when tested over multiple trials (152). Furthermore, there is a high level of concurrence in preference among multiple females for a male in a dyad (152). This is backed by the data in the previous chapter where several females in preference assays and in competitive paced mating consistently preferred infected males and their urine stimuli. Thus, female rat preference could depend on physiological traits of the male and not a random choice. Studies show that the preference is not a factor of body weight, testes size or urinary testosterone (150, 152). Instead, MUPs were enough to recreate *Toxoplasma* mediated mate choice effect and hence serve as a candidate to mediate mate choice.

In the next three chapters, I will be focusing on how MUPs function as a putative sexual signal in rats. To this end, in the following chapters I have moved outside the infection paradigm to investigate role of MUPs in female mate choice, the costs and dynamic nature of MUPs expression in male rats. I illustrate the necessity and sufficiency of MUPs to act as a female olfactory attractant. The previous chapter indicated a possible correlation between MUPs levels and female preference or choice, which I will demonstrate in a non-infection context.

Females show preference towards intact urine

To begin with, I had to rule out the non-olfactory modes like vocalization of rats as a source of attraction. I first demonstrated that whole intact urine, as a stimulus on its own, was capable to elicit female attraction. Females ($n=12$) showed a clear preference towards the intact urine stimulus compared to the empty arm (no stimulus), spending on average $683s \pm 51s$ and $273s \pm 37s$ respectively (Wilcoxon signed ranks test: $p= 0.003$; Effect size: Cohen's $d= 4.60$). The average preference score is 4.05 ± 1.24 (one sample t-test: $p= 0.032$) (Figure 10) and 11 out of 12 females showed preference for intact urine (score >1). All box plots in this report depict 25th percentile, median and 75th percentile.

Females show preference towards MUPs fraction

Then urine was competitively displaced for bound volatiles and fractionated into LMW and HMW or MUPs fraction as described in the previous chapter. Each fraction was tested for preference against menadione. Females ($n=12$) showed no preference for the LMW fraction (50 μ L of stimulus). They spent on average $424s \pm 57s$ near the LMW stimulus compared to $509s \pm 65s$ near the menadione stimulus (Wilcoxon signed ranks test: $p= 0.308$; Effect size: Cohen's $d= 0.49$). The average preference score is 0.93 ± 0.25 (one sample t-test: $p= 0.798$) and only 4 out of 12 females showed preference for LMW fraction (score >1) (Figure 11A).

Figure 10. Females show preference towards intact urine (n=12). The y-axis depicts the preference score, which was calculated by dividing the time spent in the intact urine arm by the empty arm for each female. The filled black circles represent data obtained from individual females. 11 out of 12 females showed preference for intact urine (score>1). The solid black circle with whiskers is the mean $\pm$ SEM; 4.05 ± 1.24 (one sample t-test: $p = 0.032$).

Figure 11. Necessity of high-molecular weight urinary fraction to attract females. The y-axis depicts the preference score, which was calculated by dividing the time spent in the respective stimulus arms for each female. The filled black circles represent data obtained from individual females. (A) Females show no preference towards LMW fraction (n=12). Only 4 out of 12 females showed preference for LMW fraction (score>1). The solid black circle with whiskers is the mean $\pm$ SEM; 0.93 ± 0.25 (one sample t-test: $p = 0.798$). (B) Females show preference towards MUPs fraction (n=16). 16 out of 16 females showed preference for MUP fraction (score>1). The mean $\pm$ SEM is 2.23 ± 0.25 (one sample t-test: $p < 0.0001$).

In contrast, females (n=16) showed a clear preference towards HMW or MUPs (50 μ L of stimulus) containing fraction. They spent on average $542s \pm 26s$ near the MUPs stimulus compared to $252s \pm 22s$ near the menadione stimulus (Wilcoxon signed ranks test: $p < 0.0001$; Effect size: Cohen's $d = 5.02$). The average preference score is 2.23 ± 0.25 (one sample t-test: $p < 0.0001$) and 16 of 16 females showed preference for HMW fraction (score > 1) (Figure 11B). Thus, urine can act as an attractive stimulus on its own. Moreover, I confirmed that the active component is the HMW or MUPs fraction demonstrating necessity of MUPs.

Rat urine contains copious amounts of MUPs, in addition to volatiles- both free and bound to MUPs (135). Bound volatiles remain in urine for several weeks and known to act as pheromones (122, 133, 153-155). Thus, it is also important to rule them out as a source of attraction. The unbound volatiles would separate out during the fractionation step and the bound displaced by the menadione (122,123). To verify for the presence of residual volatiles even after displacement and fractionation, DART-MS analysis was used. Volatiles in MUPs fractions prior to and after menadione treatment were compared with vehicle samples. In the case that MUPs are bound to volatiles, extra peaks should be observed in samples without menadione displacement. However, no additional peaks were observed from urine samples alone (no menadione), indicating that bound volatiles are either not present or are present at levels too low to be detected. Some of the peaks were a result of background signals from sample tubes and pipette tips (supplementary Figure 3). A drawback of this analysis is that no positive control was included which shows displacement of volatiles and detection by DART-MS.

Sufficiency of FPLC purified MUPs to elicit attraction

To demonstrate sufficiency of MUPs to mediate female preference, HMW or MUPs were fractionated using size-exclusion fast protein liquid chromatography (shaded region in

representative trace in Figure 12A). Peaks of smaller absorbance that were eluted before MUPs (fractions preceding the shaded region) were revealed to contain rat serum albumin (70.6kDa) and rat serotransferrin precursor protein (78.5kDa) using mass spectrometry. Shaded region of the chromatograph contained two adjacent peaks marked by an arrow. Separation of these fractions on SDS-PAGE revealed two co-migrating bands (Figure 12B). Mass spectrometry and western blots (Figure 14A) identified these bands as MUPs (more details in next section). DART-MS analysis did not reveal extra peaks in the FPLC-purified MUPs fractions in comparison to vehicle (saline).

Female preference of the FPLC purified MUPs fraction was tested. Purified MUPs (1.66 $\mu\text{g}/\mu\text{L}$ - within physiological range of adult male urinary MUPs) were resuspended in 100 μL castrated male urine and compared against castrated urine alone. The females ($n=12$) showed a clear preference towards FPLC purified MUPs fraction spending on average $490\text{s} \pm 37\text{s}$ compared to $342\text{s} \pm 34\text{s}$ near the castrated urine stimulus (Wilcoxon signed ranks test: $p=0.005$; Effect size: Cohen's $d=2.08$). The average preference score is 1.6 ± 0.2 (one sample t-test: $p=0.015$) and 11 out of 12 females showed preference for FPLC purified MUPs (score >1) (Figure 13). To rule out any effect of the castrated urine that was used to suspend the FPLC-purified MUPs it was tested against PBS and no attraction was observed ($n=6$; $398\text{s} \pm 59\text{s}$ versus $349\text{s} \pm 46\text{s}$ respectively; Wilcoxon signed ranks test; $p=0.56$).

Urine samples from castrated males were analyzed with DART-MS and did not contain signals corresponding to menadione or extra signals aside from background signals (supplementary Figure 3). Furthermore, castration decreases MUPs expression due to androgen dependency and does not elicit attraction from females. Expression of MUPs in male urine fell below the detection sensitivity of western blot (Figure 14A; 4 weeks post-castration). In addition, absorbance of MUP peak in FPLC was severely reduced two weeks after castration, and it dropped more after four weeks (Figure 14B).

Figure 12. Characterization of MUPs. (A) Size-exclusion fast protein liquid chromatography (FPLC) showed two dominant peaks (shaded area) adjacent to each other (arrow at the point of inflection). Urine was lyophilized and concentrated in PBS and 250 μ L was loaded. Elution buffer was PBS. (B) These peaks contained two co-migrating protein bands of $\sim$ 17 kDa (coomassie blue stained gel) and identified as MUPs by mass spectrometry and western blots. Red arrow indicates the peak that was characterized as rat serum albumin.

Figure 13. Sufficiency of FPLC purified MUPs fraction to attract females (n=16). The y-axis depicts the preference score which was calculated by dividing the time spent in FPLC purified MUPs stimulus arm by the castrated urine stimulus arm for each female. The filled black circles represent data obtained from individual females. 11 out of 12 females showed preference for FPLC MUPs stimulus (score>1). The solid black circle with whiskers is the mean $\pm$ SEM; 1.6 ± 0.2 (one sample t-test; $p=0.015$).

Figure 14. (A) Castration reduced the expression of urinary MUPs as seen with Western blot (4 weeks post-castration). First five lanes contain urine from intact male, serially diluted up to a factor of 16. The amount of MUPs in urine from castrated male was lower than a 16-fold dilution of the intact male urine. (B) FPLC revealed reduction in the peak containing MUPs at two (dark gray trace) and four weeks (light gray trace) post-castration, compared to intact urine (black trace) ($n = 2$ for each trace). For each sample, urine was lyophilized and concentrated in PBS and 250 μ L was loaded. Elution buffer was PBS. Red arrow indicates the peak that was characterized as rat serum albumin.

Figure 15. (A) Western blot confirming identity of rMUP10 and rMUP13. Maltose binding protein has a molecular weight of ~ 42 kDa, so rMBP-MUP fusion protein is around 60kDa. Lane 1: FPLC purified MUPs; Lane 2: rMUP13 and Lane 3: rMUP10. (B) Sufficiency of a combination of recombinant MUPs (rMUPs 10 and 13) to attract females ($n=10$). The y-axis depicts the preference score, which was calculated by dividing the time spent in rMUPs stimulus arm by the castrated urine stimulus arm for each female. The filled black circles represent data obtained from individual females. 8 out of 10 females showed preference for rMUPs stimulus (score >1). The solid black circle with whiskers is the mean $\pm$ SEM; 1.35 ± 0.15 (one sample t-test: $p = 0.047$).

Sufficiency of recombinant MUPs to elicit attraction

To confirm the identity of the two closely related MUP isoforms detected by FPLC, MUPs were cloned and expressed from cDNA library of the rat liver. DNA sequencing of thirty clones ⁶ revealed two distinct MUPs isoforms: LOC259246 (alpha-2u globulin PGCL1, 99% homology) and LOC298109 (alpha-2u globulin PGCL2, 100% homology). These DNA sequences matched with amino acid sequences from mass spectrometry of FPLC purified MUPs (Supplementary Figure 4). The more abundant of the two bands from FPLC-derived MUPs (Figure 12B, MUP_{lower}) was identified as PGCL1 and less abundant band was identified as PGCL2 (Figure 12B, MUP_{upper}). Following previously published *in silico* (145) and *in vivo* (123) data, these proteins will be referred as MUP-13 and MUP-10, respectively.

Recombinant MUP-13 and MUP-10 proteins were expressed in fusion with maltose-binding protein (rMBP-MUP; confirmed with western blot- Figure 15A). rMUP 10 and rMUP 13 could not elicit attraction from females on their own (supplementary Figure 5). Since FPLC purified MUPs contained both isoforms, the two choice preference task was repeated by combining rMUP 10 and rMUP13 (in a 1:2 ratio; 1.66 ug/ μ L) and suspended in 100 μ L of castrated male urine. Females (n=10) showed preference towards rMUP spending on average 533s $\pm$ 36s compared to 388s $\pm$ 23s near the castrated urine stimulus (Wilcoxon signed ranks test: p = 0.038; Effect size: Cohen's= 2.40). The average preference score is 1.35 $\pm$ 0.15 (one sample t-test: p = 0.047) and 8 out of 10 females showed preference for rMUPs (score>1) (Figure 15B). To rule out any effect of the MBP tag, it was tested against castrated urine alone and no attraction was observed (n=5; 419s $\pm$ 40s versus 498s $\pm$ 43s respectively; Wilcoxon signed ranks test: p = 0.37). It is interesting to note that despite the high sequence identity of the isoforms, both were not sufficient on their own to elicit attraction. This could

⁶ Done by Vineet Kumar

mean that small variations in the protein sequence or differences in folding pattern are critical such that attraction requires complete or whole signal.

Sufficiency of renatured MUPs to elicit female attraction

Previously, I demonstrated that MUPs fraction from urine was able to maintain higher attractiveness even after menadione treatment, which supports the ideas that MUPs instead of volatiles bound to it were the active component. I conducted a subsequent experiment to assess the possibility of residual volatiles still bound to MUPs in trace amounts undetectable by DART-MS. FPLC-purified MUPs fraction was denatured, dialyzed and transferred to PBS for renaturation. Denaturation was done with 6 M guanidinium hydrochloride which has been used (156) to cause release of volatiles bound to MUPs when it loses its conformation. Dialysis with excess buffer was done to remove any residual volatiles and for refolding of proteins to native state. Renaturation was confirmed with Native PAGE (supplementary Figure 6). This MUPs fraction (in PBS) was used in mate choice assay (tested against PBS) and 10 out of 12 females showed attractiveness towards this fraction (Figure 16; $604s \pm 37s$ versus $280s \pm 29s$; Wilcoxon signed ranks test: $p = 0.002$; Effect size: Cohen's $d = 4.87$).

Figure 16. Females exhibited attraction to FPLC-purified MUPs fraction even after its denaturation in 6M guanidinium hydrochloride, subsequent dialysis and then renaturation in buffered physiological saline to remove all bound volatiles. Females ($n = 12$) showed preference towards renatured MUP spending on average $604s \pm 37s$ compared to $280s \pm 29s$ near the castrated urine stimulus (Wilcoxon signed ranks test: $p = 0.002$)

Dose-dependence of MUPs elicits attraction

Data until here shows that MUPs are sufficient and necessary to elicit female attraction. In the previous chapter, we saw that females preferred *Toxoplasma* infected rats that could be due to increased MUPs levels post infection. As such, it is important to understand how MUPs signal attractiveness- is it dose dependant or presence of certain isoforms? Precedent work in mice indicates MUPs in male contests operate through competitive scent signaling, requiring males to express unique MUPs signature (132, 157-160), however, there have been reports of limited variation in MUPs expression pattern in lab and wild mice strains possibly due to inbreeding (161-163). Previous work (135, 164, 165) and the 2D gels of urine samples (in chapter 3) I tested appear to indicate that Wistar male rats (strain used in my work) do not express unique MUPs variants. Data so far suggest that in rats, MUPs may have limited applicability for recognition of individuals. As such, it is highly likely female preference for males is MUPs dose dependent.

To test this, a two choice preference task was done where females were tested for attraction to different concentrations of FPLC purified MUPs- 1X (1.66 $\mu\text{g}/\mu\text{L}$) or 3X (~ 5 $\mu\text{g}/\mu\text{L}$). Females ($n=11$) showed more preference towards the higher concentration (3X) of MUPs. They spent on average $334\text{s} \pm 40\text{s}$ near the 3X stimulus compared to $208\text{s} \pm 24\text{s}$ (Wilcoxon signed ranks test: $p= 0.016$; Effect size: Cohen's $d= 1.91$) near 1X stimulus. The average preference score is 1.53 ± 0.24 (one sample t-test: $p= 0.054$) and 8 out of 11 females showed preference for 3X FPLC purified MUPs (score >1) (Figure 17).

Figure 17. Dose-dependent attraction of MUPs by females ($n=11$). The y-axis depicts the preference score which was calculated by dividing the time spent in 3X ($\sim 5 \mu\text{g}/\mu\text{L}$) stimulus arm by 1X ($\sim 1.66 \mu\text{g}/\mu\text{L}$) stimulus arm for each female. The filled black circles represent data obtained from individual females. 8 out of 11 females showed preference for 3X stimulus (score >1). The solid black circle with whiskers is the mean $\pm$ SEM; 1.53 ± 0.24 (one sample t -test: $p = 0.054$).

Figure 18. Attractive members of a male dyad exhibited greater amounts of MUPs compared to less attractive members (A; $n = 11$ dyads). Boxes represent the 25th, 50th, and 75th percentiles. Dot-and-whisker represents the mean $\pm$ SEM. Ordinate depicts amount of MUPs in arbitrary units (pool of all samples arbitrarily placed at 100 AU). $**P = 0.01$, paired t -test. The relationship between MUPs and number of pups sired was not statistically significant (B; $n = 11$ dyads).

Correlation of female preference and MUPs levels

To further establish the dose dependent nature of MUPs; one can pose the question whether female preference can be determined or predicted based on male MUPs levels? That is, if MUPs level of males in a dyad are known can we predict preference on the basis of MUPs? For this, I received urine samples of 11 dyads/pairs of males (Long-Evans) from our collaborators who were repeatedly tested for female preference (methods can be seen in (134, 150, 151)). Based on the multiple trials, an attractiveness ratio was assigned for each male. It was calculated by dividing the number of tests a male was preferred out of all of the mate choice tests that each pair was used in. The mean attractiveness ratio for the attractive males was 0.73 ± 0.04 , in other words, the females in 73.4% of all mate choice tests preferred the attractive male in each dyad. This is significantly higher than chance (0.50 or 50%) level ($|t_{10}| = 5.30, p = 0.01$, one-sample t -test). This created two groups of males- the attractive and non-attractive- from which urinary MUPs levels were measured which would answer the question whether attractive males expressed more MUPs. Across the board, urine of attractive males had greater levels of MUPs, about twice as much as non-attractive partner (Figure 18A, $|t_{10}| = 3.02, p = 0.013$, paired t -test). The 25th percentile of MUPs quantity in the attractive male rats was greater than the 75th percentile of respective non-attractive partners. Creatinine levels between the groups were not significantly different ($t = 0.12, p = 0.90$, paired t -test).

Furthermore, we assessed the male pairs with respect to the number of pups sired. In line with previous report (150), the group of males with fewer progeny had higher MUPs levels (Figure 18B, $|t_{10}| = 1.90, p = 0.086$; one-sample t -test). Thus, there is no apparent relation between attractiveness (MUPs levels) and reproductive success (pups sired). In other words, mate choice assay does not reflect or predict number of progeny. This could be due to cryptic sperm competition in utero upon ejaculation, either in terms of sperm count (166), storage capacity of sperm (167), competition among sperm from different males (167) and effective

deposition of sperm (appropriate placement of copulatory plug) (168). Moreover, studies with laboratory strains raise questions about suitability of mate choice experiments that use proxy measures like odor (urine or bedding). For example in (169), it is shown that though wild-derived female mice avoid infected (with *Salmonella enterica*) males in the beginning, they do not avoid mating with them. They also reported no difference in reproductive success.

Interestingly, in one dyad where females showed equal preference (i.e. attractive ratio of 50%), MUPs levels were nearly identical between both males. To make this clearer, I created a MUP ratio, which is the ratio of MUPs of attractive or preferred male divided by MUPs of non-attractive male. A MUP ratio >1 would mean that the attractive male (in mate choice assay) also had greater expression of MUPs. In 9 pairs, female preference correlated with the male with higher MUPs (Figure 19, MUP ratio >1). More interestingly, in one pair where females showed equal preference for both males, MUPs level were nearly identical (Figure. MUP ratio =1; highlighted with a red circle).

A significant positive correlation was seen between MUPs levels and attractiveness ratio of each individual male rat (Figure 20A, Pearson correlation coefficient = 0.43, $n = 20$, $P = 0.048$). This could mean that females would deem males with greater level of MUPs more attractive. Subsequently, to analyze the data independently of dyad grouping, I carried out my own preference assay for each male's urine against a pooled sample (a pool of urine from all males). Samples were again displaced with menadione and fractionated. Once again, a significant positive correlation was observed between time spent by females ($n=4$) near stimulus and MUPs levels (Figure 20B, Pearson correlation coefficient = 0.62, $n= 20$ $P = 0.004$). As such, this data lends credence to the possibility of dose dependence and using MUPs levels to predict female preference.

Figure 19. Females preference correlates with greater MUPs levels ($n=11$). The y-axis depicts the MUP ratio, which is the ratio of MUPs of attractive male divided by MUPs of non-attractive male. The filled black circles represent ratio obtained from each male pair. In 9 out of 11 pairs, female preference correlated with the male with higher MUPs levels (MUP ratio >1). The solid black circle with whiskers is the mean $\pm$ SEM; 3.28 ± 0.95 (one sample t-test: $p = 0.038$). More interestingly, in one pair where females showed equal preference for both males, MUPs level was the same (MUP ratio = 1; red circle).

Figure 20. Attractiveness of individual male rats was positively correlated with the amount of MUPs, both in terms of attractiveness displayed in (A) competitive setting within a dyad ($n = 22$ males) and (B) olfactory attraction measured against standard pooled urine sample ($n = 20$ males). Abscissa depicts amount of MUPs in arbitrary units (pool of all samples arbitrarily placed at 100 AU). Ordinate depicts attractiveness ratio (A) or time spent by females near menadione-displaced HMW fraction of the individual urine samples (B)

In this chapter, I have demonstrated the necessity and sufficiency of MUPs to elicit female attraction using urinary MUPs fraction, FPLC purified MUPs and recombinant MUPs. With the use of menadione displacement and DART-MS, I have shown that MUPs without bound volatiles retain ability to create olfactory-based attraction acting as a probable sexual signal. Moreover, by denaturing and renaturing MUPs I could further to rule out a role for volatiles and demonstrate the MUPs on their own are sufficient to elicit attraction. However, similar to MUPs, volatiles in urine are also sexually dimorphic and androgen dependent (170, 171). The addition of volatiles to castrated urine can increase attractive quality of the castrated urine though not to the levels of intact urine (170). Thus, the role of MUPs needs to be placed beside the function of urinary volatiles. The data also points toward a dose dependent function of MUPs in mate choice as seen by the female preference for higher concentration of purified MUPs and with the positive correlation of MUPs levels with attractiveness ratio. In other words, MUPs could act as a physiological feature to distinguish attractiveness of male rats and possibly predict female preference.

Various sexual selection theories state that sexual signals are honest reflectors of male quality as the expression of the signals is costly in terms of resources needed for it (172). This cost creates a handicap in survival or viability of an individual (115, 173). This is the case with male rats (who secrete MUPs at times in orders of magnitude greater than females) and the cost could come in part from its testosterone dependency (139, 143, 174-176). Thus, only fit males can express and maintain ‘costly’ sexual signals, which often require testosterone that could impose costs in metabolism, immunosuppression and life-history conflicts (177, 178). The handicap hypothesis was initially proposed by A Zahavi who stated that “waste can make sense, because by wasting one provides conclusively that one has enough assets to waste and more. The investment –the waste itself- is just what makes the advertisement reliable” (179). However, common criticism, of this hypothesis included that it relies too heavily on

modeling and less on empirical evidence. Moreover, often it is hard to determine the true costs of signaling and to differentiate marginal and strategic costs. Additionally and contrary to common predictions, honest signals are not necessarily handicaps and that showing that a signal is costly is not enough to understand the handicap mechanism. In (180) and (181), they provide a thorough examination of the handicap hypothesis and its limitations

As fitness levels vary due to factors such as diet, age, social or health status, this determines how an individual copes with the costs and demands of a sexual signal. This would lead to variation or dynamic expression of sexual signals that in turn, conspecifics use indirectly to assess fitness. This story is made more interesting as *Toxoplasma* infection subverts the idea of sexual signals accurately reflecting infection status (as overall indicator of male quality) as described in chapter 3. By increasing expression of MUPs, females might be deceived to mate with infected males thereby being infected themselves, however, it is not known if this correlates with reproductive success. It is unknown how exactly *Toxoplasma* affects MUPs expression and a study has demonstrated that depending on the strain or host, infection can increase or decrease testosterone (182). The experiment in this chapter tentatively suggests that males with higher MUPs levels do not gain an advantage in reproductive success (Figure 18B). In the next two chapters, I will be looking into possible costs of increased MUPs signaling and the factors that could affect its signaling. In conclusion, MUPs role as a sexual signal involves the ability to elicit female olfactory attraction and be of predictive value in female mate choice.

5. Kairomonal communication in mice

This chapter includes work from two papers I published

1. Vasudevan, A., and Vyas, A. (2014) *Toxoplasma gondii* infection enhances the kairomonal valence of rat urine. *F1000Res.* 3:92. **Figure 22 and 23 are reproduced from this paper under the terms of the Creative Commons Attribution License.**

2. Vasudevan, A., and Vyas, A. (2013) Kairomonal communication in mice is concentration-dependent with a proportional discrimination threshold. *F1000Res.* 2:195. **Figure 24 and 25 are reproduced from this paper under the terms of the Creative Commons Attribution License.**

In nature, sexual signaling has developed in a myriad of forms to target the various sensory systems. In humans with our well-developed visual senses, colors (183-185), facial features (186, 187) and body shapes (188-192) are among the varied sexual signals. However, there is growing evidence among humans for a pheromonal sexual signal (193-196). Similarly, signaling with feathers of peacocks, songs of canaries and urinary pheromones (MUPs) acts on the visual, aural and olfactory systems respectively. One of the advantages of such modes of display is that the signaling by an individual can act or communicate from a distance due to its open broadcasting nature (41). In this chapter, I will be focusing on a cost of increased levels of signaling (observed in the previous chapters) upon this open olfactory or pheromonal communication system.

Pheromones are chemical compounds secreted by an animal that alters behavior of conspecifics of the same species and fall under the category of chemical communication. In mammals, pheromones are utilized to signal dominance and influence mate choice (122, 133, 197) and among insects, they can communicate danger, sources of food and sexual receptiveness (198-203). In addition to the advantage of being an open broadcast system, pheromonal communication through urine marks (MUPs) also does not need the individual to be physically present as urine or scent marking can remain stable in the environment for many days (133). However, unintended receivers like predators can intercept this open communication system of pheromones (204-206). This is similar to dangers of brighter

coloration on birds that make them more visible to both mates and predators. Thus, though greater expression of pheromones (MUPs) allow the male rat to benefit from increased attractiveness (as seen in *Toxoplasma* infected rats (chapter 3) and its dose dependent effect on mate choice (chapter 4)) and possibly reproductive opportunities, it could be at the cost of greater predation. To test this, one would need to compare predation rates between rats that have different levels of MUPs or between control and *Toxoplasma* infected animals (shown to increase MUPs). Due to ethical considerations of such an experiment, we can instead study the trade-off from another unintended receiver of pheromones- the prey species. The idea is that if predators are more likely to detect preys with stronger sexual signaling then vice versa should hold true. That is, prey could be able to decrease danger of predation (123, 207), raising possibility of opportunity cost.

When individuals of another species receive the same pheromones that affect their behavior, these very chemical compounds are called kairomones (208). Kairomones cues are co-opted by predators to locate prey for their food and by prey to act as warning signals of predators. Such signaling is to the benefit of the receiver at the cost or detriment for the emitter due to the open nature of pheromones/kairomones (209). Thus, odors that function as pheromones in intra-species signaling are liable to be co-opted or eavesdropped as kairomones by unintended prey or predator of that species.

In this chapter, I will be looking at rat urine and MUPs in their role as kairomones towards its prey, mice. Rats predate house mice with studies showing that around 70% of wild rats kill mice (muricide) (210, 211). This behavior is affected by rearing (212), availability of food (211), social (213) and environmental conditions (214). Due to this intense predation pressure, mice have evolved innate fear to rats (215, 216) and their urinary odors (217), with MUP13 being identified as the key kairomone, precluding the need for a live rat (123). These studies showed typical defensive behavior in addition to activation of brain pathways for such

behavior and secretion of stress hormones in response to rat odors. Thus, like female rats, mice display innate behavior (attractive vs. aversive) to rat urinary odors- possibly MUPs. Thus, I wanted to investigate if there was similar but opposite effect of increased MUPs signaling on mice avoidance behavior. First, I tested if mice could discriminate (like female rats) between control and *Toxoplasma* infected urine and second, I tested for dose dependence in avoidance behavior. Heightened avoidance or aversion behavior in mice could indicate an opportunity cost in terms of loss of predation for rats. This could be a trade-off for rats face when benefiting from increased but open signaling of pheromones (MUPs).

Increased kairomonal valence of *Toxoplasma* infected rat urine

We have seen that *Toxoplasma* infected rats have greater MUPs expression and females show greater preference towards them (96). Following this, it would suggest that mice should display greater avoidance to the enhanced pheromone levels of infected male rats. In other words, we tested if *Toxoplasma* infection increased the kairomonal valence of rat urine to mice. For this, we compared avoidance of mice to control and infected urine/scent marks using an avoidance-avoidance conflict assay. Furthermore, we tested behavior with both fresh urine marks and aged urine marks (in sequence, aged followed by fresh marks). For fresh urine testing, rat urine was collected using metabolic cages and pooled from two rats (control and infected) on the same day of testing. The purpose of aged urine was to determine role of non-volatiles (MUPs) compared to volatiles. Volatiles dissipate quickly over time (bound volatiles will take longer), while non-volatiles in urine remain stable for weeks (154). A plastic Petri dish was placed in home cage of rats to urine mark for twenty-four hours. The Petri dishes were stored at RT for three days and then was used as the stimulus in the assay. Avoidance was measured by calculating time (20 minute trials) spent by mice in two opposing bisects of arena (76×9 cm; 15 cm high Figure 21). Opposing bisects either had urine from control or infected rats (100 μ L each). In the case of aged urine, the three day old Petri dishes from the

control and infected rats were positioned at ends of opposing bisects. Avoidance was quantified as described above. The same batch of mice was used for both assays.

The behavior tests showed that mice ($n=12$) spent more time in the bisect with fresh urine from control rats (Figure 22A, 674 ± 55 s in control bisect versus 510 ± 49 s in infected bisect; paired t-test: $t_{22} = 2.13$, $p = 0.037$; Effect size: Cohen's $D = 0.909$). A preference score was calculated for each mouse by dividing the time spent in the control bisect by the infected bisect. In 9 out of 12 trials, mice spent more time in the control bisect (Figure 22B; mean preference score = 1.36 ± 0.22 ; 1 outlier removed). This was also seen when aged urine marks was tested. Mice spent more time in the bisect containing aged urine marks from control rats (Figure 23A, 715 ± 52 s in the control bisect versus 469 ± 51 s in the infected rat bisect; paired t-test: $t_{22} = 3.36$, $p = 0.035$; Effect size: Cohen's $D = 1.38$). Similarly, 9 out of 12 mice spent more time near the urine marks obtained from control animals (Figure 23B, mean preference score = 1.94 ± 0.36). ANOVA was done with time spent in control or infected bisect and fresh or aged urine as two sources of within subject factors. ANOVA showed a significant main effect for the infection status of rats ($F(1,11) = 8.049$, $p = 0.016$). There was no statistical significance in the difference between fresh and aged urine marks ($F(1,11) = 0.005$, $p > 0.9$). Similarly, the interaction between the infection status and the age of the urine marks was not statistically different ($F(1,22) = 0.314$, $p > 0.5$).

Figure 21. Diagram of the maze or arena used to observe mice movement and time spent near opposing rat urine stimuli. The dimensions were 76×9 cm; 15 cm high.

Figure 22. Uninfected mice avoid fresh scent marks obtained from infected rats. (A) Preference was quantified by comparing time spent by mice in two opposing bisects of an arena, with each containing fresh urine from control or infected rats (trial duration = 1200 s, $n = 12$ mice). Ordinate and abscissa depict time spent in infected and control bisect in seconds, respectively ($p = 0.037$, paired t-test). Mean and SEM of data used in scatter-plot are depicted by dot and whiskers (674 ± 55 s in control bisect versus 510 ± 49 s in infected bisect). (B) A preference score was computed for each subject by dividing the time spent in the control bisect by the infected bisect (chance = 1). The filled black circles represent preference score from one mouse (1 outlier removed). Box plots show median, 25th and 75th percentile. The filled black circle with whiskers is the mean $\pm$ SEM; 1.36 ± 0.22 .

Figure 23. Uninfected mice avoid aged scent marks from infected rats. (A) Preference of mice for control urine marks was observed even when aged (3 days old) rat urine was used. Mean and SEM of data used in scatter-plot are depicted by dot and whiskers (714 ± 52 s in control bisect versus 469 ± 51 s in infected bisect; $p = 0.035$, paired t-test). (B) Preference score from each mouse is represented by filled black circles. The filled black circle with whiskers is the mean $\pm$ SEM; 1.94 ± 0.36 . See Figure 20 for further details on graphs.

Thus, fresh and aged urine marks from *Toxoplasma* infected rats elicited a greater avoidance or kairomonal response in mice. With the use of aged urine, the key component in the kairomonal response appears to be non-volatiles (MUPs). This corroborates a previous study where recombinant MUPs (MUP13) was sufficient to induce avoidance behavior in mice (123), excluding a necessary role for volatile components of urine. Thus, the trade-off between male rats and *Toxoplasma* (increased MUPs production and attractiveness versus increase in transmission frequency) creates a possibility for greater avoidance by mice- a possible cost for rats. Putting this together, the data suggests this mice behavior, like with female rats, and is likely due to greater MUPs levels in infected rat urine. To test this, my next step was to assess if the avoidance behavior or kairomonal communication is dose dependent.

Dose responsivity of kairomonal communication

The neurobiological and physiological aspects of kairomonal communication in rodents have been studied (123, 218, 219) so I looked into its perceptual properties. So far, kairomonal communication has been described from the point of the predator, rat. For foraging animals like mice, they have to assess constantly the trade-off between searching for food and mates against avoidance of predators. The trade-off is often mediated by detection of predator odors or cues (kairomones) to assess the level of predation threat against the need for foraging opportunities (218, 220, 221). To balance this, kairomonal responses would lie along a continuum possibly based on intensity or dose responsivity of kairomones instead of a binary all or none response. Many pheromonal responses are dose dependent ((222-224) and in this thesis) so it follows that kairomonal responses would be similar in nature. For such a system, animals must be able to estimate or detect quantitative differences in kairomonal odors.

Animals have shown the ability to quantify estimates of percepts like effort, time, reward and olfaction (225-229). These have been studied by “using a comparative representational

system that is dependent on the ratios between opposing quantities” (217). This capability to discriminate between strength/quantity of percepts allows the animal to regulate its behavior to best manage trade-offs present in its environment. In addition to testing for dose responsivity of kairomonal response, I looked at discrimination threshold or the ‘just noticeable difference’ between two stimuli of different intensities. The sensitivity to changes in the quantity of a stimulus lowers with increase in magnitude of stimulus. Thus, when opposing stimuli are weak, the discrimination threshold is smaller as compared to stronger opposing stimuli. This idea is referred to as Weber’s Law (230) and is a basic feature of a number of percepts.

First, dose responsivity was tested. Rat urine was collected, displaced with menadione and fractionated with the 3 kDa centrifugal device as described previously. Only the MUPs fraction (from now on referred to as rat urine) was used in the assays. The response of mice to increasing doses of rat urine was observed (10 minute trials). Avoidance was quantified by computing time spent by mice in two opposing bisects of an arena containing the stimuli (same setup as depicted in Figure 21). Opposing bisects contained either rat urine or phosphate-buffered saline. The amount of rat urine was methodically varied from 1X to 16X (3.125, 6.25, 12.5, 25 and 50 μ L). X was arbitrarily defined as 3.125 μ L of rat urine. The various doses were made by a twofold serial dilution to maintain a constant volume of stimuli (50 μ L) while the concentration varied. The stimuli was placed at the terminal ends of the bisects and the mice had direct access to the stimuli. The same set of mice ($n=10$) was used in testing for all doses (from lower to higher doses) with a 24 hours gap between two consecutive trials. ANOVA showed that time spent near rat urine decreased with an increasing amounts of rat urine (Figure 24; $n = 10$; $F(4,45) = 6.9$; $p = 0.0002$). Mice spent significantly more time near the lowest dose (1X), compared to the strongest (LSD, Fisher's least significant difference, $p = 0.0002$). The stimulus-response curve displayed a strong fit to

sigmoidal curve (Figure 24; $R^2 > 0.99$; $p < 0.01$), exhibiting a monotonic linear response between doses- 2X to 8X. To rule out the possibility of carry-over effects during repeated trials, a separate set of five mice was tested repetitively at a single dose (4X) across five days. These mice showed a comparable aversion to rat urine over the trials, showing a lack of habituation, sensitization or conditioning during repetitive testing (one-way ANOVA; $F(4,20) = 0.314$, $p > 0.8$).

Second, discrimination threshold was tested with the same set up. The only difference being that both bisects now had rat urine as stimuli. The stimuli were made as before to only alter the concentration but keeping an equal volume. We tested five equidistant doses (Log2 scale) covering the linear part of the dose-response curve (2X-8X or 6.25-25 μL). One bisect contained these doses as stimuli. The discrimination for each dose was investigated in two consecutive trials by providing more rat urine in the opposing bisect, that differed by a ratio of either 1.2 or 1.3. The mice ($n=15$, same mice that were used in the dose response experiment) were observed for time spent in each bisect. Again, the same set of mice was used in testing for all doses (from lower to higher doses) with a 24 hours gap between two consecutive trials. A positive discrimination was observed as a smaller amount of time was spent in the arm containing the greater amount of rat urine. A two-way ANOVA for dose and ratio showed a significant main effect of the ratio (Figure 25A; $F(1,138) = 32.1$, $p = 0.00000008$). The main effect of doses did not achieve statistical significance ($F(4,138) = 1.347$, $p = 0.256$). Moreover, the interaction between ratios and doses was not significant ($F(4,138) = 1.214$, $p = 0.308$). Therefore, irrespective of the dose, discrimination threshold was always proportional to the kairomone strength (Figure 25B) between a ratio of 1.2 and 1.3. In other words, the discrimination threshold was smaller for weaker doses and greater for stronger doses.

Figure 24. Kairomonal communication in mice is dose-dependent. Aversion of mice to increasing doses of rat urine was quantified by comparing the time spent in arm containing increasing doses of rat urine and the other with PBS (trial duration = 600s, $n = 10$ mice). The graph depicts mean time spent in arm with rat urine (mean $\pm$ SEM). The gray line depicts sigmoidal fit. Abscissa shows dose of rat urine used ($\log_2$ scale; x set as 3.125 μ L of rat urine).

Figure 25. Discrimination threshold in mice is proportional to kairomone strength. The discrimination threshold at different doses of rat urine was tested with an avoidance-avoidance conflict, where bisects contained either lower or higher quantity of rat urine. The higher dose was of either a 20% (white bars) or 30% (shaded bars) greater magnitude (e.g. 120% or 130% of 2X). (A) A significant main effect of ratio is observed with mice overall spending greater time (mean $\pm$ SEM) near the lower magnitude or ratio for each dose. (B) Abscissa represents the lower dose used in each of the comparisons (e.g. 2X). Ordinate represents time spent (mean $\pm$ SEM) in arm with the greater amount of rat urine divided by the sum of the time spent in both arms (gray line = 50% chance). $N = 15$ mice for all comparisons. $\log_2$ scale; arbitrarily set as 3.125 μ L of rat urine.

I have shown that mice perception of kairomones in rat urine (MUPs) is dose dependent. The regulation of avoidance behavior indicates an ability to discriminate quantities or strength of kairomone signal. This would allow mice to calibrate behavioral responses with information on predation threat. As such, a weaker kairomone signal could indicate that a longer time has passed since urine mark was deposited, lowering chances of presence of a predator and would elicit less avoidance. A stronger signal would suggest a more recent threat of predator in the environment. A similar dose response system has been studied in perception by rats towards cat stimulus (219). Such discrimination of kairomones appears to rely on relative quantitative estimates of opposing stimuli and not on an absolute scale. A discrimination threshold characterizes the relative estimation, which is a constant proportional fraction of stimulus strength (Weber's Law). Prior studies have shown that other similar olfaction percepts follow Weber's Law in humans (volatile odors (229)) and Argentine ants (224). With mice, the kairomonal communication follows this principle with a discrimination threshold valued between 1.2 and 1.3 times the stimulus.

Until this point, I have shown that in rats, MUPs serve as both a source of pheromones and kairomones depending on the identity of the receiver. Pheromones used in intra-species communication are open to be exploited as kairomones as conspecific communication needs strong expression of chemicals. This makes them ideal candidates for eavesdropping by other species (116). Mice show increased avoidance behavior from urine of *Toxoplasma* infected rats and this kairomonal response to MUPs fraction is dose dependent. This is a similar but an opposite effect to attractive behavior of female rats towards MUPs. Therefore, the benefit of increased MUPs signaling is at a possible cost of loss of predation opportunity due to the open nature of pheromonal and kairomonal communication. This goes back to the idea of honest nature of sexual signals due to the cost incurred by males to sustain its expression despite the handicap imposed (115, 172). In this possible scenario, rats that benefit from

greater female preference through increased MUPs levels could have to deal with the cost of easier detection by mice. Of course this hypothesis must be tempered till it can be more directly tested. Moreover, it can be argued that the cost of eavesdropping is an inherent and inescapable constraint of such signaling. As such, it might not be a 'true' handicap when compared with more traditional examples like lower survival or reduced immune function. My aim was to suggest that costs arise from the fact that males producing more MUPs could be at a greater disadvantage as prey can more easily detect such individuals and lead to loss of feeding opportunity. How individuals respond to costs of sexual signals depends on their fitness level, which is affected by internal (health, age), and external (social status) factors that is termed as condition dependence of sexual signals (95, 231). The interplay of these factors determine the extent an individual can allocate resources to sexual signaling that in turn would serve as readout or proxy for fitness. In the next chapter, I look into these factors and study how MUPs signaling varies because of condition dependence of the individual.

6. Condition dependence of MUPs

One of the main themes of sexual selection puts forward that sexual signals reflect the current state or condition of the individual (115, 172). Factors like health, age, diet and social environment together affect physiological and endocrine processes that determine signaling and condition. In other words, expression of traits is dynamic in nature and can accurately reflect condition as signaling often depends on the very same internal and external factors - hence the phrase condition dependence (231). As such, expression of signals would vary in different contexts and be restricted when the cost to sustain the signaling becomes too high for the individual (173). For instance, signaling can be up regulated during mating season or down regulated during times of environmental stress (e.g. lack of food) or sickness. Display of traits would allow an individual to showcase its ability to manage such costs and thereby ones fitness or genetic legacy (112). Moreover, expression of sexual signals would be affected by the needs of diverse situations and relay information on the condition of an individual. In this chapter, I will be studying three common factors (age, health and social environment) which affect an individual's condition. I studied whether expression of MUPs is similarly dynamic and open to manipulation by condition altering factors.

Longitudinal analysis of MUPs expression

The first factor I studied was the effect of aging on MUPs. Aging is characterized by senescence, which is the decline in normal physiological functions like motor impairment, muscle loss, neuron degeneration and lower hormonal levels (232-234). An idea behind evolution of female mate choice is that decline in these and other physiological functions would lower overall male condition or fitness which would be reflected in a similar decline in male displays (115, 172). Evolutionary ideas of ageing suggest that a tradeoff occurs between increased reproductive investments (which include sexual signaling) earlier in life at the cost

of lower physiological functions later in life (235-238). Thus, phenotypes that benefit the individual early in life to pass on its genes are selected despite their contribution to senescence (239, 240). It is expected that males would exhibit a more drastic tradeoff given that they can possibly sire more offspring and incur greater risks involved with male competition and reproductive behavior (241, 242). Expression and sustaining costly sexual signals are also part of greater reproductive investment by males (115). Evidence for expression of traits causing faster senescence or aging has been seen in houbara bustards. In these birds, males that invest more in sexual display show lower sperm quality or “spermatogenic burn-out” sooner than others (235). Interestingly, males that excessively spent more time and energy in sexual displays during their younger ages maintained the levels later in life but had lower sperm count (85% lower), less motility and morphological defects. This calls into question the honesty of sexual signals to reflect viability and fitness of males (235).

Another common effect of aging documented in rats is lowering of testosterone levels due to possible defects in leydig cells and steroidogenesis pathway (243-247). Thus, I was interested to see how androgen dependent sexual signals like MUPs vary with age. It is known that at puberty, as levels of testosterone increase and a juvenile male rodent matures into an adult, MUPs start to be synthesized in liver and secreted in urine (248-250). This transition phase of males is critical as it is now a potential mate, which is signaled to conspecifics by presence of huge quantities of MUPs in adult male urine. So the question is what happens after that- do males continue to maintain MUPs levels (sexual display) like houbara bustards or will it decline? For this, I tested MUPs levels of 10 Sprague-Dawley rats at four time points- 6 (T1), 9 (T2), 12 (T3) and 18 (T4) months. The average lifespan of a brown rat is 2-3 years so the time span I have used covers up to half its lifespan. These rats were part of an experiment conducted by a collaborator so my analysis was restricted only to urine collection and MUPs

measurement. A One-way ANOVA for the different time points showed a significant within subject effect ($F(1, 8) = 9.118, p = 0.017$). The data shows highest average of MUPs levels is at 6 months (T1) and it decreases by 51% at 18 months (T4) (Figure 26, $n=9^7$; 6 months = 115 ± 30 (CI); 18 months = 56 ± 24 (CI); paired t-test: $p < 0.01$; Effect size; Cohen's $D = 0.983$). In 8 of the 9 animals, MUPs levels were lower at 18 months compared to 6 months. In addition, a ratio was calculated by dividing MUPs levels of individuals at 18 months by 6 months which gave a mean ratio of 0.38 ± 0.1 ($n=8$ (1 outlier removed); one sample t-test: $p < 0.001$) further indicating that MUPs levels were significantly lower at 18 months. There was a decrease in MUPs levels between 6 and 9 months though not significant. Thus, it is clear that MUPs levels decrease with respect to aging and could act as an indicator of aging. Reports in mice have shown that aging affects odor (differential volatile production) and can be used to discriminate (251). In addition, it was reported that with age MUP expression increased instead and also attractiveness to females (252). But another report demonstrated that aging in mice is correlated with reduced MUPs expression and that their scent could be less attractive to females (253). Given the dose dependence of MUPs, female preference could be influenced by this decline in MUPs levels. However, as I was not able to measure other reproductive parameters for these animals, it is hard to conclude whether MUPs are honest indicators of fitness as the individuals aged. As seen in the example of bustard birds, faster reproductive ageing of “showy” males caused an “uncoupling of expected relationships between elaborate male display and quality of sperm” (235). Thus, the relation between male sexual signals and fitness might not always be tight over time or as individual ages. Studies on honesty of sexual signals to reflect viability or fitness should take into account that aging could be a possible confounding factor.

⁷ We were not able to collect urine from one animal at time point 18 months

Figure 26. Expression of MUPs levels decreased over four time points- 6 (T1), 9 (T2), 12 (T3) and 18 (T4) months (n = 10 except n = 9 at 18 months (T4)). The grey dot and whiskers are the mean $\pm$ confidence interval. MUPs levels at 6 months= 115 ± 30 ; 9 months= 87 ± 27 ; 12 months= 87 ± 19 ; 18 months= 56 ± 24 ; paired t-test: $p < 0.01$.

Effect of health on rodent olfactory cues & signaling

The second factor studied was the role of health in signaling. For many social animals, poor health status from parasites and infections affects their social behavior (254-257). This is because social behavior promotes direct contact between conspecifics (258-260). Thus, it could serve as a direct transmission route of parasites or infections between conspecifics (29, 254, 256, 261). A function of female mate choice, in addition to choosing a fit partner, could be to detect and avoid parasitized males in preference to healthy mates (112). For such a strategy, it is necessary to detect infected animals from a distance to prevent direct contact and transmission. To this end, many females show aversion and avoidance to odors of parasitized animals (see Table 3 for examples in rodents). These studies suggest that infections could alter chemosensory signals (odors) like urine to reflect one's health condition and quality to conspecifics (257, 262). This goes back to the Hamilton and Zuk hypothesis that states that sexual signals (in case of rodents, urinary odors) are indicators for parasite infections and resistance. However, certain reports indicate that avoidance to odor of infected males is not always correlated with females' final mate choice in terms of sexually receptive behavior (169, 263-265). Though female rodents could determine and avoid odor of infected males, they displayed receptive behavior equally to both (266-268). Moreover, when parasitized females are used, they show decreased aversive behavior towards infected male odors (269). In light of the role of health-associated odors in mate choice and as females usually exercise mate choice, male preference for female odor signals are understudied. Male mice show a similar avoidance of *Trichnella spiralis* infected females (270, 271) and males (271). Thus, information from health-related odors provides information on conspecifics.

The identity of rodent olfactory cues that are involved in the avoidance behavior towards infected males remains unidentified. It is thought that the changes in the odor are a result of immunopathological effects of infection- the production of cytokines and other pro-

inflammatory responses to prime the body's defense system (272-274). Alternatively, the changes could be an adaptive response mechanism to manage the infection (56) as seen in parasites that manipulate endocrine system, behavior and possibly odor of the hosts (73, 270, 275). Given that a wide range of infections affect odors, there may be a shared mechanism behind infection related odor changes. Cytokine signaling controls the expression of sickness behaviors which is defined as behavioral changes needed for recovery and survival (276-278). The behavioral changes encompass a lowering in social and sexual motivation (279, 280), decreased exploration (281), hypothermia (282, 283) and loss of appetite (284-286) which could viewed as a means to reduce social interactions and spread of infections. Thus, the host immune response would cause a cascade of molecular changes to prepare the defenses that affects behavior of sick individuals and could cause release of illness related odors (287). As a wide range of infections leads to reduced attractiveness of the infected male rodents, and given MUPs significant role in chemical communication I was interested how infection or sickness affected MUPs signaling. For this, we investigated if treatment with lipopolysaccharides (LPS- a bacterial cell wall antigen and potent activator of inflammation (255, 277, 286)), reduced expression of MUPs in male rats and if this resulted in an associated decrease in the attractiveness of the LPS-treated males.

As rats utilize odorant communication to signal health condition (288), this system is open to manipulation by condition dependent signaling. Similarly, females of several lizard species show preference to establish or spend more time in areas scent marked by males with compounds signaling a better health (289). On the same note, female rats prefer area vacated and urine marked by high testosterone males (290). This raises possibility of condition dependence of urinary pheromones like MUPs. Condition dependence is possible as signaling by pheromones could be accurate reflection of physiological and endocrine processes controlling production of the very same pheromones. As testosterone levels control MUPs

expression (139, 176, 291), factors like infection or sickness that lower testosterone could reflect in MUPs signaling. I have used LPS in this study to stimulate infection like status. LPS is known to activate the innate immune system (including cytokines signaling (292)) along with behavioral and physiological changes that characterize sickness behavior (see Table 6, p. 128). Both male and female rats demonstrate avoidance behavior to airborne odor of LPS-treated animals (293). Moreover, when LPS was infused into the third ventricle of the brain, the avoidance behavior was maintained which ruled out the possibility of LPS excretion (when peripheral administration is used) into urine mediating the avoidance response (294). LPS and testosterone appear to have opposing influences on each other. Systemic LPS has shown to reduce circulating testosterone levels (294). This could be due to a decrease in expression of enzymes in steroidogenesis and spermatogenesis pathways (295, 296) possibly by increasing oxidative stress and pro-inflammatory mediators (297, 298). In return, exogenous testosterone administration alleviates LPS induced inflammatory responses, lowering cytokine production (177, 299-303) and more importantly, it decreases avoidance behavior of healthy conspecifics towards bedding of LPS-treated animals (294). Thus, it appears that a trade-off occurs in adult males between testosterone levels (for sexual attraction and competition) against immunity (inflammatory responses, suppression of testosterone). Therefore, condition-altering factors like LPS treatment can divert resources (physiological substrates i.e. testosterone) and tilt the balance in the trade-off. Fitness of the male would determine if the individual could maintain expression of 'costly' MUPs.

LPS negatively affects MUPs expression and mate choice

In this setup, animals were treated with 3 doses of LPS (0.5mg/kg, *i.p.*) or saline over 3 weeks ($n = 8$ in each group). LPS-treatment significantly reduced body-weight gain of animals (body weight gain at the end of experiment compared to day zero body weight; $p = 0.003$). The urine of LPS-treated animals had significantly lower levels of MUPs (Figure 27,

placebo: 155 ± 30 (CI), LPS-treated: 54 ± 31 (CI); $z = -2.89$, exact Mann-Whitney test: $p = 0.002$; Effect size: Cohen's $D = 2.35$). The 25th percentile of placebo group MUPs levels is greater than 75th percentile of LPS-treated animals. MUPs levels in creatinine-adjusted urine samples were determined using Western blots. Both groups exhibited comparable filtration rates of urine from the kidney ($p > 0.25$).

As described previously, LPS negatively affects testosterone levels which was reflected in this experiment. Testosterone levels were measured from serum of LPS-treated and placebo group. The latter had approximately 3.5 times more testosterone (Figure 28, placebo: 0.77 ± 0.30 ng/mL (CI), LPS-treated: 0.23 ± 0.10 ng/mL (CI); $z = -2.52$, exact Mann-Whitney Test: $p = 0.01$; Effect size: Cohen's $D = 1.20$). This decrease in testosterone is concomitant with the reduction in urinary MUPs levels.

Next, we tested whether females ($n = 12$) showed preference towards urine of placebo-treated males in comparison to LPS-treated males, as would be expected from higher MUPs levels in the former. The setup was the same as used in previous female preference assays (50 μ L stimuli of urine displaced of volatiles, pooled from 8 control and 8 LPS-treated males). The females spent less time in bisect containing urine from LPS-treated males. The mean time spent in each bisect was $545s \pm 48s$ (placebo-treated) and $320s \pm 46s$ (LPS-treated) (Figure 29; $z = -2.04$, Wilcoxon Signed Rank test: $p = 0.042$; Effect size: Cohen's $D = 1.40$). Out of the 12 females tested, 9 preferred urine from placebo-treated males with an average reference score of 2.48 ± 0.57 (one sample t-test: $p = 0.02$).

Figure 27. Condition-dependant nature of the male urinary MUPs expression. LPS treatment greatly reduced the amount of MUPs in the urine (A). The grey dot and whiskers are the mean $\pm$ confidence interval ($n = 8$ animals for control and 7 animals for LPS-treated animals). The ordinate depicts the amount of MUPs in arbitrary units (exact Mann-Whitney test: $p = 0.002$).

Figure 28. LPS treatment significantly reduces serum testosterone levels. The grey dot and whiskers are the mean $\pm$ confidence interval ($n = 8$ animals for control and 8 animals for LPS-treated animals). The ordinate depicts the amount of serum testosterone in ng/mL (exact Mann-Whitney Test: $p = 0.01$).

Figure 29. Condition-dependant nature of MUP-induced attraction to the females. The urine from LPS-treated males also evoked reduced attraction to the estrus females, compared to urine obtained from control animals ($n = 12$, Wilcoxon Signed Rank test: $p = 0.042$).

Prior literature mainly focuses on avoidance behavior towards airborne or volatile odor of LPS-treated animals (287) so this study shows that non-volatile components (MUPs) can also signal similar information. Thus, LPS treatment significantly reduced expression of MUPs and as expected by dose-dependence nature of MUPs signaling, it led to decrease in the attractiveness of the LPS-treated males. This is in contrast to *T. gondii* infection that increases MUPs expression, which could be due to differences in the course of both infections. *T. gondii* infection moves from an acute phase (up to 3-4 weeks) to a chronic latent phase (>6 weeks) (73) and it is at this point when behavior assays and MUPs measurement were done. Thus, it is possible that testosterone and MUPs expression could be decreased (like LPS) in acute phase and increase during chronic stage when the peripheral infection is cleared. We were interested in the chronic phase as that is when the behavior manipulations were observed. In the chronic phase, *Toxoplasma* encysts in immune-privileged organs like the brain and testes which could allow it to affect steroidogenesis as reported in (28), though exact mechanisms are still to be determined. The LPS effects point to condition dependence of MUPs due to its androgen sensitivity, which was affected by health status. An individual would have to mediate trade-off between testosterone (for signaling) against initiating immune responses, (lowers testosterone), for sickness or infections.

Response of conspecifics to illness (LPS) related odor cues

We have seen that LPS treatment reduces MUPs expression and given MUPs role in chemical communication, we were interested what effect this would have upon healthy neighbors or co-housed animals. That is, how do conspecifics respond (in terms of MUPs expression) to illness related odor cues from LPS-treated animals? Previous reports (293, 304, 305) and the female preference assay done above show that healthy conspecifics can discriminate them. When a healthy and a LPS-treated animal are co-housed with a partition allowing for social investigation without direct physical contact, healthy male rats showed reduced sniffing and

greater burying behavior towards LPS-treated rats (293). On the other hand, in a setup without any barrier allowing for direct social interaction, healthy males did not show any decrease in contact with LPS-treated animals (306). Therefore, airborne odor cues from sick animals appear to initiate avoidance in a setup that does not allow for direct contact. In the same study (306), healthy mice neighbors did not demonstrate any agonistic behavior with treated animals but did initiate behavioral changes towards LPS animals. This included “increased inter-individual distance, decreased physical contacts and changes in the modalities of social exploration (increased proportion of muzzle sniffing and decreased proportion of ano-genital sniffing)”.

Another factor that would affect interaction and expression of sickness behavior between healthy and sick conspecifics is the social dynamics (dominance-subordinate relationship) that exists within the dyad/group (307). In one study, it was observed that social hierarchy mediated sickness behavior. When dominant mice received LPS treatment, it led to a decrease in frequency of social and aggressive behaviors displayed. However, a similar decrease was not seen when submissive mice were given LPS (308). Because of their higher position in social hierarchy, dominant mice could “prioritize recuperative behavior” and reduce aggressive behaviors towards submissive cage mates (they do not pose a serious threat of aggression). However, submissive mice continued to focus on defensive behaviors as they still were in the presence of dominant mice (308). This agrees with another study that showed LPS treatment of dominant mice increases social instability in a dyad (309). Together, these studies provide evidence that interaction between healthy and sick (LPS treated) animals depends on social hierarchy.

Until now, I have talked about MUPs in context of female preference and mate choice but the LPS model allowed me to study the interaction between MUPs signaling and competition. For this part of the study, male Wistar rats (n=24) were body weight matched and divided

into two groups (housed two per cage) - one that would receive LPS/placebo treatment and the other with no treatment (healthy). Twelve males were randomly grouped into either LPS-treatment ($n = 6$) or placebo-treatment ($n = 6$) and its partners are referred to as co-house LPS or co-house placebo. The co-housing period was for 10 days. Each cage was divided by a perforated partition enabling sniffing and investigation but prohibiting direct physical interaction. LPS-treatment (1 mg/kg body weight; *i.p.*) was administered every three days over the 10 days (total 3 doses) to maintain the sickness effect throughout the co-housing period while placebo-treated animals were given saline. As with prior LPS treatment, body weight gain over the co-housing period was significantly lower compared to placebo treated animals ($n = 6$ each, $z = -2.887$, exact Mann-Whitney test: $p = 0.002$). There was no significant difference in body weight gain for both co-housed groups of animals over the 10 day period ($n = 6$ each, $z = -0.480$, exact Mann-Whitney test: $p = 0.669$).

Urine samples of co-housed animals were collected at two intervals; once prior to co-housing and then after co-housing with placebo- or LPS-treated partners. Urine for MUPs quantification was creatinine adjusted as done previously. MUPs levels for each co-housed animal were normalized to its own baseline level (i.e. urine collected prior co-housing). Co-housing led to some interesting effects. Those co-housed with LPS-treated animals ($n = 5$, 1 outlier) showed greater expression of MUPs compared to those co-housed with placebo-treated animals ($n = 6$) (Figure 30, co-house LPS: 142 ± 79 (CI); co-house placebo: 41 ± 25 (CI); $z = -2.19$, exact Mann-Whitney test: $p = 0.03$; effect size: Cohen's $D = 1.63$). Once again, the 25th percentile of MUPs levels of co-house with LPS-treated animals surpassed the 75th percentile of co-house with placebo-treated animals. Therefore, healthy conspecifics responded to lower levels of MUPs in LPS-treated animals by increasing their levels of MUPs.

Given that LPS treatment initiates sickness behaviors, it is possible that these animals divert resources from competition (in terms of MUPs production) with their neighbor, which in turn causes co-housed animals to increase their MUPs levels. As such, by increasing levels of MUPs it could act as a proxy for display of dominance to males (intra-sex signaling) and females (inter-sex signaling). Given the lack of polymorphism of MUPs expression among male rats (135, 164, 165) and female dose dependent preference of MUPs, it follows that male competition could also depend on MUPs levels.

Figure 30. Co-housing of healthy conspecifics with placebo treated ($n = 6$) and LPS treated ($n = 5$) animals caused a change in MUPs levels. Those co-housed with LPS treated increased MUPs significantly. The grey dot and whiskers are the mean $\pm$ confidence interval ($n = 8$ animals for control and 7 animals for LPS-treated animals). The ordinate depicts the amount of MUPs in arbitrary units (exact Mann-Whitney test: $p = 0.03$).

Figure 31. Characterization of dominance behavior with approach-avoidance to cat odor behavioral assay. Conspecifics co-housed with LPs treated animals (n = 6) showed (A) greater time spent near cat odor (exact Mann-Whitney test: $p = 0.017$) (B) and higher number of approaches (exact Mann-Whitney test: $p = 0.052$) towards cat odor than conspecifics co-housed with placebo group (n = 5). The grey dot and whiskers are the mean $\pm$ confidence interval

To characterize the possible dominance behavior of co-housed animals, we tested the behavioral approach-avoidance responses of these animals toward predator (Bobcat urine- 2 mL) stimulus. Rats were habituated in arena measuring 76 length $\times$ 9 width $\times$ 15 height in cm for 10 min consecutively for 9 days prior to actual testing (20 min trial). 15% of space immediately bordering the odor stimulus was defined as the interaction zone. Approach-avoidance responses were determined by amount of time spent sniffing the cat odor and number of approaches within the interaction zone. Data was analyzed using ANY-maze (Stoelting, USA). Animals co-housed with LPS-treated group (n = 6) spent greater amount of time sniffing the cat odor in comparison to those co-housed with placebo-treated group (n = 5;

1 outlier) (Figure 31A, co-house LPS: $105s \pm 46s$ (CI); co-house placebo: $17s \pm 12s$ (CI); $z = -2.37$, exact Mann-Whitney test: $p = 0.017$; Effect size: Cohen's $D = 2.07$). Concomitantly, the former group ($n = 6$) had more counts of 'head-in' approaches toward the interaction zone while animals co-housed with placebo-treated group ($n = 5$; 1 outlier) stayed away from the interaction zone (Figure 31B, co-house LPS: 6.8 ± 2.8 (CI); co-house placebo: 2.6 ± 1.2 (CI); $z = -2.04$, exact Mann-Whitney test: $p = 0.052$; Effect size: Cohen's $D = 1.56$). In both parameters, the 25th percentile of animals co-housed with LPS-treated group surpassed the 75th percentile of co-housed with placebo-treated group. A representative manual tracking plot of one animal from each group (Figure 32, L: co-housed with placebo-treated, R: co-housed with LPS-treated) helps to visualize the behavior in both parameters.

Figure 32. A representative manual tracking plot towards predator stimulus of one of animal from each group (L: co-housed with placebo-treated, R: co-housed with LPS-treated)

In addition, a positive correlation is observed between the amount of time spent near the predator stimulus and their MUPs expression levels (Figure 33; Spearman's $\rho = 0.709$; $p = 0.022$; $n = 10$; 2 outliers). Together this data supports previous reports that dominant rats exhibit greater exploratory behavior (as reported in (310)) and is possibly correlated with production of MUPs.

Figure 33. A positive correlation is observed between the amount of time spent near the predator stimulus and MUPs expression levels of each individual (Spearman's $\rho = 0.709$; $p = 0.022$; $n = 10$; 2 outliers)

The honesty of sexual signals is due to fact they are expensive or costly to produce and maintain, therefore ensuring that only fit males can afford to engage in sexual display (95, 115, 172, 231). Here we saw that LPS, an immunological proxy for bacterial infection decreases production of MUPs. This is in line with the idea that MUPs acts as an olfactory trait used by male rats to signal health condition. More interestingly, healthy conspecifics responded to presence of sick (LPS treated) neighbors by increasing expression of MUPs. This possibly implies that competition or dominance in male rats could also be a dose dependent effect. Taken together, the condition dependence of MUPs on androgen could force animals to trade-off between testosterone-mediated behaviors against immune responses. Therefore, MUPs possible role in inter- and intra-sexual signaling lends to credence to the idea that it is sexually selected.

Effect of social environment on MUPs expression

Until now, I have studied MUPs role in mate choice and competition/dominance in isolation. In this last part, I have tried to bring these two components together to assess how their interaction affects MUPs signaling. More specifically, the experimental setup is designed to

understand how varying social environment influences signaling. To begin with, I needed a clearer way to recreate a dominance-subordinate relation instead of using LPS due to myriad of possible side effects. To artificially create such a relationship, castration followed by testosterone supplementation was used. Social status consists of competitive behavior that is established via aggressive behavior influenced by testosterone (310, 311). Dominant males also lead attacks on strangers in a colony (312, 313) and display greater exploratory behavior than subordinates (310). Higher social status has been correlated with greater testosterone levels (314). Testosterone is a mediator of aggression, which can be negated by castration (315, 316) and rescued with testosterone implants (317). Moreover, greater levels of testosterone enable rats to better respond to stressful environments. Socially dominant males have been observed to emit “22-kHz calls” (to indicate a stressful situation to the brood (318, 319)) which correlated with their testosterone levels (320). On the other hand, lower social status can cause chronic stress in rats that leads to physiological damage including cardiovascular defects (321).

So the main idea is to co-house healthy, intact rats (henceforth referred to as experimental rats) with either with castrated (C), with castrated+supplemented (C+T) or with sham (S) animals (Table 4; n = 8 each ‘surgery’ group co-housed with 8 experimental rats). This time no barrier was used allowing for direct physical interactions. The purpose is to assess how different co-housing conditions (male competition) affect expression of MUPs in the experimental rats. The castrated rats would replicate a subordinate animal with low or no MUPs while the supplemented rats (with increased MUPs) would create an aggressive or unstable environment in the dyad and the sham animals would serve as a control group. That is, how does the relationship within a dyad affect resource allocation and level of signaling?

Table 4. The different groups and housing conditions used in the social co-housing experiment

Castrated	Each group co-housed with intact males (experimental animals)
Castrated + supplemented	
Sham	

After castration and supplementation, basal levels of MUPs of experimental rats (n= 24) were measured and they were divided into three groups of 8 based on MUPs levels. This is to ensure that there were no underlying differences to begin with which could skew the results. The first co-housing period was for 10 days, after which urine was collected for MUPs quantification. To further study the behavioral effects of co-housing, a test was used to assess for dominance (food competition test). Further, co-housing was continued for another five days but this time, the experimental rats were exposed to a novel estrous female for 5 minutes each day in home cage (the ‘surgery’ rats were removed from cage during this interaction). The presence of receptive female provides a reproductive opportunity so we were interested how this change in social environment will influence MUPs signaling (Figure 34). The presence of reproductive capable females is known to increase intermale aggression (322) which could be due to increased testosterone levels when males encounter novel females (323, 324). Moreover, as the experimental rats are still co-housed with treatment rats we can see the interplay between co-housing and female exposure. Thus, we hypothesized that by altering social environment and status of male rats, we could influence how much resources they would invest in sexual signaling.

Figure 34. Experimental flow and time points for measuring MUPs levels.

First, we measured basal MUPs levels of the experimental rats to divide them into 3 groups to be housed with either C, C+ T or S animals. They were sorted so the mean $\pm$ SEM of each group were not significantly different as seen below (Table 5, One way ANOVA: $F(2,23)=0.061$, $p = 0.941$). Henceforth, the 3 experimental groups will be named as C group, C+T group and S group ($n = 8$ each group).

Table 5. Basal MUPs levels of experimental rats co-housed with 3 treatment groups sham (S), castrated (C), or with castrated+supplemented (C+T) animals ($n = 8$ each group).

Group	S	C	C+T
Mean $\pm$ SEM	89 $\pm$ 22	84 $\pm$ 22	79 $\pm$ 18

After the initial 10 days of male co-housing, MUPs level were measured once again to see effect of social stimulus (competitor or subordinate). The C+T group had highest mean net change (Figure 35, MUPs level post male co-housing divided by basal MUPs level) of 2.58 ± 0.85 ($n=8$; one sample t-test: $p= 0.05$), followed by S group ($n=8$; 1.69 ± 0.70 ; one sample t-test: $p= 0.1$) and C group ($n=7$, 1 outlier; 1.43 ± 0.32 ; one sample t-test: $p= 0.2$).

At this time point, I also measured testosterone levels of experimental rats to see the effect of male competition (Figure 36). A One way ANOVA showed a significant effect of co-housing- ($F(2,21) = 9.02, p = 0.001$)- on testosterone levels. Concomitantly to the MUPs levels, the C+T group had highest mean testosterone levels ($n=8; 1.27 \text{ ng/mL} \pm 0.52 \text{ (CI)}$) followed by S group ($n=8; 0.42 \text{ ng/mL} \pm 0.31 \text{ (CI)}$) and C group ($n=8; 0.20 \text{ ng/mL} \pm 0.07 \text{ (CI)}$). The food competition test was also conducted to analyze their aggression or competitive behavior. For this assay, the rats were starved for 6 hours prior to start. Then, 7 standard food pellets were placed in the cage and their interaction was video recorded to analyze how many pellets each rat took. Across the 3 groups, there was no difference in the mean number of pellets by the experimental rats compared to their cage mates (One way ANOVA: $F(2,22) = 0.329, p = 0.723$).

Figure 35. MUPs level net change after co-housing with male (white bar) and female exposure (grey bar). Bars depict mean $\pm$ SEM. Healthy intact rats ($n=8$ each group) were co-housed with either sham (S) castrated (C), or with castrated+supplemented (C+T) animals.

Figure 36. Serum testosterone levels post 10 days of male co-housing. The 3 groups were co-housing with sham (S; filled circles), castrated (C; circles with line), or with castrated+supplemented (C+T; hollow circles) animals ($n = 8$ each group). The grey dot and whiskers are the mean $\pm$ confidence interval. The ordinate depicts the amount of serum testosterone in ng/mL. C+T group had highest mean testosterone levels ($1.27 \text{ ng/mL} \pm 0.52$) followed by S group ($0.42 \text{ ng/mL} \pm 0.31$) and C group ($0.20 \text{ ng/mL} \pm 0.07$). A One way ANOVA showed a significant effect of co-housing- $F(2,21) = 9.02, p = 0.001$ - on testosterone levels.

Co-housing was continued but this time the experimental rats were exposed to estrous female for 5 minutes on five consecutive days. MUPs levels were measured once again, and surprisingly, the C+T group had a decrease in net change of MUPs levels (Figure 33). On the other hand, the other two groups saw an increase in net change of MUPs levels ($n=7$, 1 outlier each; C+T group: 0.51 ± 0.12 , one sample t-test: $p = 0.01$; S group: 2.81 ± 0.80 , one sample t-test: $p = 0.09$; C group: 2.77 ± 0.48 , one sample t-test: $p = 0.01$). This net change was calculated by dividing MUPs level post female exposure by MUPs post male co-housing.

However, this time I was not able to measure testosterone levels after female exposure as the rats underwent an approach-avoidance assay toward predator (Bobcat urine- 2 mL) stimulus

similar to conspecifics housed with LPS-treated animals described previously. Exposure to cat odor would interfere with testosterone levels and skew measurement. This assay could possibly characterize dominance behavior of co-housed animals. A One way ANOVA ($F(2,21) = 4.231$ $p = 0.03$) showed significant difference among time spent near cat stimulus. The C and S group spent similar amount of time while the C+T group spent significantly less (C: $n=8$, $40.2\% \pm 3.6$; S: $n=7$, $38.5\% \pm 3.9$; C+T: $n=7$, $23.6\% \pm 5.5$). As was the case with conspecifics housed with LPS-treated animals, the groups (C & S) that had higher MUPs levels (post female exposure) also spent more time near cat odor. This “emboldening” effect has been previously reported in male mice where brief exposure to female odors reduces predator mediated behavioral and hormonal effects (325).

To summarize, MUPs levels post male co-housing increased across the 3 experimental groups with C+T group having the greatest net change followed by S and C groups. This was reflected in their testosterone levels. However, after female exposure, MUPs levels fell significantly for the C+T group while the other 2 groups increased significantly. Therefore, the presence of supplemented animals whose role was to create more aggressive cage mates and produce more MUPs caused the experimental rats to also upregulate their testosterone and MUPs levels to possibly compete. While in the other 2 groups, the presence of sham and castrated animals appear to have been not as a strong environmental or social factor to cause the experimental rats to increase their MUPs levels by as much. Thus, it appears that the upregulation occurs when challenged by an aggressive or dominant competitor and in absence of such stimuli, the increase is to a lesser degree. Moreover, the benefits of increasing signaling to compete with aggressive males, that is, ‘keep up’ or ‘fight back’ would appear to outweigh the costs.

However, the exposure to female rats led to interesting changes. It was expected that the presence of reproductive opportunity would further increase MUPs levels. This is because

presence of novel females increases testosterone levels and aggressive behavior (322-324). Therefore, an increased in MUPs would be expected due to its androgen dependency and dose dependence preference shown by females. Accordingly, the C & S group showed a marked increase in MUPs but the complete opposite was seen in the C+T group. A possible explanation is that since the experimental rats are still co-housed with the surgery rats, the effect on MUPs is due to interplay between varying male competition and female stimulus. For the C+T, the reproductive opportunity presented must be balanced with the cost of possible increased aggression from the supplemented cage mate. This is because female exposure occurred in the home cage (the 'surgery' rats were removed during the 5 min trial) but her odor either from shed fur or urine would remain in the cage. Therefore, though the surgery rats were not exposed to actual female rats, they would detect other stimuli. In addition, given that odor based communication is vital (female prefer area vacated by high testosterone males (290, 326)), such cues can also signal female presence and possibility of reproductive opportunity. Thus, male competition or aggressive behavior in the C+T group might have increased further such that the cost of increasing MUPs levels was greater than the possible benefits of reproductive opportunity. This is similar to subordinate rats reducing urine marking or volatile synthesis in presence of dominant individuals to reflect their social status (327-330). However, in the C and S groups without the presence of as a strong competitor or aggressor (compared to C+T), these animals could afford to increase signaling levels. This implies that MUPs are dynamically regulated and change based on environment or social situations. This fits into the idea of an individual increasing signaling only in conditions or contexts where the benefits outweigh the costs. In this way, the dynamism of signaling can accurately reflect condition or status of the individual and ability to manage the cost-benefit associated.

7. Discussion

In most cases, the link between a genotype and expression of a phenotype is limited to individuals. In contrast, there are unique examples such as the selection of wasp genes that lead to irregular or non-orb weaving by spiders (331). Clearly, the genotype of the parasite (wasp) extends its reach and is expressed in the behavior or phenotype of the spider, that is, in another individual. This phenomenon has been termed as extended phenotype (37) and parasitic manipulation of host behavior and its underlying physiological mechanisms fits in this paradigm. The *Toxoplasma gondii* and rat association is a well-documented example of behavioral manipulation in which infected rats lose fear towards predator (cat) odor (72, 73, 93). Taking this a step further, in this thesis I have shown that the extended phenotype manifests in not only the host but also affects behavior of a conspecific. This is characterized by the manipulation of uninfected female mate choice towards *Toxoplasma* infected males.

The dual manipulation (loss of fear and increase female preference for infected males) is an interesting case as it raises the question if they are parasite induced effects, are there two different mechanisms at play or can both be explained by a single physiological process? Occam's razor would suggest that an explanation for both manipulations that requires only one parasite induced mechanism, would be more parsimonious. The loss of fear has been more widely studied with various hypotheses put forward. In one perspective, tropism of *Toxoplasma* cysts to specific brain areas like amygdala or nucleus accumbens could play a part (73, 125); however, at best there appears to be only a weak and inconsistent association. Alternatively, inhibition of NMDA channels by an increase in kynurenic acid from interaction of *Toxoplasma* and astrocytes (332) has been thought to be involved. Another possibility comes from the observation of two parasitic genes with high homology to mammalian tyrosine hydroxylase (333, 334). The enzyme is involved in the rate-limiting step

in dopamine production (335), a neurotransmitter responsible in signaling of reward and motivation. To this end, *Toxoplasma* increases release of dopamine from mouse brain slices (334) and drugs that inhibit dopaminergic transmission, rescue infection induced behavioral changes (336). The theories suggested so far are brain centric and treat changes occurring in the brain in isolation from rest of the body. At the very least, they cannot account for the mate choice manipulation directly.

However, there is an extensive two-way communication between the brain and periphery with the HPA (hypothalamic–pituitary–adrenal) and HPG (hypothalamic–pituitary–gonadal) axis. This communication mainly occurs via hormones, for example in the HPA axis, the hypothalamus secretes corticotropin-releasing hormone that acts on the pituitary gland to release adrenocorticotrophic hormone (337). This in turn acts on the adrenal cortex to produce cortisol/corticosterone that also can loop back to affect the hypothalamus and pituitary in a classical negative feedback cycle (338). Similarly, in the HPG axis, luteinizing hormone and testosterone are the key mediators (339). There are regions of the brain that are rich in androgen receptors and testosterone is known to influence behavior as it affects brain structure and functions (340). Thus, it is possible for *Toxoplasma* infection to hijack or ride on such pathways to effect changes in the brain and behavior from the periphery of the host. In chapter 4, I described such a model that our group has put forward in which *Toxoplasma* invades testes, increasing testosterone synthesis (28) that crosses the blood brain barrier to upregulate AVP (via epigenetic modification at its promoter) in the medial amygdala (MEA) (129, 130). The MEA is thought to play a role in maintaining the balance between defensive and sexual behavior (341, 342)s- and increased AVP tilts behavior to the latter. This in turn could cause a shift from a defensive to a more approach behavior even in response to cat odors. In light of these observations on the role of testosterone in the fatal attraction hypothesis, I was interested how else *Toxoplasma* affects behavior of infected rats. As

testosterone plays an important function in reproductive/sexual investment and behavior, this could explain the mate choice manipulation (96) and increased sexual advertisement observed (343).

In this thesis, I demonstrated that *Toxoplasma* creates a shift in female preference or mate choice towards the infected males, which could be due to increased expression of a possible sexual signal- MUPs. The manipulation could serve to further increase parasite transmission through sexual and congenital modes, thereby increasing potential population susceptible to trophic transmission. It would be interesting to study if progeny that show presence of cysts in brain from vertical transmission (as seen in this thesis), maintain infection status into adulthood and exhibit the loss of fear phenotype.

This manipulation could be a product of the arms race between the host and parasite, with both players attempting to outmaneuver the other resulting in continuous co-evolution. This kind of a relationship has been termed the Red Queen hypothesis (95). Thus, parasites that provide its hosts with either sexual or reproductive advantage are favored which falls under the “compensatory responses” hypothesis (21, 41). The possible trade-off between increased attraction of infected males against the cost of loss of fear acts to negate the otherwise strong selection pressure from hosts to build immunity from the parasitism. To better characterize the manipulation, I showed that *Toxoplasma* infected rats had a greater expression of MUPs at both the liver transcript and urine protein levels (343); however no change was seen to expression pattern of MUPs isoforms. Additionally, I demonstrated that MUPs fraction of urine from infected males was sufficient to establish the mate choice manipulation. As a whole, this points towards female attraction being influenced by MUPs probably in a dose dependent manner rather than qualitative nature of certain MUPs. There have been previous reports of parasitic infections affecting MUPs production- *Schistosoma mansoni* infection led to decrease in MUPs mRNA levels in mice (344) and the activation of immune responses

with foreign antigens (sheep red blood cells) also reduced male MUPs production and attractiveness of males scent (299). However, there is a report that the use of LPS actually increased MUPs expression and increased scent attractiveness of male urine though this was seen in non-estrous females (345). Moreover, it was previously suggested in (262) that MUPs regulation could explain how infection could decrease attractiveness of male urine to females. Next, I will be discussing MUPs role in chemical signaling and the kind of information it can convey as a possible sexual signal.

Major Urinary Proteins (MUPs)

In rodents, social information is transmitted by odors, often via urinary cues (133, 288, 346, 347). A wide range of information on an individual can be communicated, from sex, social status, health and age (288), all necessary to assess potential suitability of a mate. Odors of male rats have been shown to be sufficient to elicit sexual attraction. For example, an area occupied by a high-testosterone male retains its attractiveness (290) and soiled bedding from males is sufficient to induce female mate choice (96). Clearly, female preference for males does not need physical presence of males, thus precluding the role of non-olfactory modes, such as ultrasonic vocalizations. In this light, a putative candidate for a rat sexual signal is **Major Urinary Proteins (MUPs)**. MUPs is secreted in copious amounts in the urine, a common communication means in rodents (123, 132, 133, 154, 158) and hence a good way to advertise ones suitability as a mate. It is a member of the lipocalin protein family that transports small hydrophobic volatile molecules (pheromones), slowing their degradation and facilitating delivery to recipient olfactory system (133, 154). Previous work done in mice has also shown MUPs to act as a pheromone by themselves, in absence of bound volatile ligands (122, 123, 133, 153). Given that mice and rat MUPs evolved independently and in parallel since diverging from their last common ancestor (145), it is highly likely that MUPs in rats also function as a sexual signal.

MUPs in chemical signaling

Currently, the most well understood function of MUPs is its role in chemical signaling, though, there have been reports of a physiological role for MUPs in regulation of glucose and lipid metabolism in mice (348, 349). MUPs are synthesized mainly in the liver, filtered in kidneys and excreted into the urine (176, 350). MUPs possess a conserved β -barrel structure with a hydrophobic pocket in the center. These pockets allow MUPs to act as carriers for volatile molecules, slowly releasing them and increasing longevity of volatile signals (133, 154). In addition to its role as a carrier, MUPs can act as pheromones triggering adaptive behavioral responses. MUPs are capable of inducing estrous (351, 352) and urine from an unfamiliar male can cause a female to terminate her pregnancy (Bruce effect (353, 354)). For females, it allows them to conserve reproductive resources in search for a 'fitter' male. For males, it is a way to improve their fitness to achieve the most offspring. MUPs also mediate sexual attraction in female mice and a specific MUP (*Darcin*) (153), was isolated that maintained the effect. In addition, MUPs were implicated in stimulating aggressive behavior (122), indicating its salient role in sexual selection related behaviors.

What kind of information do MUPs transmit?

Sexual signals provide information about an individual that conspecifics utilize to assess the suitability of the signaler as a potential mate.

Sex of an individual

In rats, as females are the limiting gender for they carry offspring till birth and are more involved in parental care, it is usually the males who extravagantly display the signal. Thus, this becomes a quick and efficient way to distinguish between the sexes. Sex of a rat is displayed as adult male urine contains much greater levels (in orders of magnitude) of MUPs than females. MUPs are hormonally regulated, mainly by testosterone and as males tend to have much higher levels of testosterone, MUPs expression is accordingly greater (139, 140,

175, 176, 350, 355, 356). This is collaborated by the fact that upon castration, MUPs expression is reduced dramatically to levels similar to females (143, 174). Thus, MUPs plays an important role in differentiating the sexes.

Age of an individual

MUPs expression pattern varies over time from birth to adulthood in rats. These temporal variations are a result of its regulation by hormones that similarly vary across ages. As such, each growth phase has its own distinct tissue and sex specific MUP expression pattern. This helps to distinguish between juveniles and adults, and critically it relays reproductive status of the individual to others. MUPs are secreted from other tissues like salivary glands and lachrymal glands (136, 137). Salivary MUPs are not hormonally regulated, with comparable levels between males and females. They are highest before weaning, decreasing until expression is undetectable in adults. Lachrymal MUPs are under a degree of hormonal control but are secreted before puberty (136). At puberty, as testosterone levels start to increase, a juvenile male matures into an adult and begins to synthesize MUPs in the liver that is secreted in the urine (248, 249). This transition of a male is important as it now has the ability to reproduce and is a potential mate. Castrating an adult male causes a sharp decrease in MUPs in the liver and urine, mirroring expression profile of a juvenile male (175, 176). Females do not show any attraction to urine or bedding from castrated males (357) compared to intact males, in essence considering them as juvenile males. Supplementation with exogenous testosterone rescues MUPs secretion in urine and attraction. MUPs are also sufficient to induce aggression between males. In a resident-intruder assay, the dominant male mice would not attack a castrated male, also considering them as juvenile males. Although when the latter is streaked (or its urine supplemented) with MUPs it instigates an aggressive response (122). Thus, MUPs aid in signaling reproductive availability of a potential mate to females and has a possible role in dominance contests between males.

Ownership Identity

A sexual signal must be able to transmit ownership identity. This is because a conspecific has to associate the information displayed with a specific individual. In examples such as deer antlers, peacock plumage and nightingale song; the sexual signal can be associated with the individual directly- visually or acoustically. Urine marks are unique, as the signaler does not have to be physically present. Hence, identity must be coded intrinsically in the sexual signal.

Certain features of MUPs (work done in mice) allow for this:

First, genetically coded MUPs show constancy throughout adulthood (137). The volatile signature may change depending on status (327), health and diet (288) but the MUPs signature remains constant.

Second, MUPs signals remain stable in the environment for weeks resistant to degradation, preserving information on identity (133, 153). This allows the owner to advertise to conspecifics even in their absence.

Third, MUPs are polymorphic in nature that allow for unique signature for individuals (157-160). MUPs variation could arise from differences on its surface and differential ligand binding pockets (358-360). The diversity of the MUPs signal is due to its combinatorial or polymorphic expression pattern. The expression resembles a bar code of sorts where a MUP type is defined as the overall pattern of the various MUPs (157, 158, 160, 162). This increases the number of possible combinations greatly. The genetically coded MUP type is used to assess genetic heterozygosity of potential mates by females (158). The idea is females choose a mate that provides greater genetic variation to produce fitter offspring (361). However, there have been reports of limited variation in MUPs expression pattern in lab and wild mice strains possibly due to inbreeding (161-163).

The combinatorial expression pattern is a result of evolutionary distinct events. The MUP gene cluster is thought to have developed from a single ancestral MUP gene from which it expanded independently and in parallel. This occurred after divergence of rat and mice lineages (post speciation) as seen by the different structure and organization of MUP genes (145). The MUP gene then expanded by paralogous gene duplication generating polymorphic variations. As a result, rats and mice have 9 and 22 MUP genes respectively (145). Such variety allows for diversity of behaviors to be mediated by MUPs alone (362). Similarly, the dynamic regulation of MUPs expression in various tissues and its temporal variations described before can possibly be explained by the variety in MUPs isoforms. Clearly, MUPs have evolved specifically in context of rodent behaviors with features to enable an efficient mode of intra-species communication as would be expected from a sexual signal.

Necessity & sufficiency of MUPs

To implicate MUPs as a putative sexual signal in rats, I have demonstrated the necessity and sufficiency of MUPs to elicit female attraction or preference. The displacement of volatiles and fractionation of urine followed by DART-MS, allowed me to demonstrate that urinary MUPs fraction serves as an attractant on its own. Moreover, with the use of FPLC purified MUPs and recombinant MUPs, I was able to shown that MUPs still maintained its olfactory based attraction, corroborating its role as a probable sexual signal. The fact that *Toxoplasma* increased MUPs levels in infected males and not the expression pattern it points towards dose responsive signaling. Similarly, data from chapter 4, also points to a dose dependent function of MUPs in mate choice as seen by the female preference for higher concentration of FPLC purified MUPs. More interestingly, I showed a positive correlation of MUPs levels of males with attractiveness ratio and time spent by females in a two choice preference assay. Such dose dependence was also observed in chapter 6, where LPS decreased MUPs expression and subsequently female preference. In other words, MUPs could act as a physiological feature to

distinguish attractiveness (based on secretion levels in urine) of male rats and could be predictive of female preference.

MUPs: qualitative or quantitative signaling?

As described previously, MUPs are genetically coded and highly polymorphic allowing an individual male to express a unique identity or barcode (363). A model has been proposed to describe the role of MUPs in dominance contests between male mice. In mice, urine marking by an owner in its territory stimulates investigation by females and competitive counter-marking by competitor males (132, 363-365). However, subordinate males would not engage as they could incur opportunity costs through retribution by more dominant males. The ability of the owner to continuously secrete fresh scent marks in response to counter-marks from other males serves as a display of its dominance or status (132). This system requires a unique ownership signal so an individual can be associated with urine marks and this is provided by MUPs. In addition to establishing dominance status, this elegant system (described in (132)) allows for reproductive advantage by attracting females. The temporal and spatial nature of scent- and counter-marking let females create a picture of a male's scent marking and ability to resist counter marking. With this information, the female can decipher status. Thus, MUPs are involved in determining dominance status, which females 'eavesdrop' on, affecting mate choice, underlying its role in sexual selection.

However, even though rats have nine MUP genes and express multiple isoforms there is a lack of variation or polymorphism between individual rat MUPs expression pattern when compared to mice (Figure 8 & (135, 164, 165)). Thus, it may have limited applicability for recognition of individuals, thus undermining the possibility of competitive scent marking. It is likely that male rats instead contest other males through amount of MUPs expression and that females prefer males with greater MUPs. This situation is similar to use of plumage badge by house sparrows, where males with larger badges signaled dominance (366-368). In

contradiction to the above described qualitative model, there has been a report on female mice preference for increasing urinary MUPs concentration (369) and a dose dependence relation (specifically Mup20) with dominance status in mice (370). Moreover, *Darcin* (a MUP involved in sexual signaling (153) in mice) expression level was predictive of dominance status with dominant males having higher expression before competition (371). This effect was transient as there was no difference post competition. Contrasting such dose-dependency, female mice are not influenced by dilution of *Darcin* (153). So how would a potential quantitative based MUPs signaling function? That is, what kind of information can be conveyed by a male rat to probable mates when it secretes greater MUPs? Next, I will discuss how variation in sexual signals can provide information on males that other conspecifics use to assess suitability of a potential mate.

Sexual signals

What does the tail of a peacock, the antlers of a deer and pheromones in rat urine have in common? These are sexually dimorphic signals, primarily seen in males that have evolved through sexual selection with the purpose to attract mates (372). In the competition of sexual selection, individuals of a species have to display their superiority or advantage over other members to be able to reproduce (373). As such, sexual selection gives rise to such signals that aim to maximize individual reproductive success. Moreover, sexual selection is a form of natural selection and both are often in conflict due to the demands or costs that each places on the signaler. This is a result of the opposing motivations of natural and sexual selection, with the former limiting the elaboration of sexual signals for the survival of the individual (374, 375). They exert a continuous push and pull dynamics on an individual, and the outcome relies on how well an individual can balance these opposing forces (the immediate (reproduction & sexual selection) vs. the future (survival & natural selection)). An example of such a cost is illustrated by looking at the bird- King of Saxony, which uses its long

plumes to attract females. However, bowerbirds found in the same area hunt for these plumes to build bower structures to attract females too. Moreover, the local tribesmen use these plumes as ornamentation (95). Thus, the longer plumes on King of Saxony might attract females but are also put them at a greater risk for survival. Thus, expression of sexual signals is an outcome of the battle between sexual and natural selection.

Variation of Sexual Signals

An individual's ability to cope with the costs of sexual signals is dependent on many factors, which leads to variation in their expression, for example, brightness of feathers or coloration on fishes. The costs can come in various forms such as increased chances of predation (374) as described for the King of Saxony. On the other hand, another possibility is the loss of predation opportunity as stronger sexual signals could allow preys to more easily detect its predator and avoid detection, leading to a possible loss of food sources (217, 376). Costs can also be internal in nature as expression of sexual signals depends on the physiological status of the animal to be able to invest energy and resources. Testosterone is a common mediator of sexual signals and generally greater levels of testosterone is correlated with greater expression and female preference (290, 326). However, higher levels of testosterone have some drawbacks, inflicting costs in terms of higher metabolism rates and its immunosuppressive nature (177, 178, 377). This is a part of the immunocompetence-handicap hypothesis, which put forward that the immunosuppressive effects of testosterone only allows the best quality males to express sexual signals and at same time prevent diseases.

The interplay between sexual signals, testosterone and immune system is seen with the use of carotenoids, in birds and fishes, for pigmentation of ornaments. Carotenoids are organic pigments found in many plants and photosynthetic organisms that are absorbed by animals from their diet (378). It has been shown in these animals that greater carotenoid-based

ornaments are preferred in mate choice (379, 380). Carotenoids are also thought to have a role in immunity due to its antioxidant potential but this relationship is far from clear (381-383). So it has been suggested that a “trade-off between immunity and sexual signaling is mediated by the interactions of testosterone with carotenoids” (384). Thus, both the immune system and sexual signals compete for available carotenoids and testosterone could determine where the balance lies. Increased levels of testosterone could possibly increase bioavailability of carotenoids for sexual signaling and less for immune function. Following the immunocompetence-handicap hypothesis, males with better fitness would allocate less carotenoids for immune system and instead invest in sexual signals. This is crucial as testosterone itself is known to promote oxidative stress (380), so better quality males have to be able to compensate for detrimental effects of testosterone.

Hence, the benefit of sexual signals in attracting mates is counterbalanced by the external and internal costs it imposes. Different sexual selection theories put forward that sexual signals are honest proxies of male fitness or quality as their expression is costly with regards to resources required (115, 172, 380). This cost causes a disadvantage in survival or viability of the individual. Such a scenario could arise in male rats (express MUPs in much greater amount than females) and the cost could be due to its testosterone dependency. Therefore, only fit males can produce and sustain ‘costly’ sexual displays that quite often need testosterone, which could impose handicap in its metabolism, immunosuppression or life-history conflicts. Accordingly, only some individuals can afford to manage the cost-benefit associated. As fitness levels vary due to factors such as diet, age, social or health status, together it will determine how an individual copes with the costs and demands of a sexual signal (231). This would lead to variation in expression of sexual signals that would enable conspecifics to distinguish individuals and provide information or serve as a proxy for fitness, necessary for choosing a mate.

Sexual Selection Behaviors

This information on fitness or ability to sustain expression of sexual signals is relayed in two main behaviors that drive sexual selection- mate choice (usually a female choosing a male) and dominance (competition or contest between males). Mate choice is observed in wide spectrum of animals from flatworms to mammals, where one sex exerts a choice on its mate preference of the opposite sex (385, 386). Most species are anisogamous in nature due to the asymmetry in distribution of investment by the sexes in reproductive processes and parental care (females carry offspring till birth and are usually more involved in care) (95). As such, this bias makes females the limiting gender (372) or in other words, the more invested sex is choosier. Thus, females tend to be the 'choosy' ones, selecting a male from its competitors (387). How can parental investment affect sexual selection? If the only contribution of the males is during copulation, then males will be under pressure from strong female mate choice to evolve elaborate ornamentation, as it is the only way to display suitability as a mate (387). In many bird species, lekking behavior is observed where males congregate at a certain area during breeding season and display courting/competitive behavior as females peruse the males for copulation (95). In species such as Sage Grouse and Great Snipe the males use vocalization to attract females that is very costly in terms of energy and detection by predators. Great Snipe males are known to lose body weight due to demands of greater vocalization (388). On the other hand, if both sexes invest in parental care, both now have an incentive to be choosier in deciding a mate. For example, in crested auklets, both sexes have visual ornamentation in the form of crests and size of crest is related to greater chances of mating (389-391).

Dominance or competition behavior usually occurs in the sex that has the potential to have more offspring (392). As mentioned above, females tend to incur disproportionately greater cost of parental care, thus, they cannot so easily be ready for the next mating. However, when

males are not involved in parental care they do not need to wait and as such have greater potential opportunities for mating. This biases the operational sex ratio (OSR) towards males as there are more males ready to mate than females at a given time, leading to greater intra-sexual competition among them (393-395). OSR is the ratio of sexually competing males to females who are ready to mate (i.e. sexually active, not gestating) (392, 396). This will drive sexual selection and greater pressure for male ornamentation (397, 398) where there are more sexually competing males than females. In most rodent species, females are the limiting gender leading to strong female mate choice (149, 151) and intra-sexual competition in males resulting in development of sexual signals. There are cases where a female biased operational sex ratio (more females than males ready to mate) is observed like in the Bella moth. Females compete with other females more strongly to get mates, using pheromones (399). This is because males provide spermatophore during mating which causes them to lose up to 11% of their body weight. The spermatophore contains sperms, defensive toxins and nutrients for the female (400). Post copulation, the male needs time to build a new spermatophore for the next mating while the female is ready to find a new mate (399). In this scenario, males are in greater demand, leading to female competition.

Dominance behavior is often expressed directly in contests or fights, like in baboons where they compete directly against each other. However, such competition is not always sustainable due to the cost incurred using energy, time and physical injury (368). Instead dominance is displayed indirectly via sexual signal proxies like plumage color or length of wings. Nevertheless, intra- and inter-sex selection behaviors are often intertwined, with females exerting a preference for males successful in a dominance contest. This would lead to reinforcement of the same male sexual signal by both female mate choice and male competition (401, 402). However, when male competition or sexual signal inflicts a cost on females, the relation between dominant male and female preference is not as strong. An

example can be seen in *Drosophila melanogaster* where increasing male body size is known to enhance male mating success but also more harm is inflicted on females with a negative effect on their fitness (403). Therefore, through these behaviors, sexual signals reflect fitness of a potential mate.

Kairomonal responses of MUPs

In this thesis, I looked into a possible cost of increased MUPs signaling in terms of kairomonal responses by mice. This is based on the fact that rat MUPs act as both a source of pheromones and kairomones depending on the identity of the receiver. Using the information that *Toxoplasma* infected rats secrete greater amount of MUPs (343), I hypothesized that similar to female rats, mice behavior should be affected by higher MUPs levels in infected rats. This was reflected by greater avoidance response in mice toward fresh and aged urine marks of infected rats (404). The purpose of the aged urine was to remove volatiles (either free or bound) present in the urine and implicate MUPs as the active ingredient. This fits into prior work showing that recombinant MUPs (MUP13) was sufficient on its own to induce avoidance behavior in mice (123). Thus, the increase in MUPs signaling could impose a cost in terms of greater avoidance or aversion by mice towards rat urine stimuli. To better understand this, I looked into the nature of the avoidance behavior or kairomonal communication. Similar to female rat preference, mice avoidance of kairomones (rat MUPs) is dose dependent (217). The ability to modulate such behavior points to a capacity to discriminate distinct quantities or strength of kairomone signal to adjust behavior concerning potential predators. Moreover, this kind discrimination seems to be based on relative quantitative estimates of opposing stimuli and not on an absolute scale (217). That is, the strength of a stimulus is in reference to another source of the same stimulus that an animal comes across. This comparison of relative strengths appears to have a constant discrimination threshold that is a proportional fraction of stimulus strength (Weber's Law).

As a result, the advantage of increased MUPs signaling to rats in terms of greater female preference could possibly incur a cost in loss of predation opportunity, as mice would more easily detect them. This is because MUPs are acting as both a pheromone and a kairomone and the open nature of these communication systems allows for eavesdropping (209). This follows the idea of ‘honest’ sexual signals as costs are placed on males to sustain the display in spite of the handicap in place (231). As discussed prior, how individuals react to costs of sexual displays is down to their fitness level which in turn is shaped by internal (health, age) and external (social environment) factors that is known as condition dependence of sexual signals. The interaction of such factors decide to what extent an individual can allot resources to signaling which in turn would act as a readout or proxy for fitness.

Condition dependence of sexual signals

In this thesis, I studied condition dependence of MUPs with three possible factors- age, health/sickness and social environment. The idea is that MUPs would be dynamically regulated by the interplay of such factors based on the costs imposed on individuals.

In a longitudinal study to observe effect of age, MUPs levels decreased over a 12 month period. Aging often is accompanied by loss or decline in normal bodily functions that would be reflected in overall reduced condition or fitness and male displays (232, 234, 235). For example, when men experience a decrease in testosterone levels it causes changes like lower muscle mass, reduced fertility or increased abdominal fat deposition and associated health issues (405). Individuals have to manage a tradeoff between “slow & steady” or “fast & furious”, in which investing greatly in reproductive activities (which include displays) during early stages of life comes at a cost of reduced physiological functions in later parts of life (235). Similarly, when rats transition from juvenile to adolescence and adulthood, testosterone and subsequently MUPs levels begin to rise. Over their adulthood, MUPs levels decreased to half of the initial levels (comparison between 6 and 18 months). However, the

correlation between male displays and reproductive fitness with age might not be as clear as demonstrated in bustards (235) and should be kept in mind when assessing honesty of signaling.

Similar to aging, the use of LPS as an immunological proxy to induce sickness like behavior (255, 277) was done to lower condition, which also caused a reduction in MUPs levels. Conspecifics normally detect and avoid sick other individuals and such information has been shown to be relayed in odors (259, 287, 288, 293, 294). This implies that sickness related odors could affect chemosensory signals in urine to reflect one's health condition to conspecifics. Moreover, as with aging, infection or sickness also negatively affects testosterone and thus LPS treatment caused a reduction in MUPs and female preference. In addition, as conspecifics can sense infected or sick animals, it was interesting to see effect of illness related odor cues on conspecifics. Those co-housed with LPS-treated animals increased their MUPs levels and showed more dominant like behavior (exploratory) in cat odor approach-avoidance assay. As LPS-treated animals would have lower MUPs levels and express sickness like behavior, they could have diverted resources from signaling. The co-housed animals appear to have responded with increased signaling possibly as a proxy for dominance display.

However, in the final experiment- altering social status by co-housing with castrated or testosterone supplemented animals, provided some unexpected observations. Housing with castrated animals is similar to housing with LPS-treated animals, as the treatment animal in the dyad would be the subordinate. It would follow that a similar increase in signaling should be seen in the co-housed with castrated group, but, it actually showed the smallest increase in MUPs. Instead, the group co-housed with testosterone-supplemented animals saw the greatest increase. From this, it appears that signaling is enhanced only when a strong challenger- high testosterone animal with increased MUPs and aggression- is present and the benefit of being

able to compete is greater than the costs. The introduction of female exposure reversed this trend with those housed with supplemented animals falling the most and the other groups showed a significant increase as would be expected. The huge decrease could be explained in that the supplemented animals would also increase MUPs/ aggressive behavior from residual of female exposure in home cage. As such, the costs of increased signaling might outweigh the benefits.

Due to the costs associated with sexual signals, it makes sense for an individual to be able to regulate them based on contexts, needs and their ability or fitness- condition dependence. I have showed that age, health/sickness and social environment could affect MUPs signaling. However, its relation or effect on reproductive fitness either directly or indirectly still needs to be addressed to understand whether it is really an honest sexual signal. Moreover, given that MUPs measurements in this thesis are relatively quantified, it is tough to make any conclusions on the expected optimal production in males given the costs and benefits found.

Evolution of a sexually selected signal

For MUPs to be a true sexual signal, it must accurately be a reflection of fitness or quality of the signaler. Most models of the evolution of extravagant male sexual signals and female preferences for them, posit that the male sexual signal is costly to produce and sustain (115, 172, 217). These costs ensure the honesty of sexual signals as resources are used to produce a handicap in survival or lower the fitness of males (173, 178, 231). As such, only males that are fit enough can engage in sexual signaling. The models also assume that the effect of sexual signals on fitness depends on condition dependence (diet, social interactions, disease) of the trait. This means that “males invest differentially in the sexual trait in relation to their ability to bear the associated costs” (374).

An excellent example is the bright coloration on male guppies which females show a preference towards (374). This is a sexually dimorphic signal with brighter orange coloration (carotenoid based) linked to “boldness toward predators”, less parasitization and better foraging ability. Hence, bright coloration seems to be an honest reflection of quality (374). In this study, they showed that the more brightly colored male incurred greater cost in terms of predation by cichlid fish. Thus, though females prefer brighter coloration, it also increases predation risk, reinforcing reliability of this sexual signal as an honest indicator of male quality.

Few main schools of thought describe evolution of mate choice and sexual signals. *Fisherian selection* puts forward that preference for a trait began arbitrarily with no benefit to fitness other than sexual selection. Over generations, the preference for the trait and gene(s) for the trait are inherited, leading to self-reinforcing evolution and exaggeration of the trait (runaway selection) until limited by the cost of natural selection (406). The *Good Gene* hypothesis states that sexual signals reflect the general genetic quality (health and viability) of the individual, which will be inherited by the offspring (407). Moreover, such traits are apt indicators of fitness as they are condition dependent (health status) and involve costs (handicap) so that only males fit enough can manage both, reflecting superior genetic legacy (95, 231). *Sensory bias* suggests that attraction to a trait is as a result of selection for traits that exploit pre-existing sensory bias. The initial attraction or selection could possibly be in a non-mating context and over time, mutations lead to development of a display trait that uses this bias (408). So how do MUPs as a sexual signal fit in this framework?

How to study dynamic nature of MUPs and costs associated?

Sexual signals are dynamic in nature and expression of sexual signals would alter based on needs of appropriate situations. In chapter 6, I carried out some preliminary investigations on the relation between MUPs to the costs it imposes and regulation of MUPs by challenges.

Below I propose a more formal plan (using some similar elements from chapter 6) to test the Fisherian Selection and Good gene/handicap hypothesis in context of MUPs.

Firstly, I propose to artificially induce over-expression of MUPs and study the costs (like immune function) it entails on the rat. One could induce MUPs over-expression with use of adeno-associated virus (AAV) in the liver (major production site). Another possible avenue to elevate MUPs levels is to implant testosterone capsules (due to androgen dependency of MUPs) and examine the cost of high MUPs levels. As testosterone is known to be an immunosuppressant and promoter of oxidative stress, examining immune system parameters should be apt. Other costs or parameters that can be studied are sperm count, growth rate and reproductive success.

Secondly, varying challenges can be introduced to observe effect on MUPs levels. The challenges can be in two forms, one affecting internal state and the other would be external or environmental. The internal state can be altered by using *Toxoplasma gondii* infection and LPS an immunological proxy for sickness. External challenges would be studied by introducing intruders (like a castrated male or a sexually mature male or a female) and analyzing the effect of social interactions on MUPs levels. MUPs should only increase in response to appropriate challenge and magnitude of regulation would be affected by interaction of internal state and external challenge. In addition, one can also examine the effect of challenges on various costs and parameters and link it with MUPs regulation.

To summarize, the various ways to study MUPs could be 1) artificially increase MUPs levels and study costs associated, 2) Introduce challenges and measure MUPs levels (regulate 'naturally') and 3) by introducing challenges that regulate MUPs levels, study costs associated.

Experimental Predictions

These experiments will provide insights into how rats cope with the costs in relation to MUPs levels and the combined effect on fitness. As each school of mate choice would expect a different relationship, this information would help to elucidate how MUPs evolved as a sexual signal.

The *Good Gene* School would predict that as MUPs levels increase the associated costs would also increase. Moreover, MUPs would be condition dependent on factors like health status (in this case, LPS) that affect ability to divert finite resources. The *Fisherian selection* School suggests that at lower levels MUPs would provide certain physiological benefits which would peak and then steadily decline with increasing MUPs levels. The decline will occur when the benefits of MUPs is outweighed by the costs. *Sensory bias* theory puts forward that males and females had an initial preference for MUPs in a non-mating context which had some selectable benefits. This was subsequently exploited by one side for reproductive means. This is seen by the fact MUPs is sexually dimorphic with males expressing in much greater amounts in urine compared to females. To test this, I would have to investigate if similar to females, do males show preference for MUPs?

In Figure 37, I have plotted hypothetical curves for MUPs regulation by the various challenges that I would expect to observe. The x-axis represents the challenges introduced that would increase in terms of its potency. The y-axis represents MUPs levels. The use of LPS will allow me to study condition dependency of MUPs. In theory, the rats injected with LPS should be less able or fit to respond to the challenges by increasing MUPs levels. In contrast, the use of *Toxoplasma gondii* infection increases basal level of MUPs and it would interesting to see how these rats regulate in response to internal challenges.

Figure 37. Hypothetical curves for MUPs regulation by the various challenges based on internal state of the animal. Challenges introduced would increase in terms of its potency (e.g. castrated male would be closer to the origin on x-axis while a female would be towards the right end of the x-axis).

Figure 38 depicts the relation between MUPs levels and costs or physiological parameters based on predictions of each school of mate choice as described. The x-axis is MUPs levels while the y-axis measures the physiological parameter being investigated. For this, MUPs will be artificially increased and MUPs level will be regulated by ‘natural’ challenges.

Figure 38. Hypothetical relation between MUPs levels (x-axis) and fitness or physiological parameters (y-axis) based on predictions of each school of mate choice.

Parasites & sexual signals

At this point, I would like to bring together the two main topics that I have discussed- parasites and sexual signals. Previously, I mentioned in brief that sexual signals could serve as proxy for parasite infection and disease resistance, which form part of the heritable or genetic legacy of a male that can be inherited by offspring (112, 113, 273). This idea is part

of the seminal Hamilton-Zuk hypothesis that puts forward “health and resistance to parasites were indicators of condition that could drive the evolution of secondary sexual characteristic [sexual signal]”(112) . This hypothesis falls under the Good Gene School as traits are thought to relay information on genetic quality of the signaler and are condition dependent on health and parasitic resistance.

To understand the Hamilton-Zuk hypothesis, it is important to understand the question it was trying to answer. For this, we have to go back to the lekking behavior that I described previously. In species with lekking, it has been observed that mating success is highly skewed to only a few males. For example, in the Sage Grouse, the dominant male has been seen to mate with 30 females in one morning and a single male might be responsible for half of all the matings at one lek (409). If this occurs over generations, there will be loss of genetic variability in offspring as only few of the males are fathering most of the next generation. This also would lead to loss of female choosiness as benefits of being choosy diminish (95). Thus, the question of how genetic variation and choice is sustained in sexual selection has to be answered by the hypothesis (for that matter any sexual selection theory) for it to be valid.

For this, we have to look at the idea that one of the purposes of sex (and sexual recombination) is to fight off diseases. Hosts and parasites are in a constant battle trying to outsmart each other by continuously altering their “genetic locks and keys” (95). This would start a co-evolutionary arms race between the parasite’s virulence and the host’s resistance. If in one generation a host strain is most resistant to the prevalent strain of parasite, then it will be the most prevalent host strain in the next generation. Similarly, the most common host strain will be the one that the parasite strain can attack the easiest in the next generation (95). These are examples of negative frequency dependent selection where fitness of a genotype/phenotype increases as it becomes rarer in the population (410). Therefore, as genes for resistance will continually evolve with virulence genes (generating co-evolution cycles), it

would maintain heritable variation in fitness over generations (411). The lek paradox is solved as by selecting the healthiest male in each generation, females would be choosing different combination of genes in each generation. In this way, both genetic variability and choosiness are maintained (95). However, as resistance genes cannot be directly assessed for, females would continue to use sexual signals, which would reflect health status, but the genes underlying would continue to vary (411). Specifically, the Hamilton-Zuk hypothesis “argued that genetic variance in fitness could be maintained when the traits under sexual selection are associated with genes for resistance.” In other words, sexual signals could act as a proxy for health and parasitic resistance. Thus, host-parasite co-evolution would lead to continuous genetic variation associated with sexual signals.

In this way, parasites could influence sexual selection of sexual signals that signify parasite resistance, possibly explaining why some species have more exaggerated ornamentation. The Hamilton-Zuk hypothesis predicts that amongst species with greater selective pressure of parasites, females can honestly rely on sexual signals when choosing a mate leading to positive correlation between ornamentation and parasite load. And within a species, the opposite would occur with a negative relationship implying that greater ornamentation is a signal for lower parasite load (112).

“If you cannot beat them, join them”- if sexual signals have evolved to be honest indicators of parasite infections, it would be an incentive for a parasite to hijack and use it to its own advantage. Thus, parasites such as *T. gondii* would manipulate putative sexual signals like MUPs in rats to increase transmission to conspecific intermediate hosts directly and possibly (indirectly) enhance transmission to definitive hosts.

Table 6. Effects of LPS-treatment on behavioral, physiological and hormonal aspects

Gender & strain	Age	Paradigm studied	Dose of LPS	Effects of LPS treatment	Reference
Male Sprague Dawley	Adult	-Social interaction test	-from 1 to 125 µg/kg	-Decreased in time spent in social interaction	-Differential behavioral, physiological, and hormonal sensitivity to LPS challenge in rats International Journal of Interferon, Cytokine and Mediator Research 2009:1 1–13 (PMID number not available)
		-Home cage activity	-5 to 250 µg/kg	-Decreased locomotor activity	
			-15, 50, and 125 µg/kg	-Decreased locomotor activity in both distance and duration travelled	
			-15 µg/kg and above	-Rearing behavior decreased -Immobility increased	
			-50 and 125 µg/kg	-Decreased in grooming duration	
		-Saccharin preference test	- 1, 5, 15, 50, 125, or 250 µg/kg -5 and 15 LPS µg/kg	-Decreased in saccharin preference -Water intake increased	
		-Body weight	-1 µg/kg and 15 µg/kg -50, 125, or 250 µg/kg	-Decreased 4 h after the injection, but not 24 h - decreased BW at both 4 and 24 h following injection	
			-250 µg/kg -From 15 to	-Decreased thermal response at 2 h post injection -Increased in plasma	

		-Serum cytokine levels	250 µg/kg -Starting at 5 µg/kg -1 µg/kg -All doses	ACTH levels within 2 h of injection -IL-1 β and IL-6 levels were increased - IL-10 increased -TNF- α levels increased	
Male & Female Sprague–Dawley	Adult	Physiological and behavioral effects of LPS	100 µg/rat, i.p	- Demonstrated hyperthermia, anorexia, anxiety, depression like phenomenon and reduction in body weights -Serum levels of IL-6 and TNF- α level in LPS treated were increased	25715113
Male Sprague-Dawley	Adult	Investigated a behavioral and metabolic paradigm with chronic dose of LPS	75 or 300 µg/kg/day osmotic pumps	LPS-infused rats showed acute signs of sickness behavior, but chronic LPS infusion did not induce behavioral or metabolic changes	25697068
Male Sprague–Dawley	Adult	Effect on hippocampal pattern separation, context object discrimination	167 µg/kg i.p.	LPS-treated rats memory retrieval was strongly impaired but not in tasks like novel object recognition and spatial memory in the water maze	25451612
Male Wistar	Adult 12-week-old	Effect on cognitive performance (Barnes maze) and inhibitory avoidance tests	1 mg/kg	-Deficits in cognitive performance in the Barnes maze and inhibitory avoidance tests - Effects were attenuated with intermittent fasting Prevented LPS-	24886300

				induced elevation of IL-1 α , IL-1 β and TNF- α levels, reduction of BDNF levels in the hippocampus. Also significantly attenuated LPS-induced elevations of serum IL-1 β , IFN- γ , RANTES, TNF- α and IL-6 levels	
Male Sprague-Dawley	Adult	Effect on social behavior	100 μ g/kg i.p.	-Reduction in social interaction of juveniles -Reduction in social investigation (barrier) of another male	19414023
Female Sprague-Dawley	Pups	Effect of neonatal LPS on adulthood	1mg/kg intracerebral postnatal day 5	-Neonatal LPS exposure causes persistent injuries to the hippocampus and results in long-lasting learning disabilities -Chronic inflammation in the rat hippocampus -Less anxiety-like responses in EPM	23298854
Male and female Long-Evans	Pups & adult	Effect on anxiety behavior	50 μ g/kg on postnatal day 3 and 5	- In the light-dark test, neonatal treatment decreased adult anxiety-like behavior in females, but not males	23280058
Wistar rats male	Adult	long term effects of single dose of LPS (7 days & 10 months)	5 mg/kg i.p.	-LPS induced a TNF- α increase in the hippocampus and frontal cortex (from 7 days onward) and cerebellum (at 10 months) - Enhanced IL-18 expression in these	22642744

				areas at 10 months	
Female Wistar	Pups & adult	Effect of neonatal LPS treatment on the female reproductive development	0.05 mg/kg, i.p.	<p>-Neonatal exposure caused increased tyrosine hydroxylase phosphorylation in adrenals</p> <p>-Treatment increased the plasma corticosterone concentrations of females as juveniles, adolescents and adults, and reduced FSH in adolescence</p> <p>-Increased catch-up growth and earlier onset of puberty in LPS-treated females</p> <p>-Diminished follicular reserve was observed in neonatally LPS-treated females along with the advanced reproductive senescence</p> <p>-While fertility rates were not affected, higher mortality and morbidity were observed in litters born to LPS-treated mothers</p>	22366707
Male Wistar	Adult	Testing range of doses and a battery of tests	10, 50, 200 and 500 µg/kg, i.p.	- Dose-dependent increase in anxiety-like behaviors (EPM), forming an inverted U curve peaked at 200 µg/kg	22059515

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Appendix

Appendix A: Supplementary figures

Figure S1. Western blot of 2D gel of rat urine for MUPs proteins. This demonstrates that the MUP antibody used in this work identifies multiple MUPs isoforms or variants in rat urine.

Figure S2. Preferences of sexually-naïve estrus females for bisect containing either control or infected male urine marks. Data from each unique male pair is depicted in a separate panel.

Figure S3. DART-MS analysis of urine samples with and without menadione treatment. (A) Volatiles in MUPs fractions prior to and after menadione treatment were compared with vehicle samples. In the case that MUPs are bound to volatiles, extra peaks should be observed in samples without menadione displacement (red peaks). However, no additional peaks were observed from urine samples alone (no menadione), indicating that bound volatiles are either not present or are present at levels too low to be detected. **(B)** Some of the peaks were a result of background signals from sample tubes and pipette tips. **(C)** Urine samples from castrated males were analyzed and did not contain signals corresponding to menadione or extra signals aside from background signals.

Chapter 8

#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
MUP10	M	K	L	L	L	L	L	L	C	L	G	L	T	L	V	C	G	H	A	E	D	A	S	S	T	R	G	N	L	D
PGCL-2	M	K	L	L	L	L	L	L	C	L	G	L	T	L	V	C	G	H	A	E	<u>D</u>	A	S	S	T	R	G	N	L	D
MUP13	M	K	L	L	L	L	Q	P	S	S	G	L	T	L	V	C	G	H	A	E	E	A	S	S	T	R	G	N	L	D
PGCL-1	M	K	L	L	L	L	L	L	C	L	G	L	T	L	V	C	G	H	A	E	E	A	S	S	T	R	G	N	L	D
#	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
MUP10	V	D	K	L	N	G	D	W	F	S	I	V	V	A	S	N	K	R	E	K	I	E	E	N	G	S	M	R	V	F
PGCL-2	V	<u>D</u>	K	L	N	G	D	W	F	S	I	V	V	A	S	N	K	R	E	K	I	E	E	N	G	S	M	R	V	F
MUP13	V	A	K	L	N	G	D	W	F	S	I	V	V	A	S	N	K	R	E	K	I	E	E	N	G	S	M	R	V	F
PGCL-1	V	<u>A</u>	K	L	N	G	D	W	F	S	I	V	V	A	S	N	K	R	E	K	I	E	E	N	G	S	M	R	V	F
#	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
MUP10	M	Q	H	I	D	V	L	E	N	S	L	G	F	K	F	R	I	K	E	N	G	E	C	R	E	L	Y	L	V	A
PGCL-2	M	Q	H	I	D	V	L	E	N	S	L	G	F	K	F	R	I	K	E	N	G	E	C	R	E	L	Y	L	V	A
MUP13	M	Q	H	I	D	V	L	E	N	S	L	G	F	E	F	R	I	K	E	N	G	E	C	R	E	L	Y	L	V	A
PGCL-1	M	Q	H	I	D	V	L	E	N	S	L	G	F	K	F	R	I	K	E	N	G	E	C	R	E	L	Y	L	V	A
#	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
MUP10	Y	K	T	P	E	D	G	E	Y	F	V	E	Y	D	G	G	N	T	F	T	I	L	K	T	D	Y	D	R	Y	V
PGCL-2	Y	K	T	P	E	D	G	E	Y	F	V	E	Y	D	G	G	N	T	F	T	I	L	K	T	D	Y	D	R	Y	V
MUP13	Y	K	T	P	E	D	G	E	Y	F	V	E	Y	D	G	G	N	T	F	T	I	L	K	T	D	Y	D	R	Y	V
PGCL-1	Y	K	T	P	E	D	G	E	Y	F	V	E	Y	D	G	G	N	T	F	T	I	L	K	T	D	Y	D	R	Y	V
#	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
MUP10	M	F	H	L	I	N	F	K	N	G	E	T	F	Q	A	M	V	L	Y	G	R	T	K	D	L	S	S	D	I	K
PGCL-2	M	F	H	L	I	N	F	K	N	G	E	T	F	Q	<u>A</u>	M	V	L	Y	G	R	T	K	D	L	S	S	D	I	K
MUP13	M	F	H	L	I	N	F	K	N	G	E	T	F	Q	L	M	V	L	Y	G	R	T	K	D	L	S	S	D	I	K
PGCL-1	M	F	H	L	I	N	F	K	N	G	E	T	F	Q	<u>L</u>	M	V	L	Y	G	R	T	K	D	L	S	S	D	I	K
#	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
MUP10	E	K	F	A	K	L	C	E	A	H	G	I	T	R	D	N	I	I	D	L	T	K	T	D	R	C	L	Q	A	R
PGCL-2	E	K	F	A	K	L	C	E	A	H	G	I	T	R	D	N	I	I	D	L	T	K	T	D	R	C	L	Q	A	R
MUP13	E	K	F	A	K	L	C	E	A	H	G	I	T	R	D	N	I	I	D	L	T	K	T	D	R	C	L	Q	A	R
PGCL-1	E	K	F	A	K	L	C	E	A	H	G	I	T	R	D	N	I	I	D	L	T	K	T	D	R	C	L	Q	A	R
#	181																													
MUP10	G																													
PGCL-2	G																													
MUP13	G																													
PGCL-1	G																													

Figure S4. Amino acid alignment between MUP-10, PGCL-2, MUP-13 and PGCL-1

Figure S5. Females did not show attractive behavior towards rMUP-10 and rMUP-13. (A) Females spent on average $467s \pm 57s$ near rMUP-10 and $435s \pm 54s$ near castrated urine (n- 13; Wilcoxon signed rank test: $p= 0.75$). (B) Females spent on average $451s \pm 36s$ near rMUP-10 and $365s \pm 42s$ near castrated urine (n-10; Wilcoxon signed rank test: $p= 0.21$).

Figure S6. Native PAGE (12%) of denatured (D) (by guanidinium hydrochloride; second lane from left) and renatured (R) MUPs (third lane from left). Loading protein volume was $10 \mu\text{L}$ for each sample. The renatured sample had retarded migration due to restoration of its native conformation. This confirms that the renaturation step was successful.

Figure S7. The original 2D gel shown in Figure 8 with molecular weight marker.

Appendix B: Publications List

([±] Equal contribution)

1. **Vasudevan, A[±]**, Kumar, V[±], Chiang, Y., Yew, J., Cheemadan, S., & Vyas, A. (2015). Alpha2u-globulins mediate manipulation of host attractiveness in *Toxoplasma gondii* - *Rattus norvegicus* association. *ISME J*, 9, 2112-2115.

My contribution was the behavior assays, Western blot and real time qPCR.

2. Kumar, V.[±], **Vasudevan, A[±]**, Soh, L.J.T., Le Min, C., Vyas, A., Zewail-Foote, M., *et al.* (2014). Sexual attractiveness in male rats is associated with greater concentration of major urinary proteins. *Biol Reprod*, 91, 150.

My contribution was the behavior assays and Western blot.

3. **Vasudevan, A.** & Vyas, A. (2014). *Toxoplasma gondii* infection enhances the kairomonal valence of rat urine. *F1000Research*, 3, 92 (doi: 10.12688/f1000research.3890.1).

I did all the experiments in this paper.

4. **Vasudevan, A.** & Vyas, A. (2013). Kairomonal communication in mice is concentration-dependent with a proportional discrimination threshold. *F1000Research*, 2, 195 (doi: 10.12688/f1000research.2-195.v2).

I did all the experiments in this paper.

5. Soh, L.J.T.[±], **Vasudevan, A[±]** & Vyas, A. (2013). Infection with *Toxoplasma gondii* does not elicit predator aversion in male mice nor increase their attractiveness in terms of mate choice. *Parasitol Res*, 112, 3373-3378.

My contribution was the behavior experiments, Western blot and bio-imaging.

6. Dass, S.A.[±], **Vasudevan, A[±]**, Dutta, D., Soh, L.J.T., Sapolsky, R.M. & Vyas, A. (2011). Protozoan parasite *Toxoplasma gondii* manipulates mate choice in rats by enhancing attractiveness of males. *PLoS One*, 6, e27229.

My contribution was the behavior assays, mating studies and Western blot.

Appendix C: Conference Talks

34th International Ethological Conference- Behavior 2015

Cairns, Australia

9th -14th Aug 2015

Selected for oral presentation and awarded Australasian Evolution Society Student Award

11th TLL Symposium – Origins, Functions and Utility of Biological Variation

National University of Singapore, Singapore

28th Feb 2013

Selected for oral presentation