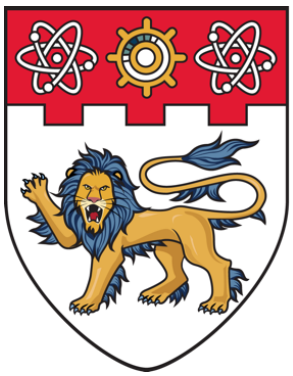

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

PARASITIC BEHAVIOURAL MANIPULATION REFLECTED AS AN EXTENDED EPIGENOTYPE IN A NATURALISTIC POPULATION

PHILIP NGO YUN XUAN

SCHOOL OF BIOLOGICAL SCIENCES

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

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

15th December 2021

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This study was carried out with financial support from the Human Frontier Science Program (HFSP) grant and the School of Biological Sciences at Nanyang Technological University for hosting this research. The culmination of this thesis would not have been possible without the mentorship and the paramount support from Associate Professor Ajai Vyas. My initial encounter with the subject of this thesis goes way back to the spring of 2019. There I was a final year undergraduate, struggling to find my place and fitting in this massive field of the biological sciences. Ajai approached the subject matter with such mastery, passion, and rigour over 13 weeks that I was convinced that this could be my calling in biology. Sure enough, I returned a year later to pursue the subject as part of my master's degree. I wouldn't dare say I was the best candidate for this project as I had numerous costly setbacks due to my carelessness and naivety throughout the way. Ajai however, was extremely supportive of my artless venture into qPCR methodologies, infection biology, and even immunobiology. I was never invalidated but instead spurred on to pursue these questions if I do believe in them. In hindsight, such experiences are truly a privilege as they stand to inculcate the ingenuous spirit of science and curiosity that is often lost with most academic practices. Beyond the professional aspects, Ajai was tremendously patient and tactful with my day-to-day struggles ranging from my interpersonal relationships to my motivation and mental wellbeing. Even when my struggles crept into my professional boundaries, such as my debilitating anxiety, Ajai would always ensure that my wellbeing is of priority even before the project. I cannot truly express how indebted I feel to Ajai for believing in me throughout this journey, giving me the chances I needed when others hesitated. I would also like to thank the

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Parasitic behavioural manipulation reflected as an extended epigenotype in a naturalistic population

Philip Y. X. Ngo

Abstract

Historically, the behavioural manipulation hypothesis is relentlessly challenged by inconsistent phenotypic profiles trying to coalesce molecules to behaviours in the laboratory. Yet, exploring the phenomena in naturalistic settings exposes the study to myriad confounders and stochasticity, making empirical evidence difficult to obtain. Herein, these challenges are addressed from a serendipitous intervention at Kangaroo Island accompanied by a longitudinal study design, allowing the dissection of the behavioural phenomenon, '*fatal attraction*', observed in rodents infected with *Toxoplasma gondii*. Comparable to laboratory findings, parasitic infection in wild mice coincides with significant epigenetic changes within the paraventricular nucleus of the hypothalamus and the posterodorsal medial amygdala. The latter of which, is previously demonstrated to sufficiently reproduce the loss of aversion towards predators in laboratory rodents. We posit that *Toxoplasma gondii* remarkably alters the epigenotype of mice in the natural wild environment, with the extension of this epigenotype steering the loss of aversion towards predatory felids, augmenting parasitic transmission. We further explore the implications of infection on other facets of mice physiology derived from Kangaroo Island and deduce plausible connotations. The 'extended epigenotype' may provide a much-needed contemporary perspective on future parasite-host associations and broad, inter-intraspecies relationships.

Introduction

1.1 Behavioural plasticity and phenotypes

In face of stochastic environments, behavioural plasticity may grant an adaptive edge to organisms attempting to thrive or overcome a novel variability[1–3]. Naturally, given identical genotypes, we can expect a wild population to be more behaviourally plastic than their laboratory counterparts[4]. After all, a phenotype enabling behavioural flexibility can have significant ecological ramifications and implications on natural selection in the wild than in sterile inbred settings[5]. Such behavioural divergence within a population can occur over shorter periods of time in lower-level clades[6].

Although behavioural phenotypes can oscillate with social cues and environmental contexts (contextual plasticity), there are a few innate behaviours that are determined by inherent limitations such as primal physiology and hardwired genetics (developmental plasticity)[7]. Such innate behaviours are often exemplified by the dichotomy between survival and reproduction. Reproductive behaviours within the animal kingdom often involve promiscuity in pursuit of suitable mates, which incurs elevated risks of predation and death[8]. On the other hand, defensive behaviours maximise survival by minimizing exploratory activities resulting in opportunistic costs of furthering its progeny[9]. These behaviours can be studied in the context of the approach-avoidance conflict, where stagnating at either end of the spectrum will negatively impact evolutionary fitness. The capacity for behavioural flexibility, therefore, becomes adaptive in the prevailing backdrop of natural selection in the wild.

1.2 Behavioural manipulation and extended phenotypes in host-parasite relationships

Behavioural manipulation enacts on prevailing plasticity and the flexibility of a behavioural phenotype. Behavioural manipulation by parasites can be also be included in the above definition[10]. A quick internet search on the terms ‘parasitic behavioural manipulation hypothesis’ often yield sensationalised results in popular media. Articles are decorated with lexicons that include puppeteering, mind control and even zombification. In actuality, there are numerous examples in nature that may justify these descriptions.

A parasitic nematomorph hairworm, *Spinochordodes tellinii*, is largely responsible for the ‘suicidal’ behaviour of its infected hosts[11]. Specifically, infected grasshoppers and crickets develop a morbid attraction to aquatic environments, drowning in the process and initiating the parasite’s life cycle of reproducing in the water. The infallible manipulation employed by these nematomorphs generated such an abundance of infected insects, that it became the main source of food for an endangered species of Japanese trout, accounting for 60% of the energy intake of the trout population[12]. In this example, parasitic manipulation is crucial to the maintenance of the energy cycle, highlighting the significance of selection pressures on host behaviour manipulation in ecology.

Another notable example of turning the infected into vectors towards definitive hosts is the *Leucocloridium* flatworm. The brood sacs of these sporocysts manifest within the stalks of terrestrial snail hosts, enacting conspicuous pulsates of colour in an attempt to entice predation by insectivorous birds[13]. Parasitic behavioural

manipulation can also manifest beyond the confines of a singular host, giving rise to aggregating effects and swarming such as the microsporidian-infected *Artemia* brine shrimp[14]. The swarming effect augments the likelihood of transmission of the parasite to its definitive host, the Greater Flamingo, through predation of the intermediary hosts[14].

Apart from predation as an endpoint of parasitism, behavioural manipulation can also be employed to directly enhance the survival of the parasite's brood. The braconid parasitoid of the Glyptapanteles wasp induces a complete castration including all faculties of survival such as feeding of its infected caterpillar host[15]. The infected host remains close to the parasitoid pupae and responds with violent head-swinging in the presence of a predator. Ultimately, the infected caterpillar does not survive till adulthood, thus conferring the benefits of the defensive behaviour exclusively to the parasitoid[15].

Perhaps the most notorious example of direct behavioural manipulation, the emerald jewel wasp is highly adapted to predate on species of cockroaches. The stinger of the parasitoid wasp doubles as a sensory organ that enables the precise introduction of a venom cocktail into two specific clusters of neurons in the brain of the cockroach[16]. The cockroach remains alive after the ordeal but is instead, blunted to apparent threats to its survival and enters a highly 'suggestive' state[17]. The predated and thus, parasitised host will then serve as sustenance and harbour the next generation of jewel wasp parasitoids[18].

In ‘The Extended phenotype’ published in the 1980s, Dawkins surmises that a behavioural phenotype of an animal maximizes the survival of the genes specific for that behaviour, unconditional to whether the genes belong to the body of the particular animal[19]. Behaviours can thus be represented as means to an end for the propagation and persistence of a gene. Similarly, it can be argued that the hosts from each of the above examples are ‘dead’ in the landscape of evolution. The salient behaviours although enacted by the host, are no more than the extensions of the parasite’s genes that inhabit them, thus ‘extended phenotype’. The endpoint of all only serves to propagate the genes of the parasite instead of the host’s.

However, deviations to this notion exist in cases where parasitism confers reproductive or survival advantages to the host’s genes. The apicomplexan parasite, *Toxoplasma gondii*, employ contrasting strategies of behavioural manipulation to ensure survival and progeny. These include augmenting transmission of the parasite between the intermediate hosts by increasing the attractiveness of male rodents demonstrated previously[20].

1.3 Aetiology of *Toxoplasma gondii* and associated behavioural comorbidities

Toxoplasma gondii (henceforth Toxoplasma) ranks as one of the most successful parasites in the world with epidemiological reports suggesting a third of the world's population being infected[21]. Seroprevalence ranges from 15% in urbanised cities of China and Korea, to 47% in Tanzania and rural regions of France[22–25], with significant correlations of infection to poverty[26]. Parasitic transmission of Toxoplasma occurs through the consumption of contaminated food and water sources, or contact with faecal matter deriving from an infected felid - the definitive host[27].

Virtually all warm-blooded animals are susceptible to infection and serve as intermediate hosts for Toxoplasma to arrive at its feline definitive hosts[28]. The unique chemistry within the digestive systems of felids allows sexual recombination of the parasite's genes, producing oocysts as a result and later excreted alongside the faeces. Specifically, the abundance of linoleic acid within the intestines of cats signals sexual reproduction in the parasite[29]. In most mammals, linoleic acid is rapidly converted to arachidonic acid by the Δ-6-desaturase enzyme. Experimental inhibition of the enzyme in murine confirms the accumulation of linoleic acid, leading to the expectant sexual reproduction of the parasite in the live mice[29].

Most clinical manifestations of Toxoplasma infection entail mild symptoms and are largely asymptomatic, except for immunocompromised individuals who are unable to control the primary infection, and thus at risk of developing toxoplasmosis and sepsis[30]. Nevertheless, an overt clearance of infection is occasionally subverted by a discreet development into chronic toxoplasmosis[31]. This latent form of toxoplasmosis develops as a result of immune pressures on the parasites, converting them into

quiescent bradyzoites that inhabit and persist within immune-privileged anatomies such as the central nervous system[32].

A literature search for potential deleterious effects of latent toxoplasmosis in humans often directs towards incidences of psychiatric disorders. Of noteworthy are studies establishing correlations of past infection with increased incidences of obsessive-compulsive disorders, bipolar disorders, and even schizophrenia[33–36]. In the case of schizophrenia, Niebuhr and colleagues reported the positive correlation specific to increasing levels of Toxoplasma IgG antibodies, and not with antibodies of other infectious agents[37]. The benign infection with Toxoplasma is also purported to associate with increased inquisitive behaviour in men and decreasing behaviours of novelty-seeking in women[35]. Prevalence of Toxoplasma infection also correspond to increased risk-perverse behaviours in both genders, reflected as probabilities of involvement in traffic accidents. Interestingly, the augmented risk-seeking behaviours are attested by elegant retrospective and prospective case-controlled studies[38–40].

The behavioural ramifications of Toxoplasma prevalence are, however, best demonstrated in rodents, the common prey of most feral cats. Berdoy and colleagues were the first to demonstrate the loss of aversion to felid odours and even the morbid attraction in Toxoplasma-infected rats[41]. This behavioural alteration consequence to infection is thought to be the result of strong selection pressures for parasitic transmission from commonly infected hosts to the felids through predation[42]. Although the implications of such behavioural alteration on infected humans are unclear, Poirotte and colleagues posit that the behavioural manipulation from Toxoplasma on

humans evolved in a time when ancestors of the *Homo Sapiens* were predated by large felids and would thus confer tangible benefits to the parasite's transmission[43].

One could simply argue that the dynamically altered behaviour was merely incidental as a result of malaise and can be reproduced with any onset of other pathogenic infection. Yet, in laboratory rodents, malaise manifests as weight loss, reduced locomotion and presentations of unkempt fur[44]. Animals with malaise do not indulge in the maintenance of 'costly' behaviours such as sexual advertisements, territorialism, or exploration as it incurs unnecessary metabolic currency indebted for purposes of recovery[45,46].

With due attention paid to avoid post facto fallacies and confirmation biases, Toxoplasma possesses an arsenal of genes intended for its cellular homeostasis that can be equally capable of subverting its environment, the brain of the host. Although parasitic tropism of specific brain regions remains debatable[47,48], the mere matter of fact that the parasite inhabits the brain becomes highly implicated in delineating the nuts-and-bolts of its manipulation strategy[49,50]. Stibbs and colleagues originally described an increase in total dopamine levels found in the brains of latent Toxoplasma-infected mice[51]. Since then, several other groups have reported similar findings and subverted the phenomena of altered behaviour using dopamine antagonists, advocating the dopaminergic pathways as the basis of behavioural manipulation in Toxoplasma[52–55]. After three decades of dopaminergic-based investigations *ad nauseum*, Gaskell and colleagues elucidated two copies of a gene from Toxoplasma encoding a rate-limiting enzyme responsible for the synthesis of the precursor to dopamine[56]. However, Afonso and colleagues succinctly rebutted by

demonstrating that in transgenic knockouts, the behavioural alterations concurring with Toxoplasma infection are independent of the parasite's gene[57]. Afonso's findings may have challenged the long-standing hypothesis, but it stands as a timely example of the dangers of misconstrued association between correlation and causation.

In the recent decade, an alternative hypothesis has gained considerable traction. The notion surrounding neuroendocrinological means of behavioural manipulation began from initial reports of Toxoplasma-infected mice preferentially selected in mate choice assays at above chance levels[20,58]. As discussed above, latent Toxoplasmosis manifests as cysts residing in immune-privileged anatomies such as the central nervous system, but also the testes in male hosts[59]. There are numerous cases of evidence pointing to the potential for sexual transmission in the life cycle of Toxoplasma. Most notably, the presence of Toxoplasma was found in the semen of three human male individuals, two of whom are serologically positive for the parasite[60]. Detection of Toxoplasma in the semen was reported in several other species of warm-blooded animals such as cats, dogs, and rodents[20,61–63]. Toxoplasma within the testes also increases the production of testosterone[64]. Testosterone is capable of crossing into the brain of the central nervous system, affecting several brain regions that possess the respective androgenic receptors[65]. Of interest, the specific introduction of testosterone within the posterodorsal medial amygdala (MePD) causes the hypomethylation of the promoter for the neuropeptide, arginine vasopressin (AVP)[66]. Infection of rats with Toxoplasma also elutes with significant hypomethylation in the exact brain regions, hinting at the sufficiency of AVP expression and the associated epigenetic modifications for reproducing the behavioural alterations[67].

1.4 Arginine Vasopressin (AVP)

AVP is an evolutionarily conserved hormone peptide that is expressed in the hypothalamus following hyperosmolality to restore fluid homeostasis[68]. A degree of pleiotropy has enabled AVP to encompass multi-faceted roles such as modulating the plasticity of defensive behaviours and aggression, to the basis of social monogamy and sexual behaviours in animals[69–72]. The nonapeptide's role can also be traced through its long phylogenic history and is seen to even regulate reproductive behaviours in birds[73] and nematodes such as the *Caenorhabditis elegans*[74].

Experimental inhibition of AVP-signalling neurons results in impaired responses specific only to reproductive cues[75]. Neuroendocrinological disruption of the AVP system can also be demonstrated through the castration of male rats as the promoter of the nonapeptide is androgen-responsive[76]. Castrated rats exhibit significant methylation of the AVP promoter within the MePD, and exogenous supplementation of testosterone reverses these epigenetic changes[77]. The appetite for sexual behaviours diminished by castration in male hamsters can also be restored through the precise introduction of testosterone within the MePD[78]. The delicate responsiveness of this epigenetic machinery surrounding the AVP promoter may just be the most adaptive target of strong selection pressures on Toxoplasma's transmission to divert its common hosts to its feline hosts.

1.5 The epigenotype

Epigenotype posits a crucial intermediary between the characteristics of an organism - i.e. the phenotype, and the fundamental genetic information represented in concerted permutations of nucleic acid bases - i.e. the genotype. The concept originally began with Conrad Hal Waddington's 'The Epigenotype' written in 1942. Waddington expanded the longstanding notion of how genotype relates to phenotype within the environment, to include 'motifs of genes' that we know today as epigenetics[79]. Although epigenetic modifications do not inhabit the core units of hereditary we know as genes, they are heritable and can be passed down directly from parent to offspring during recombination. Recently, Tyebji and colleagues demonstrated that Toxoplasma infection of the male lineage in mice corroborates with significant transcriptional differences in the sperm that are absent in uninfected controls[80]. Such findings can have significant implications on how hosts of Toxoplasma may adapt to the prevailing selection pressures of parasitism.

1.6 Aims and Hypotheses

A gene-centric perspective of behavioural phenotypes would posit that behaviours maximise selection at the fundamental level of information, for the exact gene responsible. We can then follow this train of thought, arriving at the basis of parasitic behavioural manipulation maximising selection for the genes of the parasite through the actions of its unfortunate host. In laboratory findings, rodents infected by Toxoplasma gondii exhibited profound hypomethylation of the AVP gene in the posterodorsal medial amygdala[66]. Expression of AVP within this region of the brain coincides with promiscuous behaviours specific to reproduction in rodents[81]. Moreover, mounting evidence have reported the presence of Toxo within the semen

of infected warm-blooded hosts[20,61–63], bolstering the prior notion surrounding the purposeful epigenetic modulation of the AVP in the neuroendocrine system to augment propagation. The classical trade-off between reproductive and defence behaviours sufficiently leverages on AVP signalling within the MePD[58,64]. This trade-off, however, has not been observed in laboratory settings where predation and resource delegation are not needed and absent[4]. This presents a confounding argument that the epigenesis of the AVP gene, *ergo*, the behavioural phenomenal enacted by the parasite, can be merely an artefact of the laboratory environment. As such, the true measure of the trade-off mechanism may only be measured behind the realities of the natural environment coupled with escalating consequences behind every decision the organism undertake.

In this thesis, I aim to investigate whether the purported epigenotype demonstrated in laboratory-based Toxo infected rodents can be observed and measured in natural wild settings. Specifically, profiling the methylation status of AVP in various brain regions such as the MePD and PVN between infected and non-infected wild rodents should yield convincing differences if the neuroendocrine hypothesis holds true. I hypothesize that prior laboratory notions on Toxoplasma's epigenetic modulation can be extended and observed in naturalistic populations. With the ongoing removal of the parasite's definitive host, the felids, plausible perturbations in the epidemiology of Toxo infection will also be documented and cross-referenced with existing literature for discussion.

Materials and methods

2.1 - Population sampling from Kangaroo Island

Kangaroo Island (KI) stands as the third-largest island in Australia. The government of Australia plans to remove all of the cats on the island by 2030 as part of an extensive

effort to preserve the dwindling wildlife and stem Toxo infections within livestock[82]. Cats such as the *Felis catus* remain the apex predators of the island due to low predation on the species[83]. The sampling of wild mice from KI was done in collaboration with Dr. Louis Lignereux and Dr. Ryan O'Handley from the University of Adelaide. Mice were trapped and sacrificed promptly from 12 different sites, across 16 months in KI, corresponding to areas with or without cat culling interventions. The brains, liver, fur, and testes - when applicable, were snapped frozen in liquid nitrogen and shipped to Singapore for analysis.

2.2 - Microdissection and genomic DNA processing

Indicated brain samples were allowed to partially thaw inside of the cryotome (CM1950, Leica) at −20°C for 1 hour before proceeding. The brains were sectioned at 100µm at the same temperature and immediately mounted on glass slides (HistoBond®, Marienfeld). The bregma references are found between -0.10mm to -1.94mm in the Allen mouse brain atlas with the regions of interest (ROI) annotated. The paraventricular nucleus (PVN) and the medial posterior amygdala (MePD) were visually examined, estimated accordingly, and microdissected using several visible neural landmarks[84]. Specifically, these landmarks were selected to be as proximal to the ROIs as possible (Figure 1 & 2), e.g the MePD is immediately distal from the pronounced optic tract, the PVN is located on the superior tip of the third ventricle. The medulla oblongata was harvested whenever possible from the posterior of the brain, inferior to the cerebellum. (n = 67). Microdissected regions were immediately suspended into the solution concocted within the first steps of the DNA extraction protocol (DNeasy Blood and Tissue kit, Qiagen). Isolation of genomic DNA from the

different ROIs was carried out accordingly to the manufacturer's protocol and later resuspended in RNAse-free water.

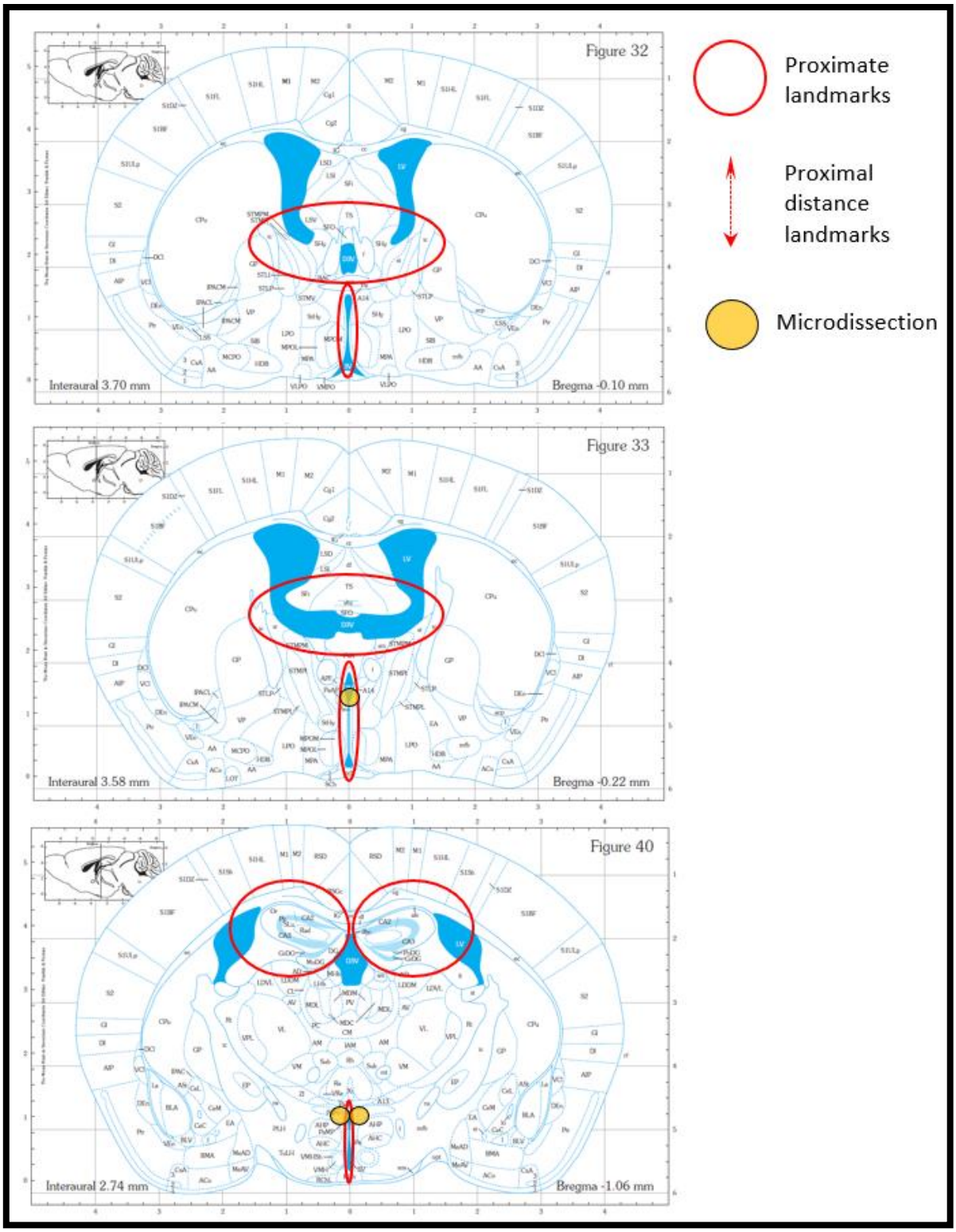

Figure 1 – References for dissecting the paraventricular nucleus

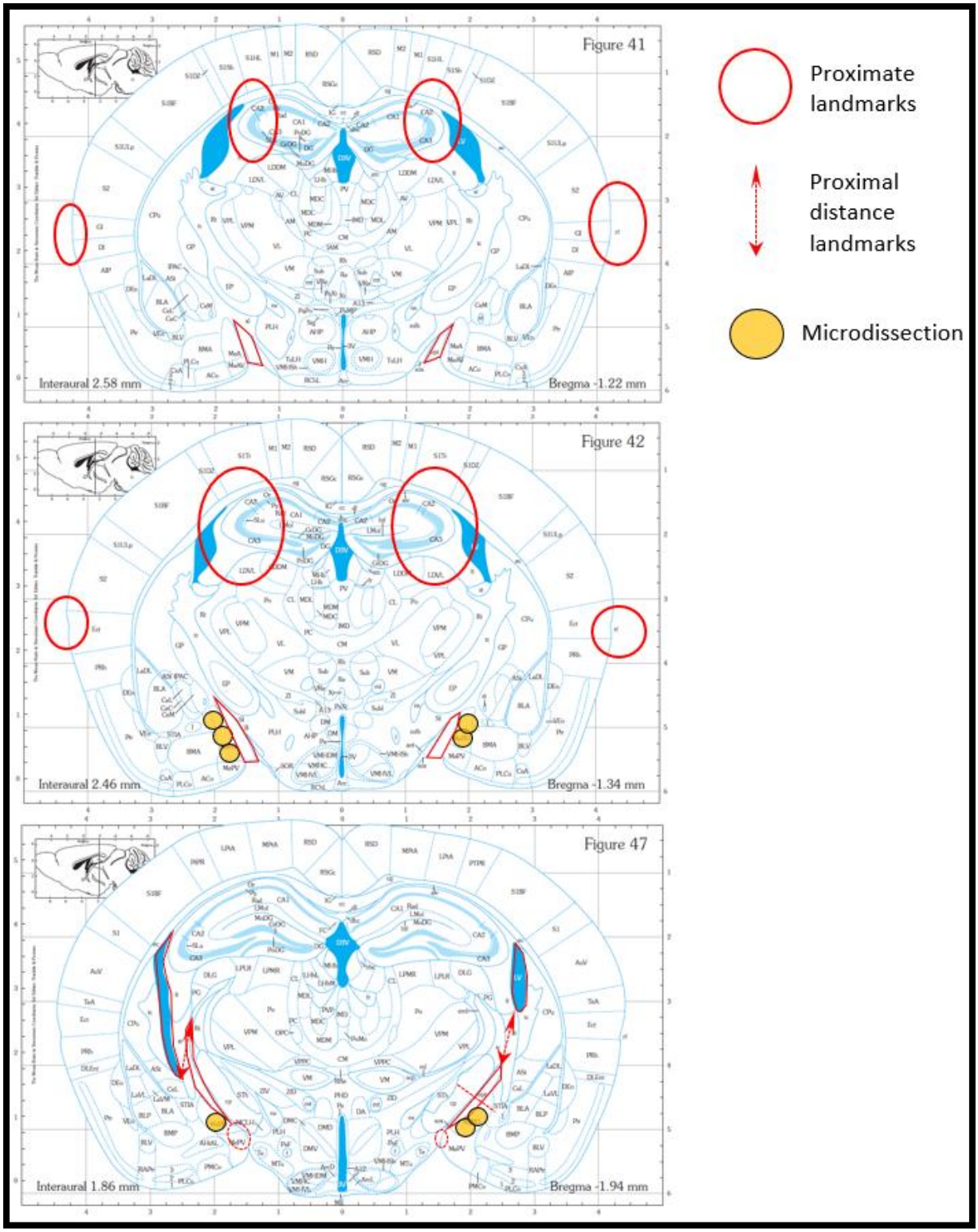

Figure 2 – References for dissecting the posterodorsal medial amygdala

2.3 - Methylation Sensitive Restriction Enzyme Assay (MSRE)

Epigenetic modifications by methylation on the AVP promoter were assessed using the methylation-sensitive restriction enzyme (MSRE) digestion assay and quantitative polymerase chain reaction (qPCR) as adapted from literature[66]. Isolated genomic DNA from each animal and their ROIs were divided equally into two tubes: *HpaII-treated* and *Sans-enzyme*. Methylation-sensitive endonucleases (Hpall, New England Biolabs, Ipswich, MA, USA) cleave specifically at CGCG sequences within the template and the nuclease activity is profoundly impeded by any methylation present on the targeted sequences. Respective tubes labelled for treatment were subjected to the HpaII endonuclease for 1 hour according to the manufacturer's protocol at 37°C. The enzymes were immediately heat-inactivated after treatment. Primers (IDT, Singapore) previously designed to flank the promoter region[66] were used to quantify the number of intact DNA sequences through qPCR[66].

AVPfw, 5' **GAATATTCAACTATGATTTCCAGGTGACCCTCCAG** 3', and *AVPrv*, 5' **CAAGCTGTCAGCAGTGATTCAGGCATCTGGGGACA** 3', were used as the forward and reverse primers respectively.

The efficiency and sensitivity of the MSRE reaction can be assessed from the qPCR melt curve (Figure 3). The melting point of the amplicon is between 85°C to 90°C, with the delta fluorescence value directly proxying the frequency of intact sequences amplified[85] (ergo, methylated and not cleaved by the restriction enzyme). The higher the Ct values, the lesser intact sequences are present within the reaction, indicating the abundance of hypomethylated sequences that were cleaved by the endonuclease[86].

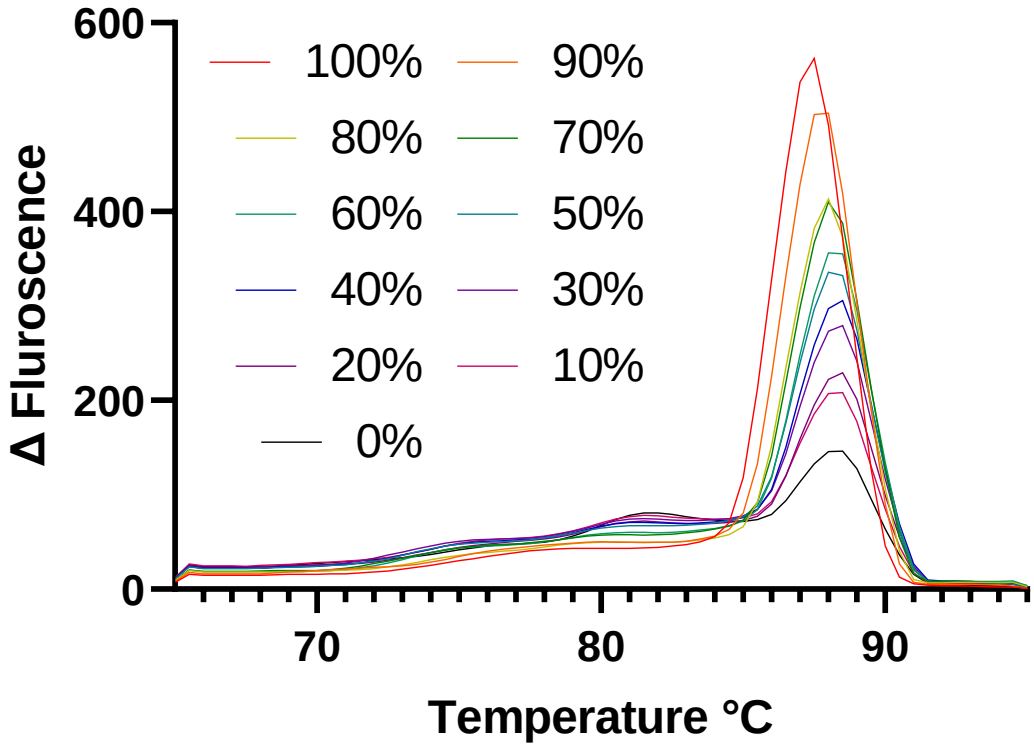

Figure 3. Melt curve reference indicating varying levels of methylated sequences present - % Values indicate the proportion of methylated sequences present within the reaction. The peak at 0% reflects subtle residual sequences that were still intact after enzymatic digestion.

Methylation on the AVP promoter manifests as intact DNA sequences during endonuclease treatment and is proxied by similar DNA abundance (in Ct values) when compared with the sans-treated counterpart after qPCR. Coefficients of variation were monitored and kept <5% within the triplicates for each discrete sample during qPCR. The delta-Ct values are obtained as a numerical subtraction of the Ct value of the sans-treated tube from the enzyme-treated tube. For brevity, the delta-Ct values were additively inversed to positive values to directly represent approximation on the degree of hypomethylation within AVP promoter between the ROIs and animals. In total, 4

discrete Ct values are arising from each of the 96 mouse samples, PVN-DNA and MePD-DNA each separated as enzyme treatment or sans-enzyme.

2.4 - PCR detection of Toxoplasma using B1

The cerebellum from the brain sample was removed for the detection of Toxoplasma using PCR. Genomic DNA was extracted similarly to the MSRE procedure (DNeasy Blood and Tissue Kit, Qiagen).

B1fw, 5' **GTGGGAATGAAAGAGACGCTAATGTG** 3', and

B1rv, 5' **CGTCACCATCAGACGAATCAACGGAA** 3',

were used as the forward and reverse primers respectively in the reactions to detect the B1 gene for the presence of Toxoplasma[87]. The PCR conditions included an initial denaturation at 95°C for 2minutes, before repeating 40 cycles of the following steps, denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and elongation at 72°C for 1 minute. The reaction ends after a final elongation step at 72°C for 5 minutes. The product was visualised on a 1.5% agarose gel with B1 detection proxied by a visible band around 474bp (shown below, lanes 1 and 3 are infected samples).

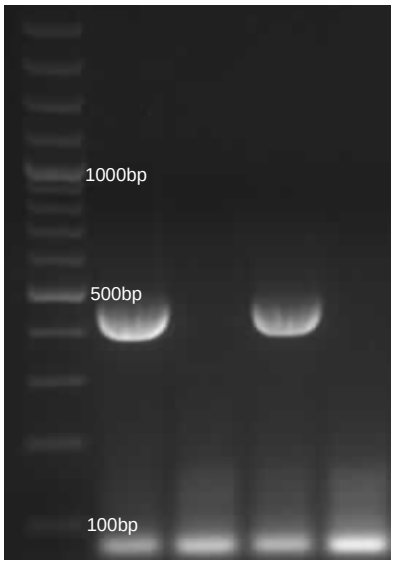

Figure 4 - The classification of Toxoplasma infection for each mouse identification is binary (1 = infected, 0 = not infected). The PCR for Toxoplasma status for a subset of samples was conducted with the help of Dr. Wen Han Tong and Dr. Sijie Tan. Base pairs of parasite positivity by B1 detection may vary (<100bp) due to unaccounted genotypic differences between Toxoplasma variants in the wild.

2.5 - Testosterone extraction and quantification

Extraction of sterols from the hairs of mice was adapted and modified from the previous literature[88]. Testosterone deposited in hair was previously reported to be chronic representations of the endocrine state instead of blood serum[88,89]. The hairs were transferred to a sample tube, submerged in liquid nitrogen, and later pulverized mechanically. The processed hair samples were then weighed before aliquoting 700 µl of 100% MeOH to each tube. The tubes were incubated at room temperature on a shaker for 24 hours, followed by centrifugation at 2000 g for 10 min. The supernatant was then transferred to new tubes and the methanol evaporated under vacuum. The testosterone was quantified in duplicates using competitive ELISA kits purchased from Enzo Life Sciences (ADI-901-065) and later detected using a microplate reader (Plate reader Infinite M200Pro, Tecan). Coefficients of variation arising from the OD values were monitored and kept <10% within the duplicates for each discrete sample and <5% accounting for intra-assay variations.

2.6 - Statistics

Datasets were analysed with GraphPad Prism 9.0 software. Inter-group differences were analysed using Mann-Whitney's U test unless indicated. General Linear Mixed Models (GLMM) were employed with help from Assoc. Prof Ajai Vyas using the JASP statistical software and R[90–93].

(*p < 0.050, **p < 0.010, ***p < 0.001, ****p < 0.0001).

Results

A total of 95 mice were microdissected for the PVN, MePD, and the medulla oblongata. An additional 7 mice were taken from the mainland of Australia as sentinels proxying the samples acquired from KI.

The delta CT values were obtained individually from each mouse which includes each of their respective brain regions, from the subtraction of the Ct values of 'sans-enzyme-treated' from the 'enzyme-treated' sample. The values were additively inversed (-1 * Z, where Z represents a data point) to represent the data in positive integers for brevity. The methylation status and ergo the degree of hypomethylation on the AVP promoter is thus represented as individual points on each graph.

3.1 - The AVP promoter is differentially methylated in the PVN, MePD, and the medulla oblongata.

All three brain regions exhibited significant differences between each other in their methylation status on the AVP promoter regardless of the year of capture, the location, or infection status (Figure 5A). The harvested medulla oblongata serves as the negative control in all experiment setups. Intragroup analysis reveals no discernible gender differences within the PVN, MePD or the medulla oblongata (Figure 5B & 5C).

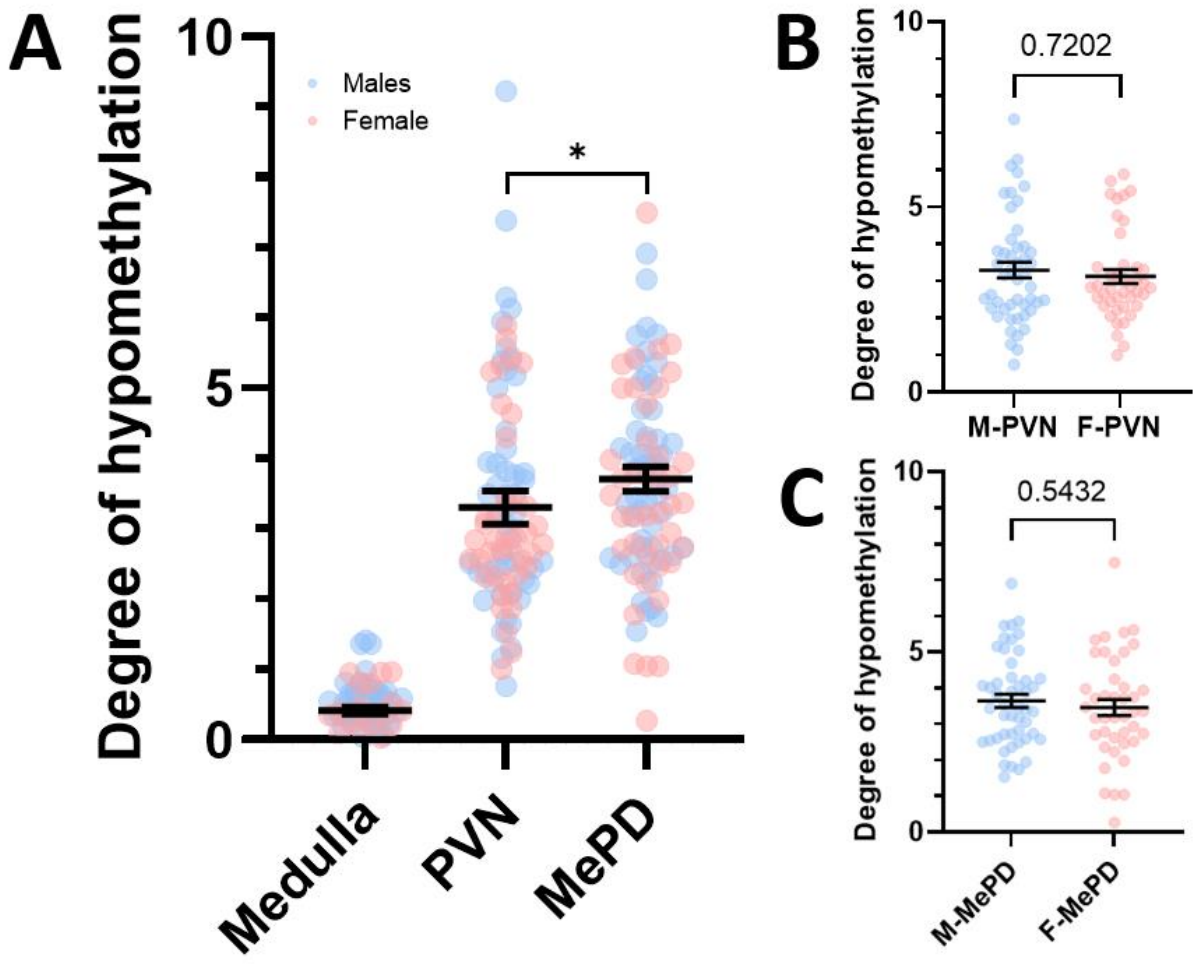

Figure 5. Methylation in the AVP promoter differs between neuroanatomical regions (A) MePD is significantly more hypomethylated than PVN and by large, the medulla oblongata. Paired t-test ($p = 0.0363$, $n = 95$). (B) There are no significant differences between gender in PVN-AVP methylation, $p = 0.7202$ and (C) no significant differences between gender in MePD-AVP methylation, $p = 0.5432$ (Males = 52, Females = 43)

3.2 - Stratification by infection status reveals differential hypomethylation in the AVP promoter of PVN and MePD in male mice

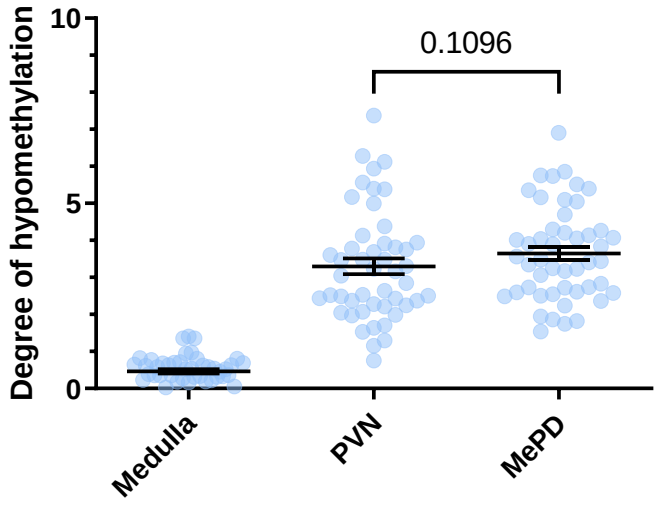

Figure 6. Neuroanatomical differences in AVP hypomethylation (male)

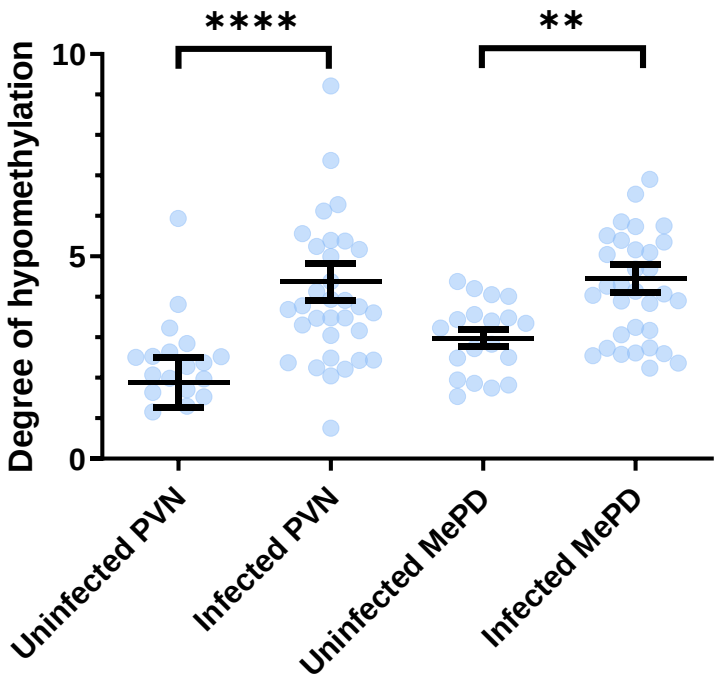

Figure 7. Infection reveals significantly hypomethylated PVN and MePD (males)

When stratifying the previous data (Figure 5A) according to gender, the prior significance between the PVN and the MePD is absent within the male mice population (Figure 6). Hypomethylation of the AVP promoter between the PVN and the MePD is marginally different within male mice (paired t-test, $p = 0.1096$). The degree of hypomethylation within the medulla oblongata also appears unremarkable within the male mice population, indicating that the AVP promoter is significantly methylated (Figure 6).

However, intra-group analysis through stratification against infection status reveals significant differences within the PVN and MePD. The AVP promoter within the PVN of infected male mice is significantly more hypomethylated compared to its uninfected counterpart ($p < 0.0001$). Similarly in the MePD, infected mice exhibit significant hypomethylation as compared to uninfected male mice ($p = 0.0013$). (Figure 7)

Interestingly, the differences in methylation between the PVN and MePD is absent between infected male mice ($p = 0.4143$, not shown in Figure 6), while the MePD exhibits significant hypomethylation as compared to the PVN in uninfected male mice (paired t-test, $p = 0.0319$, not shown in Figure 7).

3.3 - Infected female mice do not exhibit epigenetic changes in the MePD

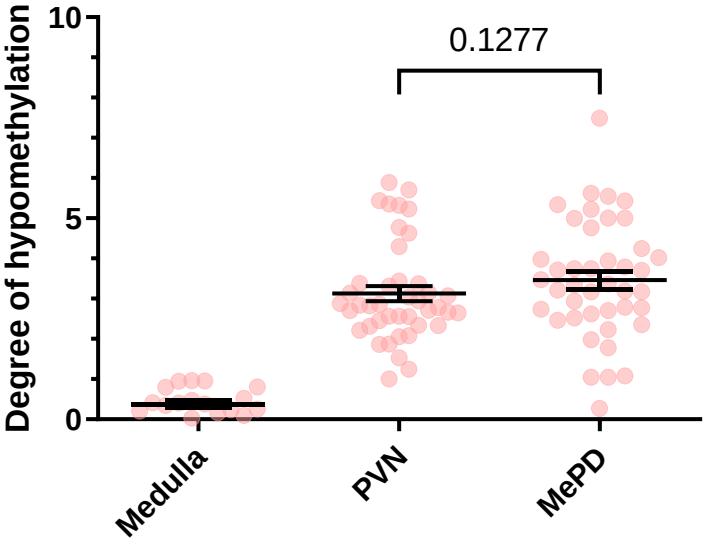

Figure 8. Neuroanatomical differences in AVP methylation (female)

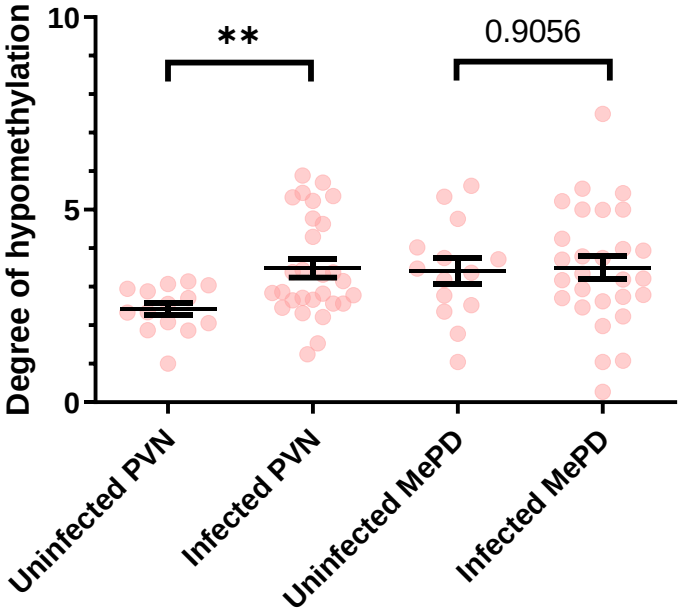

Figure 9. Infection reveals significantly hypomethylated PVN but not MePD (females)

There are marginal differences in methylation between the PVN and MePD in female mice in general (paired t-test, $p = 0.1277$) (Figure 8). The degree of hypomethylation within the AVP promoter of the medulla oblongata appears unremarkable, again demonstrating that transcriptional activity is unlikely in this neuroanatomical landmark due to profound methylation.

An intra-group analysis against infection status reveals significant differences within the PVN. The AVP promoter within the PVN of infected female mice is significantly hypomethylated than the uninfected counterpart ($p = 0.0096$). Contrary to the observation observed in infected male mice, there are no differences between infected and non-infected in methylation of the AVP promoter within the MePD ($p = 0.9056$).

Similar to male mice, there are no significant differences in hypomethylation of the AVP promoter between the PVN and MePD in infected female mice ($p = 0.8645$). However, the differences between the PVN and MePD in uninfected female mice remain significant (paired t-test, $p = 0.0141$).

3.4 - Neuroanatomical differences in AVP methylation attributed to uninfected mice.

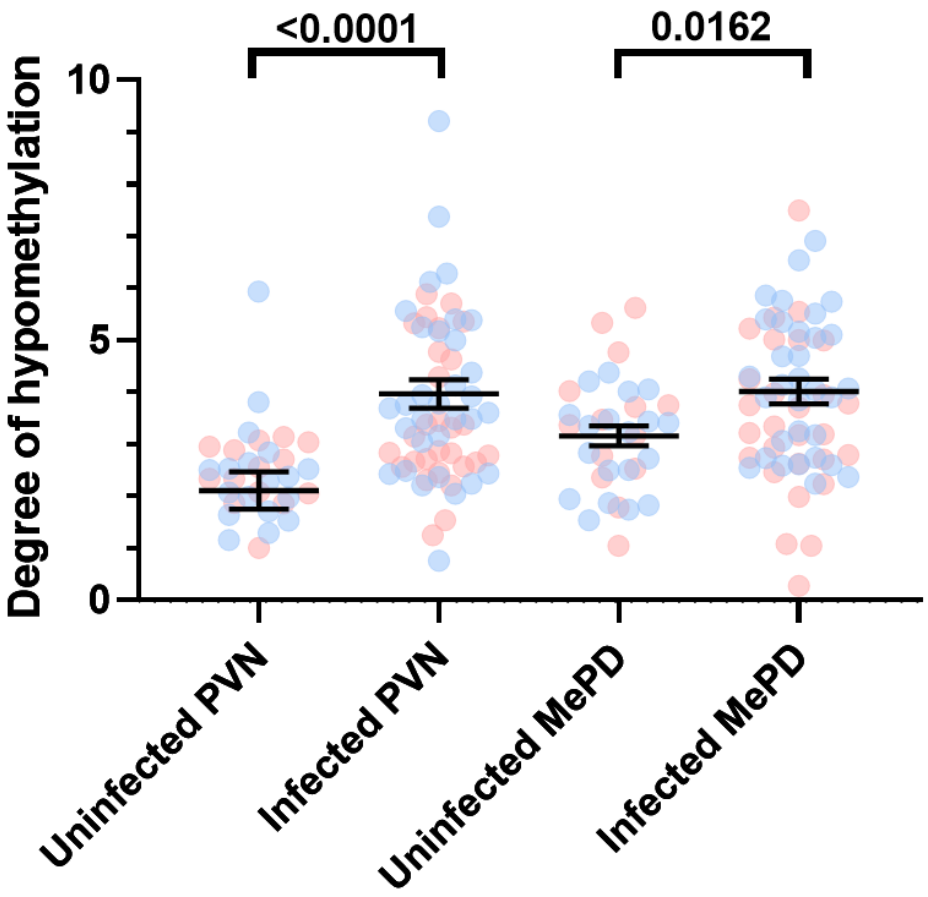

Figure 10. Infection broadly hypomethylated PVN in both gender and the MePD of males (Differences in methylation are reflected as p-values)

With gender and infection status stratified previously in the dataset, it is apparent that mice infected with Toxoplasma in the wild exhibit drastic hypomethylation within the AVP promoter in PVN (Figure 10). Hypomethylation in the AVP promoter of the MePD in the infected population is only observed in males but not females. With reference to the overall findings above (Figure 5A), the neuroanatomical differences between the PVN and MePD in methylation is mainly contributed by the uninfected mice in the sampled population (Figure 10).

3.5 - Infection likely modulates weight and testosterone in wild mice

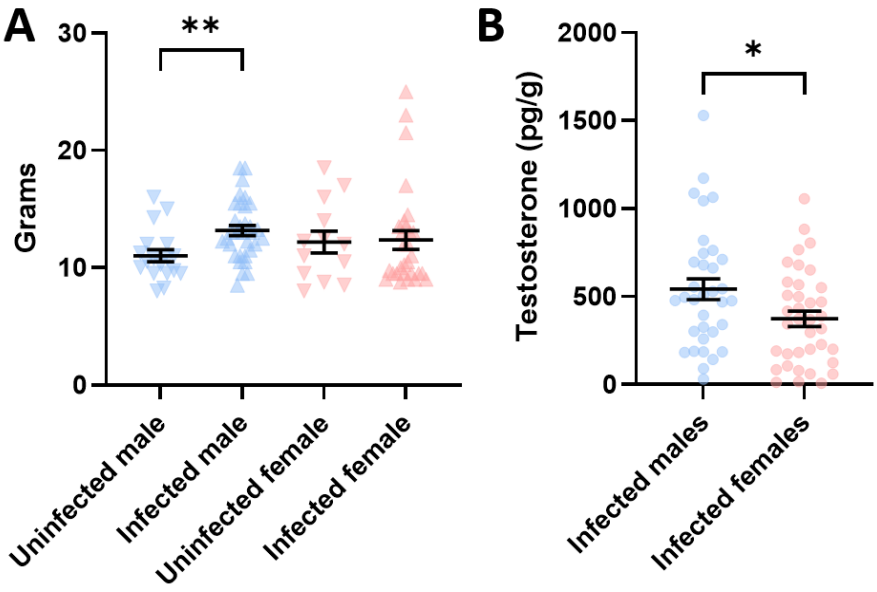

Figure 11. Infected mice exhibited diverse physiology as compared to uninfected. (A) Infected males are significantly heavier than uninfected males captured. (B) Testosterone levels of infected males are significantly higher than infected females.

Of the 95 mice captured and detailed for epigenetic analysis, the physiologies were accounted for such as weights, testosterone and corticosterone levels, and presence of another common parasite, *Taenia taeniaeformis*. Notably, uninfected male mice were significantly lighter ($p = 0.0015$), than their infected counterparts that were not observed within the population of female mice. Unfortunately, due to the unpredictability of sampling in a naturalistic population, the absence of a reliable source of hair obtained from uninfected mice hindered the ability to compare the effects of infection on the levels of testosterone. However, the methodology detailing the extraction and detection reveals significant differences in testosterone levels between infected male mice and infected female mice ($p = 0.0369$).

Discussion

This study leverages a serendipitous call for cat removal on the Dudley peninsula of Kangaroo Island (KI) in Australia. This intervention grants an unparalleled opportunity to observe if the sheer removal of Toxoplasma's definitive hosts may influence its epidemiology and the affliction on its ubiquitous vector and host - the mice. In this thesis, I have explored the laboratory correlation between infection and hypomethylation in the paraventricular hypothalamus and medial posterodorsal amygdala in naturalistic populations, providing evidence that epigenetic modulation is likely the *modus operandi* of Toxoplasma gondii in mice.

Infection with the parasite is widely known to correlate with behavioural ramifications across different species, including humans. However, the mechanism of action behind this phenomenon, ranging from subtle to drastic changes, remains ever elusive. Proposed phenotypes often fail to reproduce the behavioural alterations purported in nature. Behavioural phenotypes can be viewed as a function of negotiations between the myriad uncertainties in day-to-day life, all of which are challenging to recreate faithfully in laboratory animals. Thus, the paradigm at KI allows a pristine representation of negotiation and animal behaviour in the wild, granting invaluable insights into how parasitic behavioural manipulation may coincide and operate in nature.

Herein, we report consistent host epigenetic modifications in wild mice correlating to infection by the apicomplexan parasite (Figure 10). Specifically, we show that the promoter of arginine vasopressin (AVP) is differentially methylated by the status of Toxoplasma infection within different brain regions - the paraventricular nucleus (PVN) of the hypothalamus and the posterodorsal medial amygdala (MePD). The medulla

oblongata is not known for AVP expression and is not remarkable in the levels of methylation within its AVP promoter.

Hypomethylation of the AVP promoter likely indicates transcriptional activity. Typically, experimental infection using laboratory strains of Toxoplasma in rats produces remarkable degrees of hypomethylation on the AVP promoter within the MePD that coincides with a loss of aversion to felid odours[66]. Prior experimentation involving specific overexpression of AVP within the MePD without the concurrence of infection in rats also successfully recapitulated the loss of aversion to cat urine[67]. In parallel with these observations, ablation of the indicated AVP neurons within the MePD augments aversion to cat urine, surmising the fundamental regulation of innate predatory aversion through the mere expression of AVP within the MePD[67]. Interestingly, these behavioural ramifications consequent of infection and its epigenetics are reversed through a simple methyl-rich diet in rats[67]. All of which, exemplifies the enigmatic yet unambiguous role of epigenetics in extended phenotypes of parasitism. To our knowledge, such paradigms of 'extended epigenotype' however, has never been demonstrated in wild naturalistic conditions.

Congruent with our initial understanding, I report significant hypomethylation in the AVP promoter within the MePD concurring with the presence of Toxo infection in nature, and moreover, gender dimorphic (Figure 6 & 8). The transcription of AVP within the MePD has been demonstrated to be steroid-sensitive, specifically to testosterone. Hypomethylation of the AVP promoter within the MePD because of elevated testosterone was demonstrated in earlier experiments[64]. The abundance of androgen receptors reported within AVP-expressing neurons in the MePD recapitulates this understanding[76]. In addition, laboratory rats infected with Toxoplasma exhibit increased testosterone levels coeluting with the loss of aversion to felid odours.

Experimental removal of the gonads recapitulates prominent aversive behaviours and the exogenous supplementation of testosterone within the MePD reverses the innate aversion in gonadectomized animals[94]. Unfortunately, due to the stochastic nature of sampling in the wild, adequate sample sizes may not be achievable for certain stratified sub-groups. As a result, we are unable to deduce if testosterone was indeed augmented in infected mice than the uninfected (Figure 10B). We are, however, able to determine that infected males do indeed possess elevated testosterone levels than infected females (Figure 10B). Ultimately, the gender dimorphic nature of hypomethylation in the AVP promoter within the MePD due to infection strongly suggests and hints at the involvement of the dominating male steroidal hormone, testosterone (Figure 7 & 8).

Infected male mice were also significantly heavier than the uninfected male mice (Figure 11A), although this was not observed among female mice. The weights of the mice may correspond with circulating testosterone levels which would explain the lack of differences between infected and uninfected females. Testosterone augments muscle mass and in turn, can translate to marked increases in weight[95]. However, our line of evidence is purely anecdotal as we have yet to account for the proportion of juveniles to mature mice in the sampled population that may confound the result.

In contrast with laboratory findings[94], we also report a broad increase in hypomethylation of the AVP promoter within the PVN across both genders in the infected mice population. The functional implication of increased hypothalamic AVP was recently demonstrated to be gender dimorphic and responsible for the differential responses to anxiety and social behaviour in female mice[96].

	PVN	MePD
Gender	0.9346	0.3917
Infection	**0.0271 ***	0.8314
Year of capture	0.9088	0.4284
Culling intervention	0.96	0.9802
Gender x Infection	0.2504	**0.0382 ***
Intervention * Year	0.9604	0.9804

Table 1. GLMM estimates of variables and random effects on AVP epigenetics

A linear mixed model was generated to estimate the effects of several identifiable variables and confounders on the epigenetic changes of the AVP promoter of the PVN and MePD (Table 1). Infection weighs in as a significant attribute towards the changes in hypomethylation within the PVN, while gender * infection contributes significantly to the differential hypomethylation observed in the AVP promoter of MePD. In all, the model demonstrated that the effect of epigenetic modification is largely attributed to the concurrence of Toxoplasma infection and is not significantly modified by another measurable stochasticity of nature identified in this thesis.

Future work

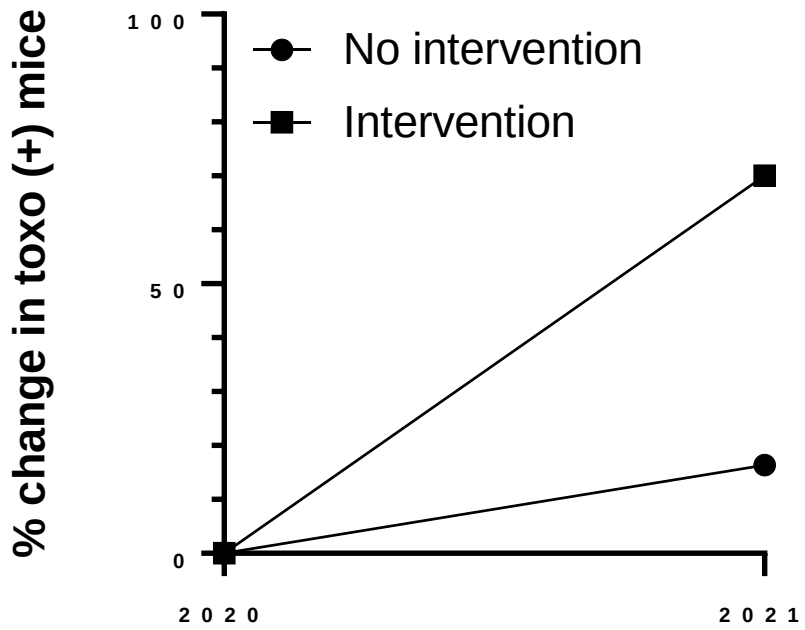

Figure 12. Anecdotal evidence suggests increased transmission of Toxo parasites between rodents observed in farms without interventional felid culling.

With the culling of cats on KI partly motivated by the negative impact of Toxoplasma infection on agricultural livestock[97], it would be interesting to delineate the success of the intervention in the first year. The data is generated as a percentage change between uninfected to infected *({[**2021's no. of infected mice** / **2021 total mice**] - [**2020's no. of infected mice** / **2020 total mice**]} / [**2020's infected mice** / **2020 total mice**] * **100**)*, from 2 different areas, with and without intervention. It is important to understand that only anecdotal evidence suggesting increased Toxo prevalence can be drawn from the rate of infection as the intervention is still ongoing as of writing (Figure 12). Moreover, the reliability of such analysis will benefit proportionately with more time points in this planned longitudinal study.

Toxoplasma gondii can be transmitted through sexual means between rodents. Infected rats were demonstrated to possess increased sexual pheromones and the loss of aversion to felid presence. The culling of the definitive host may alter the ecology to favour parasites that possess high potential for host pheromone production, over the loss of innate aversion. Natural selection would likely act on the plasticity of such extended phenotypes and optimise future strains towards the vertical transmission. Such directed selection because of man-made interventions is more common and expeditious than previously thought. Fishes are gradually decreasing in size due to fishermen releasing smaller fishes back into the seas with the larger sized killed for consumption[98]. Another notable example comes from the evolution of the deadliest fast-acting venom from *Bothrops insularis* as an adaptation to agile migratory birds as the main food source, resultant in the isolation on an island due to rising sea levels[99]. It would thus be interesting to observe the occurrence and time taken for emergence of *Toxoplasma gondii* adapting mechanisms to achieve sexual recombination without its definitive hosts. Moreover, we can also determine the dispensability of the 'fatal attraction' phenotype resulting in increased predation in ecology where felids are gradually absent.

In all, this longitudinal study provides ample opportunities for exploration and perhaps sets the stage to observe one of the crucial processes of life on earth, natural selection and evolution at play.

Conclusion

In ‘The Extended Phenotype’ written by Richard Dawkins, parasitic behavioural manipulation posits the capacity to alter the behaviour of hosts to increase the parasite’s fitness in face of natural selection. Such behavioural manipulation typically castrates the host, leaving them ‘dead’ in the eyes of evolution, i.e. the compromised propagation of the hosts’ genes in favour of the genes from the parasite. Prominent examples include the morbid attraction to water bodies in parasitized crickets to propagate the reproductive cycle of the gordian worm, to the gregarious brine shrimps parasitized by microsporidians turning bright red to entice consumption by the definitive host, a Greater Flamingo. These phenotypes are not of the hosts’ volition, but a reflection and extension of the parasites’ will. However, the medium of information that scripts the manipulated behaviour enacted by the host is often thought to derive directly from the parasite, concerted by genes and molecules.

This thesis surmises and coalesces the concept of epigenotype brought forth in 1942 by Waddington, with our current paradigm of parasitic behavioural manipulation. Specifically, we demonstrated that *Toxoplasma gondii* infection in wild mice correlates with epigenetic modifications similarly observed in sterile laboratory conditions, thereby affirming the biological significance of such parasite-host relationships in nature. The emergence of PVN-AVP hypomethylation in this study is absent in experimental infection models. This highlights the limitations of reductionists’ approach when studying a multi-faceted complex phenomenon such as animal behaviour. Parasitic behavioural manipulation is suggested to derive beyond the mere presence of genes and molecules. Such ‘extended epigenotype’ may provide the

much-needed perspective to future parasite-host associations and in broader inter-intraspecies relationships.

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