

Outcomes of *Leptospira interrogans* Infection in C3H/HeJ and C3H/HeN Mice Strains

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ABSTRACT

Leptospirosis is a significant, underrecognized global zoonosis caused by *Leptospira*. The C3H/HeJ mouse is a well-recognized lab animal model for *Leptospira* studies due to the strain's spontaneous mutation in the toll-like receptor 4 (*Tlr4*) gene and ability to develop leptospirosis. However, the full characterization of *Leptospira* infection in the C3H/HeJ model in comparison to its wild type, C3H/HeNHsd (C3H/HeN), is not available. The objective of this study was to compare the outcomes of *Leptospira interrogans* serovar Manilae infection in C3H/HeJ and C3H/HeN mice. Eight-week-old male and female mice (n = 8) from the strains C3H/HeJ and C3H/HeN were intraperitoneally infected with 6×10^6 cells *Leptospira interrogans* serovar Manilae strain L495. An uninfected group (n = 4) from each strain was kept as negative controls. Mice were observed daily post-infection to evaluate clinical scores and were humanely euthanized at Institutional Animal Care and Use Committee (IACUC)-approved endpoints. Heart, lung, liver, spleen, kidneys, and blood were collected for evaluating organ weights and histopathology, *Leptospira* colonization (PCR and culture), host response (enzyme-linked immunosorbent assay (ELISA) and microscopic agglutination test (MAT), and cytokine evaluation), transcriptomics, and metabolomics. All infected C3H/HeJ and C3H/HeN mice were susceptible to leptospirosis. The C3H/HeN strain had a higher IgM and IgG response after infection compared to the C3H/HeJ strain, and more agglutination was seen in the infected C3H/HeN group. Pro-inflammatory and macrophage-related cytokines were found in statistically significant levels in the spleen, kidneys, liver, and serum. All organs from infected mice were determined as positive via qPCR and culture. *Leptospira* infection was not detected in control mice. More pro-inflammatory- and macrophage-related transcripts were upregulated in infected C3H/HeN mice compared to C3H/HeJ mice, but the metabolite profiles of both mice strains were similar. The C3H/HeN strain had been previously described as resistant to leptospirosis, but overall, our study showed there are little differences in the outcomes of *Leptospira interrogans* serovar Manilae infection in C3H/HeN mice when compared to C3H/HeJ mice.

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CHAPTER ONE

LITERATURE REVIEW AND INTRODUCTION

***LEPTOSPIRA* AND LEPTOSPIROSIS**

***Leptospira* and its Classification**

Leptospirosis is a global, zoonotic disease affecting humans and animals caused by pathogenic species of *Leptospira*. This disease is endemic to tropical areas, but there are over 1 million human cases around the world with about 60,000 associated deaths annually (Costa et al., 2015). The discovery of leptospirosis occurred in 1886 when Adolph Weil described a disease of jaundice with “splenomegaly, renal dysfunction, conjunctivitis, and skin rashes” which was later referred to as Weil’s disease (Adler, 2015a). The cause of Weil’s disease remained relatively unknown until the beginning of the next century. *Leptospira* was first visualized and named in a suspected yellow fever patient in 1907, and in 1916, this spirochete was established as the causative agent for Weil’s disease in Japanese coal miners (Adler, 2015a; Inada et al., 1916; Stimson, 1907).

Leptospira are spirochete bacteria related to *Borrelia* (*B. burgdorferi* causes Lyme disease), *Treponema* (*T. pallidum* causes syphilis), and *Brachyspira* (an animal pathogen). It is a thin, spiral shaped gram-negative-like bacteria with lipopolysaccharides (LPS) on the surface of its outer membrane and characteristic hooked ends. Serological classification of *Leptospira* is based on the LPS structure which is unique to some *Leptospira* strains. The strains with similar serologic reactivity are classified into serovars, and different serovars with cross reactivity are loosely classified into serogroups (Levett, 2015; Wolff. J. W. & Broom, 1954). Approximately 260 different pathogenic serovars of *Leptospira* have been identified (Adler & de la Peña Moctezuma, 2010). Hosts of *Leptospira* are categorized into maintenance hosts and incidental or accidental hosts (Hartskeerl & Terpstra, 1996). Maintenance hosts (mainly animals) are characterized by chronic asymptomatic infection of the kidney with long-term shedding of *Leptospira* in their urine but no development of clinical disease (Bertelloni & Ebani, 2025). Accidental/incidental host (humans and animals) infection leads to development of clinical disease with short-term shedding in the urine (Ellis, 2015). Some serovars have a broad host

range while other serovars have developed some host adaptation. The most common serovars seen worldwide include Copenhageni, Grippityphosa, Icterohaemorrhagiae, Pomona, Australis, and Ballum, and the most common host species are brown and black rats, cattle, dogs, and pigs (Hagedoorn et al., 2024). For example, dogs are typically found carrying the serovar Canicola while Grippityphosa had quite a broad host range (Adler & de la Peña Moctezuma, 2010; Hagedoorn et al., 2024).

The discovery of genetic features of *Leptospira* led to reclassification, and now, a combination of genetic and serological classifications is used to identify the *Leptospira* species (Levett, 2015). Approximately 64 different species, including common pathogenic species *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*, of *Leptospira* have been identified and classified into different pathogenic (P) and saprophytic (S) subclades: P1, P2, S1, and S2 (Vincent et al., 2019). S1 and S2 *Leptospira* are saprophytic, cause no disease, and persist in the environment; the pathogenic species of the P2 subclade can also live in the environment but contain virulent factors (Giraud-Gatineau et al., 2024; Vincent et al., 2019). Species in the P1 subclade are further categorized in P1+ (high virulence) or P1- (low virulence) (Giraud-Gatineau et al., 2024). P1+ species are mainly host-adapted, cause the most disease in humans and animals, and have genetic signatures that help them evade the host immune system better than other species of *Leptospira*; one key P1+ species is *L. interrogans* (Giraud-Gatineau et al., 2024).

Human and Animal Leptospirosis

Risks for human leptospirosis are associated with occupations involved in agriculture, animal production, or outside activity; tropical regions with a high amount of rainfall; flooding; areas with a large presence of rodents; and outdoor recreational activities (Adler & de la Peña Moctezuma, 2010; Haake & Levett, 2015). Humans are considered accidental hosts and do not chronically carry *Leptospira*, but rodents, specifically rats and mice, are considered the major reservoir hosts of this bacteria (Ellis, 2015; Faine, 1999; Hagedoorn et al., 2024). *Leptospira* transmission mainly occurs through skin and mucous membranes (Adler & de la Peña Moctezuma, 2010). The bacteria then will disseminate through the blood and colonize the renal tubules of the kidneys where it can be shed through urine in chronic carriers (Adler & de la Peña

Moctezuma, 2010; Murray, 2015). Therefore, direct contact with the urine of an infected animal or soil and water contaminated with the urine of an infected animal is another major risk factor for contracting leptospirosis.

The presentation of leptospirosis can vary based on the serovar that infects the host, but there are some common symptoms found in this disease (Adler & de la Peña Moctezuma, 2010). The symptoms of human leptospirosis are broad and similar to those of other febrile diseases. The most basic presentation of leptospirosis includes nonspecific flu-like and/or gastrointestinal symptoms (Haake & Levett, 2015). More severe presentations of leptospirosis include Weil's disease (jaundice and renal failure), liver failure, or pulmonary hemorrhage which can lead to death (Adler & de la Peña Moctezuma, 2010; Haake & Levett, 2015). Additionally, miscarriages and stillbirths can occur in pregnant humans with leptospirosis (Cárdenas-Marrufo et al., 2016). The treatment of leptospirosis is simple if diagnosed early enough. For humans, penicillin, ampicillin, and doxycycline are commonly used antibiotics for treatment (Haake & Levett, 2015; Petakh et al., 2024). More intensive and supportive care might be needed for severe cases of leptospirosis (Faine, 1999; Petakh et al., 2024).

Leptospirosis in animals varies based on species, but most forms of transmission are similar to humans. Pathogenic *Leptospira* has been found in the reproductive tracts of horses, bovine, and sheep which raises concerns of venereal transmission (Hamond et al., 2013; Lilenbaum et al., 2008; Loureiro et al., 2017). Reproductive loss is one of the major concerns of leptospirosis in horses, swine, and cattle that lead to major economic losses (Ellis, 2015). More specific presentations of leptospirosis in animals influenced by serovars include agalactia in Hardjo-infected dairy cows; bloody urine, hemorrhaging, jaundice, and renal failure in Kennewicki-infected young swine; equine recurrent uveitis in Kennewicki- or Grippotyphosa-infected horses; and an icteric form of the disease coupled with liver, kidney, and/or vascular issues in Pomona- or Icterohaemorrhagiae-infected dogs (Ellis, 2015). Treatment of animal leptospirosis is similar to humans where antibiotics such as penicillin and streptomycin are the main choice of treatment (Ellis, 2015).

Leptospira diagnosis is done using bacterial detection via polymerase chain reaction (PCR), bacterial culture, or the detection of immune response to the bacteria via MAT and

ELISA (Ellis, 2015; Haake & Levett, 2015). PCR detection of *Leptospira* can be done with housekeeping or pathogen-specific genes like 16s rRNA or *lipL32* (Ellis, 2015; Haake & Levett, 2015; Stoddard et al., 2009). The benefit of PCR is its high sensitivity, ability to detect DNA in a variety of sample types, and quantification of *Leptospira* in certain sample types, but one downside is its inability to identify specific serovars (Haake & Levett, 2015). Detection of *Leptospira* can be done with samples from body fluids (blood or urine) or tissues (kidney or liver), but treatment, carrier status, and the duration of the disease can affect bacteria isolation and PCR results (Ellis, 2015; Haake & Levett, 2015). Cultures used for diagnosis may need to be maintained for months before any detectable level of *Leptospira* is seen, and a negative culture does not rule out infection (Ellis, 2015; Haake & Levett, 2015). MAT is considered the reference serologic test. Although reported to have high specificity for *Leptospira*, this test has a low sensitivity due to the possibility of low antibody titers early in infection and its inability to distinguish between active infection or vaccine-induced immunity (Adler & de la Peña Moctezuma, 2010; McCreight et al., 2025). ELISA tests for *Leptospira* are developed for the detection of antibodies against *Leptospira* in infected animals, but this diagnostic test faces sensitivity issues similar to MAT (Ellis, 2015; Haake & Levett, 2015).

No vaccines for leptospirosis in humans exist, but there are some vaccines available for dogs, swine, and cattle. The animal vaccines are limited because they only offer short term immunity against only a handful of pathogenic serovars and are often geographically specific (Adler & de la Peña Moctezuma, 2010). Available vaccines for animals are typically heat-killed bacterins, but scientists shifted to researching recombinant proteins in vaccines that can offer protection against a variety of pathogenic serovars (Adler, 2015b).

Leptospirosis remains an underrecognized, global zoonotic disease. The similarity of its symptoms to other diseases makes it difficult to identify early infection for the best treatment outcomes. Additionally, the lack of a vaccine that can protect against all pathogenic serovars and lack of better diagnostic methods to identify the disease at the point-of-care makes it difficult to tackle this disease. The lack of knowledge about the pathogenesis of *Leptospira* remains one of the biggest issues of leptospirosis. The mechanisms of how the bacteria can colonize some animals and cause no disease (maintenance/reservoir hosts) while infecting humans and other

animals leading to mild to severe disease or even death remains relatively unknown. The best way to investigate the pathogenesis of any disease is through animal models. Animal models are a great way to investigate pathogens by mimicking *in vivo* environments in a controlled, experimental setting. Various animal models have been used to study *Leptospira* pathogenesis, but the bacteria's unique host-adaptation and serovar diversity make the use of animal models quite difficult.

ANIMAL MODELS OF LEPTOSPIROSIS

Animal models have been used in *Leptospira* research for over a hundred years and have been critical to some of the major discoveries in this field (Gomes-Solecki et al., 2017; Inada et al., 1916). Hamsters and guinea pigs were the favored animal models for *Leptospira* research for a long time, but scientists have begun to use more mice models for their research (Gomes-Solecki et al., 2017). The biggest challenge for using any rodent model is finding a model that can display various forms of infection instead of natural resistance and carrier status. Many rodents, specifically mice and rats, are reported to be nonsusceptible to leptospirosis (Bonhomme & Werts, 2022).

Guinea Pig and Hamster Models of Leptospirosis

Guinea pigs have been crucial to major discoveries in *Leptospira* research in the 20th century (Padilla-Carlin et al., 2008). In 1915, guinea pigs infected with the blood of Weil's disease patients developed symptoms of "albuminuria, conjunctival congestion, jaundice, and hemorrhages" within seven days and helped identify *Leptospira* as the cause of this disease in humans (Inada et al., 1916). The guinea pig remained dominant in early *Leptospira* research until additional rodents, including the golden Syrian hamster, were found to be susceptible to *Leptospira* (Morton, 1942; Packchanian, 1940).

Guinea pig models of leptospirosis appear to reflect some symptoms of human leptospirosis. When male guinea pigs were infected with a 1 ml liver and kidney suspension of a pathogenic species of *Leptospira* named "*Leptospira icterohaemorrhagiae*" at the time, the animals displayed clinical signs such as hyperthermia, jaundice, and mucosal hemorrhaging observed during the course of the study and during necropsy (Higgins & Cousineau, 1977). In another study, researchers remarked that necrosis and hemorrhaging found in the organs of

guinea pigs infected with 10^7 or 10^8 cells of *L. interrogans* serovar Copenhageni was like severe leptospirosis in humans (Silva et al., 1995). Guinea pigs are a useful model for mimicking some of the presentation of human leptospirosis, but the lack of molecular reagents plus the limited genetic tools available for this model limit its usefulness in *Leptospira* research (Padilla-Carlin et al., 2008).

Golden Syrian hamsters are similar to guinea pigs with a lack of molecular reagents and genetic tools for any type of infectious disease research. However, this model has been one of the most common animals used in *Leptospira* research. One benefit of using hamsters is the remarkable correlation between challenge dose and days to 50% survival (Haake, 2006). Therefore, it can be easier to predict when the onset of symptoms or mortality might occur. However, each new challenge dose needs to be evaluated in hamsters because virulence can vary significantly between strains. For example, the lethal dose (LD_{50}) of intraperitoneally infected *L. kirschneri* serovar Grippotyphosa strain RM52 was identified to be less than 10 cells between ten and eleven days while hamsters subcutaneously infected with 10^8 *L. interrogans* serovar Icterohaemorrhagiae strain Verdun survived up to six days (Haake, 2006; Haake et al., 1999; Truccolo et al., 2002). The histopathology of leptospirosis in hamsters is relatively similar to the presentation in guinea pigs and humans. Intraperitoneal infection with 10^5 or less *Leptospira* cells from a variety of virulent strains led to damage within the kidney tubule cells, dissociation of hepatic cells, and lung hemorrhaging (Silva et al., 2008). Additionally, control hamsters intraperitoneally infected with 10^8 *L. interrogans* serovar Pomona strain NVSL 1427-35-093002 were found to have either moderate or severe renal lesions such as tubulointerstitial nephritis and glomerular shrinkage (Palaniappan et al., 2006). Strain-specific differences in survival and infection outcome appear to be emphasized in the golden Syrian hamster model. Some hamsters survive for multiple days with a high dose of a potentially virulent strain of *Leptospira* while other virulent strains can lead to mortality in only a few days (Haake, 2006; Haake et al., 1999; Palaniappan et al., 2006; Silva et al., 2008; Truccolo et al., 2002).

While hamsters and guinea pigs are valuable in *Leptospira* research, the lack of reagents and genetic tools poses an issue to properly study the pathogenesis of *Leptospira*. Additionally, research implementing the use of these animals is heavily regulated across the world. In the

United States of America, the Animal Welfare Act (7 U.S.C. § 2131–2159) imposes strict guidelines and standards on the facilities and care of these animals, with other countries having similar legislation (Vasbinder & Locke, 2017). Additionally, hamsters are banned in certain states (Hawaii) and countries (Australia). Now, hamsters are commonly used to assess or maintain the virulence of challenge strains of *Leptospira* (Pereira et al., 1998; Silva et al., 2009).

Rat Models of Leptospirosis

Rats are relatively resistant to leptospirosis and are one of the major carriers of the bacteria (Hagedoorn et al., 2024). However, there have been some studies looking into the pathogenesis of *Leptospira* in laboratory rats. In one study trying to investigate if the natural renal carrier state can be reproduced experimentally, *Leptospira* was able to be detected in the renal tubules of infected Wistar rats, and the only lesion found, interstitial nephritis, was observed in less than half of the rats (Tucunduva de Faria et al., 2007). Experimental *Leptospira* infection seems to only produce a long-term chronic renal carrier state, but the establishment of an asymptomatic carrier model is useful for studying the pathogenesis of *Leptospira* within reservoirs (Athanzio et al., 2008; Nally, Chow, et al., 2005; Nally et al., 2018; Tucunduva de Faria et al., 2007; Zilber et al., 2016).

Mouse Models of Leptospirosis

Mice have been a particularly popular animal model to use in any type of *in vivo* experiment. There are a plethora of reagents and molecular tools solely available for mice. Mouse models can practically be customized to fit the needs of the researcher with inbred, transgenic, and knockout models. Additionally, the genome of mice was fully sequenced alongside the human genome to offer a great basis of comparison (Waterston et al., 2002). One major barrier that prevents the widespread use of mice in *Leptospira* research is that mice are considered reservoir hosts of *Leptospira*, so it is hard to reproduce disease in this model without a high dose, young mice, or immune-compromised status.

Some of the major inbred strains used in *Leptospira* research are A/J, CBA, BALB/c, C57BL/6, and C3H mice (Gomes-Solecki et al., 2017). A/J mice have been used to investigate the complement system, and they showed minimal splenomegaly and suffered transient weight loss after challenge with 10^8 *L. interrogans* serovar Kennewicki strain Pomona Fromm (Bavia et

al., 2019). High doses of *Leptospira* are commonly used in experiments with immunocompetent mice. CBA and BALB/c mice only died from an infection of 4×10^8 *L. interrogans* serovar Pomona (strain unknown) after prior treatment with cyclophosphamide to immunocompromise the mice (Adler & Faine, 1982). Other studies involving BALB/c mice show evidence of colonization and significant cytokine and chemokine responses after infection but lack significant histopathological lesions and clinical disease (da Silva et al., 2012; Silva et al., 2009; Silva et al., 2020). C57BL/6 is one of the most common inbred mouse strains used in all types of research. For *Leptospira* research, these mice have been used to investigate the dissemination of bacteria and understand cytokine profiles after stimulation of their macrophages (Shetty et al., 2022; Viriyakosol et al., 2006). All the previously mentioned strains were proven to be resistant to leptospirosis with BALB/c mice showing the most resistance by evidence of being the least colonized with no tubulointerstitial nephritis found in any infected mouse from this group (Santos et al., 2010). Even though the most common inbred mice strains show resistance to leptospirosis, immuno-compromised mouse models have shown susceptibility and response to *Leptospira*.

Various transgenic mice have been used to understand the molecular pathways of *Leptospira* pathogenesis. A double knockout of two major toll-like receptors (TLRs), TLR2 and TLR4, involved in *Leptospira* pathogenesis led to mortality four to six days after infection with 2×10^8 *L. interrogans* serovar Copenhageni strain Fiocruz which was significantly different from the mortalities observed in TLR4 knockout mice (Chassin et al., 2009). Macrophages derived from TLR knockout mice have been used to characterize the pathway and cytokine response to *Leptospira* infection and LPS stimulation (Bonhomme et al., 2020; Viriyakosol et al., 2006).

Other immunocompromised mice such as C3H/SCID mice have been sparingly used in *Leptospira* research (Nally, Fishbein, et al., 2005). However, there is one strain of mice that has become increasingly common to use in pathogenesis studies. The C3H/HeJ mouse was identified to be susceptible to leptospirosis due to its spontaneous TLR4 mutation (Hoshino et al., 1999; Pereira et al., 1998; Viriyakosol et al., 2006). This is one of the few inbred strains of mice that show consistent susceptibility to leptospirosis (Abil et al., 2025; Nair & Gomes-Solecki, 2020; Nally, Fishbein, et al., 2005; Richer et al., 2015). C3H/HeJ mice present with leptospirosis

through a variety of external and internal clinical signs such as piloerection, lethargy, jaundice, hypothermia, weight loss, lung hemorrhages, inflammation, and nephritis (Abil et al., 2025; Nally, Fishbein, et al., 2005; Prêtre et al., 2011; Richer et al., 2015). The C3H/HeJ mouse's *tlr4* gene mutation and susceptibility to leptospirosis also made the model useful for treatment and vaccine studies (Barbosa et al., 2024; Chaurasia et al., 2022; Koizumi & Watanabe, 2004; Kundu et al., 2024; Potula et al., 2017).

The C3H/HeJ mouse model seems to address many of the issues that other animal models of *Leptospira* had. Both sublethal and lethal models of disease can be produced for a variety of experimental applications (Viriyakosol et al., 2006). C3H/HeJ mice have promising use for *Leptospira* research, but some issues with this model remain such as inconsistencies in survival due to age *Leptospira* strains or dose need to be addressed so that reproducibility of this model can be ideal (Koizumi & Watanabe, 2003; Pereira et al., 1998). For this model to be effective for continued use, more research needs to be done to fully explain and characterize the outcomes of *Leptospira* infection.

PURPOSE OF STUDY AND SPECIFIC AIMS

Studying the pathogenesis of *Leptospira* is critical to improve the diagnostic methods and prevention strategies against this zoonosis (Adler, 2015b; Ellis, 2015; Haake & Levett, 2015). C3H/HeJ mice prove to be a great model for investigating leptospirosis, but the understanding of differences in infection outcomes within its wild type remains limited. Additionally, the differences in the molecular mechanisms of *Leptospira* between infected and uninfected mice have not been fully characterized. We believe the assessment of infection, host response, and multi-omics analysis will give a better insight into the pathogenesis of *Leptospira* in C3H/HeJ mice. Therefore, the overall goal of this study was to compare the outcomes of infection in C3H/HeJ mice and its wild type, C3H/HeNHsd (from now on referred to as C3H/HeN) mice.

We hypothesize that the outcomes of *Leptospira* infection in C3H/HeJ and C3H/HeN will be different.

We tested this hypothesis using the following specific goals.

1. To compare clinical outcomes infection in C3H/HeJ and C3H/HeN mice following infection with *Leptospira interrogans*.

2. To evaluate the immune response in both strains to infection.
3. To analyze and compare the total transcriptome profiles of both mice strains to study the differentially expressed genes in the infected and control animals.
4. To analyze and compare the differences in the metabolites produced by infected versus uninfected animals through untargeted metabolomics.

The clinical, immunological, transcriptomic, and metabolic profiles will provide a complete picture of *Leptospira* infection in both mice models. Coupling clinical signs and immunological findings with transcriptomic and metabolic factors will help us and the broader *Leptospira* scientific community have a better understanding of what occurs during leptospiral infection in the mouse models used in this study. Additionally, the *Leptospira* serovar used for this study, Manilae, has been shown to cause lethal disease in C3H/HeJ mice (Koizumi & Watanabe, 2003, 2004). However, the L495 strain of this serovar has not been previously used to challenge C3H/HeJ and C3H/HeN mice, introducing a novel insight into the understanding of leptospirosis in these mouse models.

CHAPTER TWO

MATERIALS AND METHODS

LEPTOSPIRA STRAIN AND CULTURE

A low passage of *Leptospira interrogans* serovar Manilae strain L495 (LIM) (kindly provided by Dr. Andre Grassmann, University of Connecticut) was used for experimental infections. LIM was previously isolated from an infected C3H/HeJ mouse kidney, passaged once *in vitro*, and stored in liquid nitrogen. The culture was thawed three weeks before challenging mice and passages were carried out in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) media (BD, NJ, USA) supplemented with *Leptospira* Enrichment EMJH (BD, NJ, USA) at 29°C. The culture was passed twice in the same media formulation made with endotoxin-free water. Subcultures were done with 5 µl of the previous culture inoculated into 4.5 ml of media. The growth of *Leptospira* in cultures was assessed via dark-field microscopy. Potential bacterial contamination in the cultures was ruled out using subcultures in Columbia agar with 5% sheep blood (Remel™, KS, USA). Absence of *Mycoplasma* contamination was confirmed using a *Mycoplasma* PCR Detection Kit (Abcam, UK). The *Leptospira* counts were enumerated using a Petroff-Hausser chamber under dark-field microscopy. The *Leptospira* dose was adjusted to 6×10^6 LIM cells per 100 µl of inoculum.

ETHICS STATEMENT AND ANIMAL CHALLENGE STUDY

All animal experiments were carried out complying with an approved University of Tennessee Institutional Animal Care and Use Committee protocol (IACUC protocol #2968-0523 A5.)

Eight-week-old mice C3H/HeJ (The Jackson Laboratory, ME, USA) and C3H/HeN (Inotiv, IN, USA) mice (equal number of males and females) were purchased. Mice were acclimatized for three days before inoculation. Mice were housed separately based on sex and strain (four mice per cage in challenge groups and two mice per cage in control groups) in a standard ABSL-2 laboratory. Mice (n = 8 per group) were sedated with open-drop isoflurane and intraperitoneally challenged with 100 µl of 6×10^6 LIM in EMJH media. Four mice from each strain were kept as untreated, uninfected controls. Sample size was determined by Fisher's exact

test with 99% power in the infected groups and an appropriate number of mice in the control group for statistical significance.

Evaluation of Clinical Signs, Survival Rates, and Lesions

Temperature, weight, and clinical score were recorded at least once daily using an IACUC-approved clinical scoring criteria developed in the laboratory listed in **Appendix 1**. Once mice were given a clinical score of 2, they were evaluated every two hours to assess if their condition further deteriorated. At clinical score 3, mice were humanely euthanized by an overdose of open-drop isoflurane followed by confirmation using a secondary method (cervical dislocation).

Sample Collection

We collected blood before the challenge and at the time of euthanasia or death from the submandibular vein while mice were under open-drop isoflurane anesthesia. Sera were separated by leaving blood samples overnight at 4°C then centrifuging at 10,000 x g at 4°C and collecting the supernatant (sera). Sera was stored at -80°C until further evaluation. All mice were necropsied immediately after death or euthanasia. We weighed the heart, lungs, liver, spleen, and kidneys of all mice. Fresh tissue was collected for PCR and *Leptospira* culture. For real-time PCR, a section of fresh tissue weighing 16 to 155 mg from each organ was placed in a microcentrifuge tube and stored at -80°C for further evaluation. Additionally, an aliquot of each collected organ was stored in 1 mL of RNeasy Lysis Buffer (Qiagen, MD, USA) in a microcentrifuge for RNA extraction and transcriptomic analysis. Another section of each organ was placed in a tissue cassette and fixed in 10% neutral buffered formalin for histopathology. A section of liver, kidney, and spleen were placed in a microcentrifuge tube and stored at -80°C for further processing for cytokine analysis. A piece of liver weighing between 69 and 83 mg was stored liquid nitrogen for metabolomic analysis. Lastly, pieces of fresh kidney for each mouse were used for *Leptospira* culture. Any leftover organ samples were stored at -80°C.

Histopathology

The formalin-fixed tissues were processed for histopathological assessment with routine hematoxylin and eosin (H&E) staining at the UTCVM pathology services and evaluated

microscopically by two American College of Veterinary Pathologists (ACVP) board-certified veterinary pathologists.

EVALUATION OF IMMUNE RESPONSE IN MICE

Enzyme-Linked Immunosorbent Assay (ELISA)

96-well microplates (Thermo Fisher Scientific, MA, USA) were coated with 100 µl of 1.2×10^8 *Leptospira interrogans* extract diluted in ELISA Reaction Buffer (bioWORLD, OH, USA) and incubated at 4°C overnight. Plates were washed three times with PBS-T and then blocked with non-fat dry milk in 0.05% PBS-T. Plates were washed three times again and incubated with mouse sera diluted 1:100 in blocking buffer at 37°C for one hour. Following three washes, the plates were incubated with horseradish peroxidase-conjugated secondary anti-mouse IgM or IgG diluted 1:5000 in blocking buffer in 37°C for one hour. Following five washes, the plate was incubated with 1-Step™ Ultra TMB-ELISA (Thermo Fisher Scientific, MA, USA) for 15 minutes in the dark at room temperature and the reaction was stopped with ELISA Stop Solution (Invitrogen, MA, USA). Plates were immediately read by colorimetric analysis at absorbance of 450 nm with the BioTek 800 TS (BioTek, VT, USA).

Microscopic Agglutination Test (MAT)

25 µl of 1:10 diluted sera pooled from each group was placed in a flat bottom 96-well microplate. 25 µl of a five-day-old LIM culture was mixed in each well containing pooled sera. Polyclonal rabbit antibody against LIM and PBS were used as positive control and negative control respectively. The plate was incubated for 1.5 hours at 29°C and then evaluated for the presence of agglutination by darkfield microscopy using a 5x long objective and a dry dark field condenser. Each well was evaluated for the presence of agglutination.

Cytokine Assay

Mouse kidney, spleen, and liver were processed in the Bio-Plex® Cell Lysis Kit (Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer's protocol. Pools for liver, kidney, spleen lysates and sera were formed according to **Appendix 2**. Pools from organ lysates and sera were diluted 1:4 and used in the Bio-Plex® Pro Mouse Cytokine 23-plex Assay kit (Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer protocol. Each pool was run in technical duplicates alongside standards for each sample type (serum and organ). The

plate from this assay kit was run in the Luminex 200 (Diasorin, IT) and average concentration (in pg/ml) of the pool duplicates was given.

LEPTOSPIRA DETECTION

***Leptospira* Real-Time PCR**

Genomic DNA was extracted from the organs using the Quick DNA Miniprep Plus kit (Zymo, CA, USA). The *lipl32* primers and a Taqman probe were used for the real-time PCR assays adapted from Stoddard et al. (2009). Cycle threshold (Ct) values are used to represent the relative *Leptospira* burden in liver, kidney, spleen, lung, and heart. Genomic DNA extracted from LIM was used as a positive control and nuclease free water was used as a negative control. No reference gene as an internal control was used.

***Leptospira* Culture**

Fresh kidney pieces from each mouse were homogenized by passing through a 3 mL syringe into 4.5 mL *Leptospira* EMJH media and incubated at 29°C for one hour. 500 µl of each culture was placed in another tube of 4.5 mL *Leptospira* EMJH media. Cultures were first evaluated under dark-field microscopy at least three days later and then twice each week for the presence of live *Leptospira*. Cultures were filtered after one week to prevent contamination.

Confirmation of *Tlr4* Mutation Status in Mice Strains

The toll-like receptor 4 gene (*tlr4*) was amplified from DNA isolated from uninfected mice livers by conventional PCR using the protocol described by Krangvichian et al. (2023). The presence of the amplified product was confirmed via conventional PCR. The PCR products were cleaned up using QIAquick PCR Purification Kit for PCR Cleanup (Qiagen, DE). The purified products were sequenced by a commercial sequencing service (Eurofins Genomics LLC, KY, USA) to confirm the presence of the *tlr4* point mutation. Sequences were aligned using Clustal Omega to identify the *tlr4* point mutation (Hoshino et al., 1999).

TRANSCRIPTOMICS

Total RNA was extracted from liver stabilized in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, MA, USA) with the RNEasy Mini Kit (Qiagen, DE). Samples were homogenized using a syringe and needle. Quantification was done on the Nanodrop 2000 and

Qubit 4 Fluorometer (Thermo Fisher Scientific, MA, USA) in ng/μl. Transcriptome sequencing on three samples per group was performed by a commercial service (SeqCenter, PA, USA). Total RNA was sequenced after ribodepletion using mouse and *Leptospira*-specific probes. The samples were sequenced targeting 100 million reads for the controls and 200 million reads for infected animals.

METABOLOMICS

Sample Collection and Metabolomics Extractions

Liver samples were removed from liquid nitrogen and pulverized with a handheld motorized tissue grinder and pestles (Thermo Fisher Scientific, MA, USA) before fully thawing. The ground samples were immediately flash frozen in liquid nitrogen and placed in -80°C for storage until further processing.

Samples were sent to and processed at the Biological and Small Molecule Mass Spectrometry Core (BSMMSC), University of Tennessee, Knoxville, TN (RRID: SCR_021368), and this research is based in part on data obtained from the BSMMSC. Water-soluble metabolites were extracted using a previously described acidic acetonitrile extraction procedure (Rabinowitz & Kimball, 2007). Briefly, 1.5 ml of pre-chilled extraction solvent (40:40:20 HPLC grade methanol: acetonitrile: water with formic acid at a final concentration of 0.1 M) was added to each sample and kept at -20°C for 20 minutes. Samples were then centrifuged (13,300 x g, 5 min, 4°C), and supernatants containing polar metabolites were collected. Samples were re-extracted with 200 μl of extraction solvent, and the resulting supernatant was combined and evaporated under a stream of nitrogen. Metabolites were suspended with 300 μl of LCMS-grade water prior to mass analysis.

Ultra-High Performance Liquid Chromatography-High resolution Mass Spectrometry (UHPLC-HRMS) Metabolomics Analysis

A previously described UHPLC-HRMS-based untargeted metabolomics analysis was performed using an UltiMate 3000 RS autosampler (Dionex, CA, USA), Synergi 2.6 μm Hydro RP column (100 mm × 2.1 mm, 100 Å; Phenomenex, CA, USA), an UltiMate 3000 pump (Dionex), and Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, MA, USA) (Lu et al., 2010). The chromatographic method included a 25 min gradient elution, reverse phase

ion-pairing method using a water:methanol solvent system, and a tributylamine ion pairing reagent for chromatographic separation (Lu et al., 2010). Metabolites in the chromatographic eluent were ionized via negative mode electrospray ionization (ESI) prior to full scan mass spectral analysis. Raw mass spectral files were converted to mzML files using a package from ProteoWizard, msConverter (Martens et al., 2011). These files were then imported into an open-source software, metabolomics analysis and visualization engine (El-MAVEN) where metabolites were manually identified using an in-house library based on exact mass (± 5 ppm) and retention time (± 2 min) (Agrawal et al., 2019; Clasquin et al., 2012; Zaparte et al., 2024). Metabolite peaks were integrated and raw peak intensities for identified metabolites were exported from El-MAVEN to a csv (comma-separated values) file. Prior to statistical analysis, raw spectral data were normalized by mass of sample used for extraction.

STATISTICAL ANALYSIS

The basic statistical analyses were performed in GraphPad Prism (GraphPad Software, CA, USA). The Gehan-Breslow-Wilcoxon test was used to analyze the difference in survival between groups. A two-way ANOVA with Tukey's multiple comparison test was used to analyze the difference in antibody average optical density levels and average cytokine concentration. An ordinary one-way ANOVA with Šídák's multiple comparison test was used to analyze the difference in heart, lung, liver, kidney, and spleen weights.

All transcriptomic data analysis was performed by a collaborating scientist at INDICASAT, Panama City, Panama. HISAT2 (version 2.1.0) was employed to map cDNA-derived sequencing reads from both uninfected and infected tissue samples onto the mouse reference genome (Kim et al., 2015). Default parameters were applied for paired-end, non-strand-specific RNA-Seq data. The mouse genome (mm10, build GRCm38) was obtained from the UCSC Genome Browser (<http://genome.ucsc.edu/>). Gene expression levels were estimated from the mapped reads using FeatureCounts (version 1.6.3) (Liao et al., 2013). Genes deemed non-expressed, defined as those with zero or only a single read count across all samples, were excluded before further analysis.

Metabolomics data was analyzed by collaborating scientists in the Department of Chemistry and Biological and Small Molecule Mass Spectrometry Core at the University of

Tennessee, Knoxville, Tennessee, United States. MetaboAnalyst 6.0 was used for statistical analysis and data visualization (Pang et al., 2024). The weight-normalized data were filtered via interquartile range (IQR) and normalized using a log transformation and mean scaling.

Quality Control and Differential Gene Expression Analysis

To explore sample relationships in the transcriptomic analysis, read counts were normalized to correct for sequencing depth differences and subsequently $\log^2$ -transformed. Variance stabilization was performed using the variance stabilizing transformation (VST) algorithm, following the approach suggested by Anders et al. for negative binomial-distributed data (2010). Euclidean distances were computed based on transformed data, and sample relationships were visualized through principal component analysis (PCA).

Differential expression analysis was conducted on raw read counts using the DESeq2 R package (version 1.44.0) (Love et al., 2014). The DESeq2 algorithm normalizes for sequencing depth variations, estimates gene-specific dispersion values, and fits a generalized linear model to the data. Estimated model coefficients were visualized using log-ratio and mean-average (MA) plots. Genes were considered differentially expressed if they had a Benjamini-Hochberg (BH) adjusted p-value < 0.05 . For functional enrichment analysis, only genes with a $\log^2$ fold change ($\log^2\text{FC}$) absolute value of at least 0.58 were included. The top 50 most significant differentially expressed genes (adjusted p-value < 0.05) between uninfected and infected samples in both mouse strains were visualized as a heatmap based on the RNA-Seq counts normalized by size factor and variance stabilizing transformation (VST) using the pheatmap R package (version 1.10.12) (Kolde, 2018).

CHAPTER THREE

RESULTS

CLINICAL SIGNS, SURVIVAL RATES, AND LESIONS

Following intraperitoneal infection, infected C3H/HeN mice developed clinical signs of leptospirosis before infected C3H/HeJ mice, but infection in C3H/HeJ mice led to sudden mortality. Clinical signs in C3H/HeN mice were observed beginning on day 2 post-infection. Conjunctivitis was observed in one of the mice with no other clinical signs. On day 3, conjunctivitis, lethargy, piloerection, weight loss, and body temperature decrease were found in four more C3H/HeN mice. On day 3, five C3H/HeN reached endpoint clinical scores and were euthanized. Among these mice, we observed a decrease in temperature ranging from -0.7 to -2.4°C on day 3 before euthanasia. On day 4, five C3H/HeJ mice had succumbed to disease and were found deceased. These mice were showing normal behaviors the previous night. Four of the dead C3H/HeJ mice showed only temperature decreases ranging from -0.7 to -2.7 °C on day 3, but their previous weights and temperatures were normal. By day 4, clinical signs including hypothermia, piloerection, hunchbacked, and dark urine were observed the remaining C3H/HeJ mice. Lethargy, piloerection, pain, and decreasing body temperature were found in the remaining C3H/HeN mice. Images of clinical signs observed in mice are included in **Appendix 3**. All remaining infected groups on day 4 were euthanized based on the endpoint clinical scores. All control mice survived through the end of the study and were euthanized on day 7. Major clinical signs observed in each group of infected mice strain are listed in **Table 1** and average percent change in temperature and weight are shown in **Figure 1**. The Gehan-Breslow-Wilcoxon test was used to analyze the survival rates in mice where weight is given to earlier death events. **Figure 2** shows survival curves of the infected C3H/HeJ and C3H/HeN were significantly different when compared to their controls ($p = 0.0009$ and $p = 0.0043$. respectively). The survival curves of both infected mice were also significantly different from each other ($p = 0.0090$).

Table 1: Clinical Signs of Infected Mice. Based on clinical score sheet approved by the University of Tennessee IACUC and other outcomes related to infection. Clinical signs observed are listed as the number of mice infected out of the total number of mice per group.

Major Clinical Signs	Number Affected / Total Mice	
	C3H/HeJ	C3H/HeN
Body Temperature Decrease ($>0.5^{\circ}\text{C}$)	6/8	1/8
Piloerection	2/8	4/8
Lethargy	0/8	6/8
Conjunctivitis	0/8	4/8
Jaundice	1/8	2/8
Hemorrhaging in Thoracic Cavity	3/8	1/8

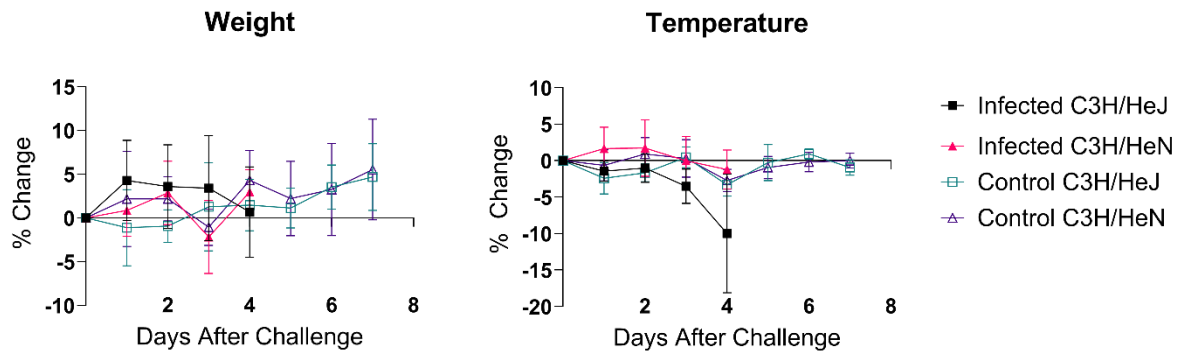


Figure 1: Average Change in Weight and Temperature of Mice. (Left) average percent change of daily weight from original weight and (right) average percent change of daily temperature from original temperature for each group. Bars represent standard deviation.

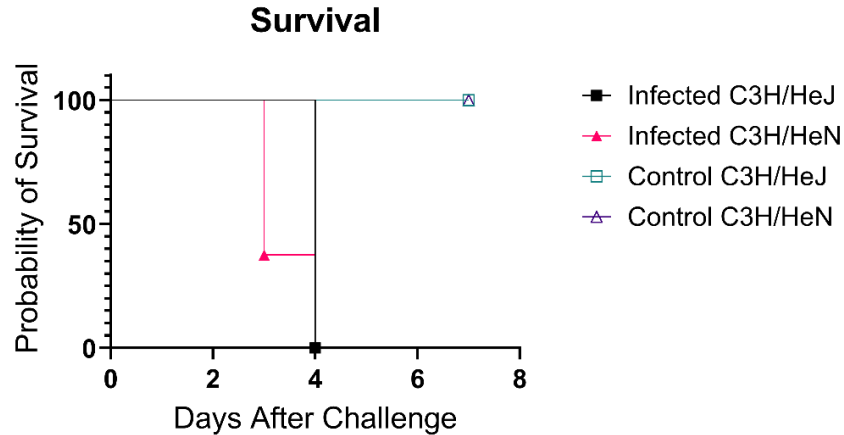


Figure 2: Survival Curve. Probability of survival for the infected and control C3H/HeJ and C3H/HeN groups. Day 0 is the day of challenge.

Organ Weight Comparison

Since an increase in size of liver and spleen (splenomegaly and hepatomegaly) were observed in infected mice at necropsy, we weighed kidney, liver, spleen, lung, and heart of each mouse. Organ weights transformed to percent body weight of the mice are shown in **Figure 3**. For the kidney, lungs, and heart, the infected C3H/HeJ and C3H/HeN groups had a significantly increased weight when compared to each of their control groups. Supporting our visual observations, the infected C3H/HeJ and C3H/HeN groups had significantly heavier livers ($p = 0.001$ and 0.005 respectively) and spleens ($p < 0.0001$ each) when compared to each of their control groups).

Histopathology

Heart, lung, liver, spleen, and kidney were stained with the common hematoxylin and eosin (H&E) technique and evaluated by two board-certified veterinary pathologists. The infected C3H/HeJ and C3H/HeN groups had similar histopathological findings, and the infected groups had only minor differences in comparison to the controls. No major histopathologic findings were found in the heart, and alveolar hemorrhaging was observed in the lungs of both infected and control mice. Mild hepatocellular necrosis and disassociation of the hepatic cords were observed in the liver of infected mice. Within the spleen of infected mice, mild lymphoid hyperplasia with tangible body macrophages was observed in the white pulp. Red pulp

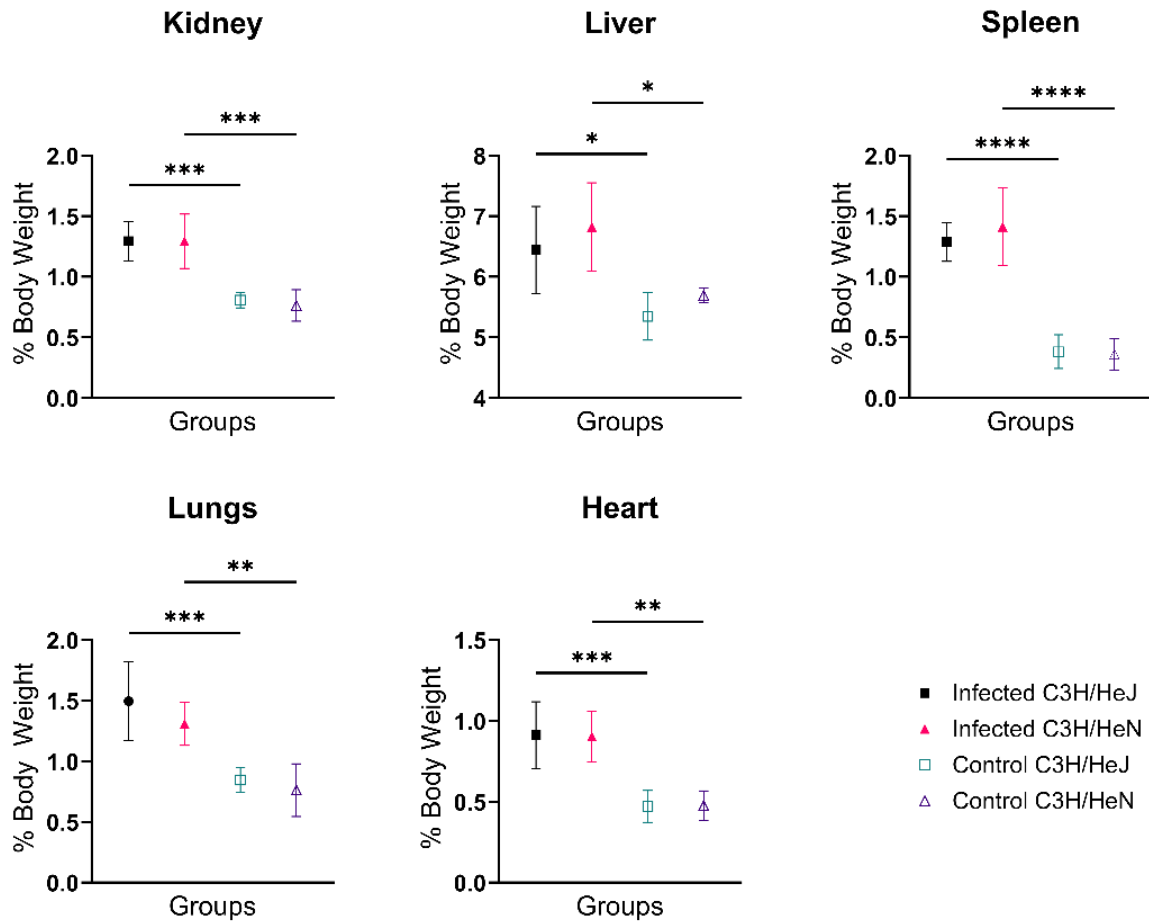


Figure 3: Percentage Weight of Organs. Whole organs collected from each infected and control C3H/HeJ and C3H/HeN group. Kidney represents the average weight between the left and right kidney. Bars represent standard deviation. A one-way ANOVA with Šidák's multiple comparisons test was done to assess the statistical significance between each group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

histiocytosis with erythrophagia and lymphocytolysis along with moderate extramedullary hematopoiesis was also observed in the spleens. Tubular necrosis as well as multifocal basophilia within the tubular lumen occurred within the kidneys of infected mice. Representative photos of histopathological changes in the heart, lung, liver, spleen, and kidney are listed in **Appendix 4**.

RESULT OF IMMUNE RESPONSE IN MICE

Enzyme-Linked Immunosorbent Assay (ELISA)

Overall, infected C3H/HeN mice had a significantly higher antibody response than C3H/HeJ mice as shown in **Figure 4**. IgM and total IgG levels were tested in the sera of control and infected C3H/HeJ and C3H/HeN mice on day 0 (before infection) and at the time of euthanasia. Infected C3H/HeJ and C3H/HeN mice had a significantly higher antibody response from day 0 to euthanasia for IgM ($p < 0.0001$ each) and IgG ($p < 0.0001$ each). At euthanasia, both the infected groups had a significantly higher IgM ($p < 0.0001$ each) and IgG ($p < 0.0001$ each) response at euthanasia when compared to their respective control groups. Overall, the IgM response for both infected strains was higher than the IgG response.

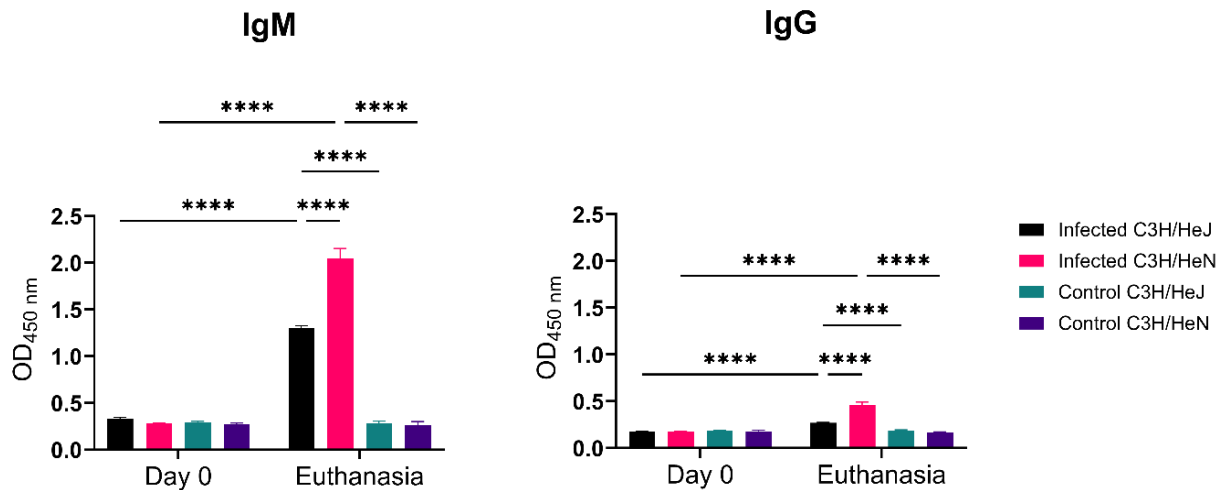


Figure 4: ELISA Results. IgM and IgG antibody response assessed from the pooled serum of each group of mice measured at 450 nm optical density. Bars represent standard deviation. Groups ran in technical triplicates. Two-way ANOVA with Tukey's multiple comparison test was performed to assess statistical significance between groups. **** = $p < 0.0001$.

Microscopic Agglutination Test

The microscopic agglutination test (MAT) results showed more agglutination in the infected C3H/HeN group when compared to the infected C3H/HeJ group. The infected C3H/HeJ group had little to no agglutination, similarly to the control of the same group. However, mild nonspecific agglutination was seen in control C3H/HeN mice.

Cytokine response of C3H/HeJ and C3H/HeN Mice

Results from a multiplex cytokine assay (23 cytokines) performed with pools as described in the methods of kidney, liver, and spleen lysates and sera from infected and control C3H/HeJ and C3H/HeN mice strains were compared. We observed a significantly higher average MCP-1 concentration in infected C3H/HeN mice compared to the control in all four sample types (kidney: $p < 0.0001$, liver: $p = 0.0171$, spleen: $p < 0.0001$, serum: $p = 0.0097$). Comparisons were also made between C3H/HeJ and C3H/HeN control groups to assess if any natural differences in cytokine concentrations between strains exist. The only statistical difference between control groups observed was eotaxin in the serum where C3H/HeN mice controls had a statistically higher average concentration cytokine when compared to C3H/HeJ mice controls ($p < 0.0001$). Overall, average concentrations of multiple cytokines tested were higher in infected animals compared to control samples, but many of these differences were not statistically significant. For example, the average kidney concentration of the cytokine IL-12p40 in 32.53 pg/ml in infected C3H/HeN mice while only 9.26 pg/ml in control C3H/HeN mice, despite no statistical significance being found. All tested cytokine concentrations are listed in **Appendix 5**. The rest of the cytokine concentrations with statistically significant comparisons between groups are shown in **Figure 5**.

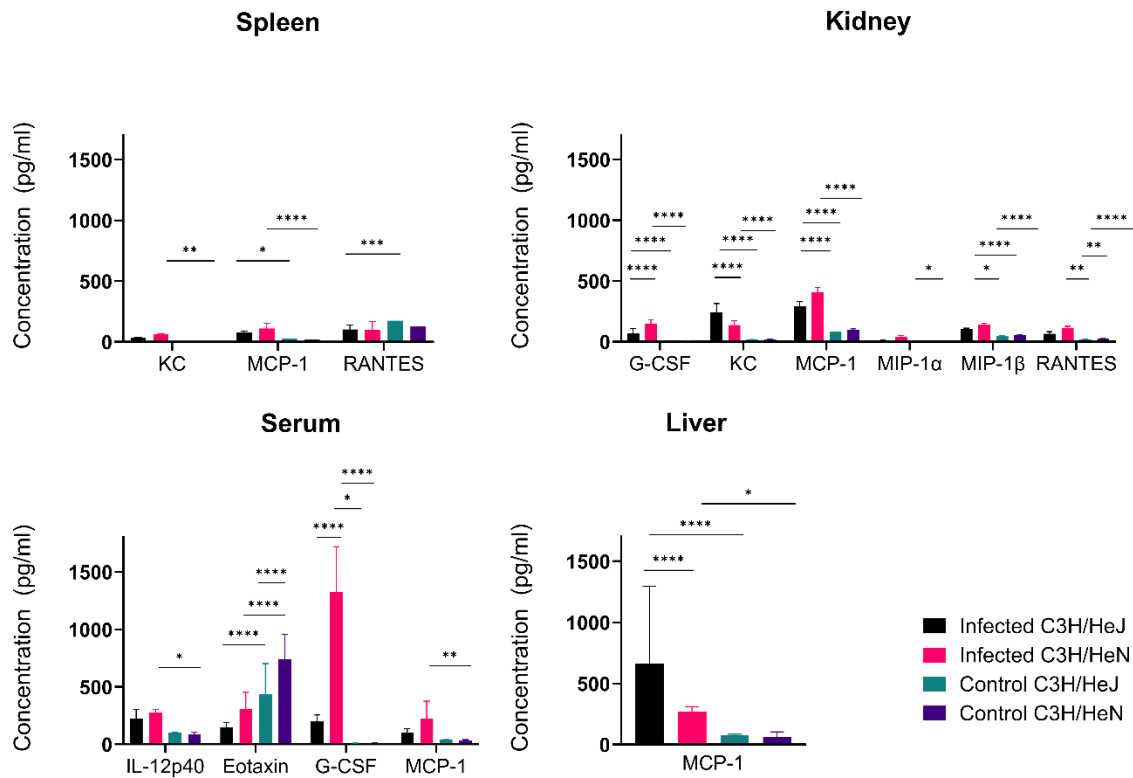


Figure 5: Cytokine Results. Average concentration (in pg/ml) of cytokines showing a significantly different response between groups in the kidney, liver, spleen, and serum. Bars represent standard deviation. A two-way ANOVA with Tukey's multiple comparison test was performed to assess statistical significance between groups. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

LEPTOSPIRA DETECTION

Leptospira Colonization of C3H/HeJ and C3H/HeN Mice

Seven of the kidney cultures in the infected C3H/HeJ group were determined positive by observing live bacteria in the culture. The last infected animal in this group was determined positive by a real-time PCR of the culture. Live *Leptospira* was seen in all the infected C3H/HeN cultures. All control kidney bacterial cultures were confirmed negative. Additionally, all the organs from infected mice in both C3H strains were positive for the presence of *Leptospira* by real-time PCR, and all the negative controls from each strain were negative. Each average Ct value from the tested organs is listed in **Appendix 6**.

Confirmation of the *Tlr4* Mutation

Since we did not find resistance to *Leptospira* infection in infected C3H/HeN mice and severity of the disease was higher in this strain, we decided to confirm the presence/absence of *tlr4* point mutation in both strains. The sequence used to identify the presence or absence of the point mutation was the 717-bp region of *tlr4* PCR amplified with primers previously mentioned in Krangvichian et al. (2023). The point mutation from cysteine to adenine is seen in the *tlr4* gene of C3H/HeJ mice and not present in the C3H/HeN mice (**Figure 6**) (Hoshino et al., 1999).

TRANSCRIPTOMICS

Highly Expressed *Leptospira* Transcripts

A total transcriptomic analysis of *Leptospira* transcripts was done on the liver tissue of infected mice. 3,279 different *Leptospira* genes were identified in the infected groups with copy numbers ranging from 1 to 16,171 copies on average to identify what genes are expressed during infection. There were little differences in the amount of expressed *Leptospira* gene copies detected in each infected group. Key identified highly expressed transcripts with average copy number are listed in **Appendix 7**. Highly expressed possible housekeeping genes for *Leptospira* are shown in **Appendix 7, Table 9** with the highest expressed gene being a HEAT repeat domain-containing protein (locus tag: LIC_RS02125) at 6,293 copies. In the analysis of *Leptospira* transcripts, high expressions were found in five genes that code for proteins of unknown function (**Appendix 7, Table 10**). The highest expressed transcript of these unknown proteins is the hypothetical protein (locus tag: LIC_RS19390) with a mean copy number of 16,171 copies across both infected groups. This hypothetical protein has approximately 2.5x more expressed copies than the highest expressed housekeeping gene transcript. A gene coding for a protein involved in membrane synthesis, murein biosynthesis integral membrane (locus tag: LIC_RS10570), was found to be expressed at an average of 568 copies (**Appendix 7, Table 9**). A few key low expressed transcripts in comparison to the highly expressed transcripts were also identified: major surface lipoprotein LipL32 (locus tag: LIC_RS06940) at 418 average copies, lipoprotein adhesin LigA (locus tag: LIC_RS02420) at 71 average copies, and lipoprotein adhesin LigB (locus tag: LIC_RS02415) at 212 average copies (**Appendix 7, Table 11**). The *lipl32* gene is the target used in real-time PCR to detect the

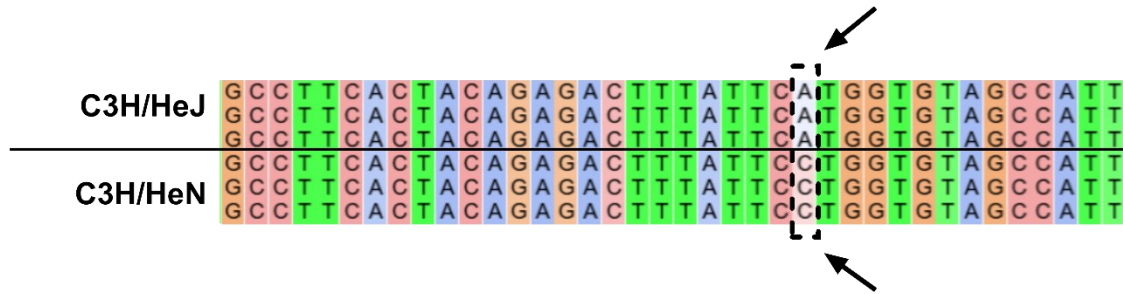


Figure 6: *Tlr4* Alignment. Multi-sequence alignment of *tlr4* gene in control C3H/HeJ and C3H/HeN mice. Alignments performed in Clustal Omega. Dash-box and arrows show the point mutation of the cysteine to adenine.

Leptospira in our study. LigA and LigB have been previously researched as potential *Leptospira* vaccine targets (Koizumi & Watanabe, 2004).

Differential Gene Expression

Several mouse-specific immune transcripts coding for cytokines and cytokine receptors were identified to analyze the differential gene expression between infected groups and their respective control. Both infected C3H/HeJ and C3H/HeN had upregulation of some pro-inflammatory and macrophage-related cytokine genes such as *Ccl2* (MCP-1), *Ccl5* (RANTES), and *Cxcl3* (MIP-2 β). All immune transcripts found upregulated in both groups in comparison to their controls are listed in **Appendix 7, Table 12**. However, there were a number of genes found upregulated only in the infected C3H/HeN group with no upregulation seen in the infected C3H/HeJ group (**Appendix 7, Table 13**). Particularly, *Ifng* (IFN- γ) and IFN- γ related genes were found only upregulated in the C3H/HeN group in comparison to its control. Additionally, two macrophage related genes, *Ccl19* (CCL19) and *Ccl3* (MIP-1 α), were also found to be only upregulated in this group. *Ccl24* (Eotaxin-2) and *Ccr3* (CCR3) were two related transcripts found to be downregulated in the infected groups (**Appendix 7, Table 14**). *Ccl24* was similarly downregulated in both infected groups while *Ccr3* was only found to be downregulated in infected C3H/HeJ mice.

METABOLOMICS

Preliminary metabolomic analysis was performed on the liver of infected and control mice. The metabolite profiles of the infected groups and control groups are distinctly separated,

but there is a lot of variation in the profiles of the infected groups due to the spread of data points as shown in principal component analysis (PCA) plot **Figure 7**. The VIP (variable importance in projection) scores of the top twenty identified metabolites show how much each metabolite is involved in the clustering of groups in the PCA plot. In the VIP score graph, upregulation and downregulation of metabolites in each group can be identified (**Figure 7**). For example, guanidoacetic (acid) is a metabolite made in the liver involved in kidney function that is appropriately upregulated in infected groups of mice because of *Leptospira*'s colonization of the kidney. A volcano plot comparing each infected group to their respective controls and each infected group to each other was created to show the upregulation and downregulation of certain metabolites (**Appendix 8, Figure 17**). The overall metabolic profile of the infected mice in comparison to their respective controls are similar to each other, with a lot of upregulated and downregulated metabolites in the infected animals in comparison to their respective control. There are less differences in the upregulation and downregulation of metabolites between infected groups when looking at the volcano plot comparing the infected C3H/HeJ and C3H/HeN, showing the high similarity of the metabolic profiles between these strains. The same trend is observed in the top 25 metabolic pathways identified in the infected groups compared to their respective controls and the infected groups compared to each other (**Appendix 8**).

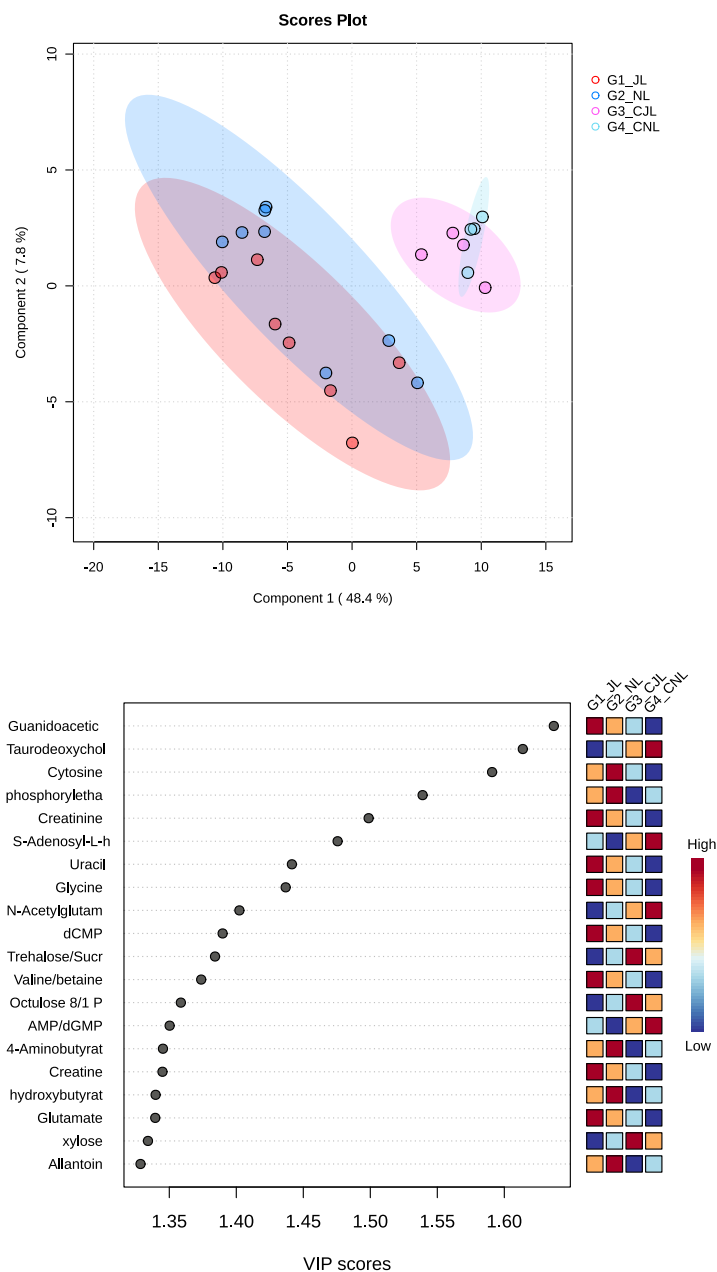


Figure 7: Metabolite Profiles. All group comparison of the metabolic profiles in liver samples. Partial least squares discriminant analysis (PLS-DA) in a principal component analysis (PCA) plot (top). Variable importance in projection (VIP) score plot (bottom) shows the twenty metabolites with the highest VIP scores for each comparison. A VIP score >1 indicates that a metabolite significantly contributes to the overall metabolic profile differences in the PLS-DA model. G1_JL = infected C3H/HeJ, G2_NL = infected C3H/HeN, G3_CJL = control C3H/HeJ, G4_CNL = control C3H/HeN.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

CLINICAL SIGNS AND HISTOPATHOLOGY

Both C3H/HeJ and C3H/HeN mice were susceptible to LIM infection, but their immune response to infection was different. The higher IgM response observed in C3H/HeN mice compared to C3H/HeJ mice is likely due to the difference in toll-like receptor 4 (TLR4) signaling between each strain. Confirming this observation, a previous study found a direct link between TLR4 signaling and B cell activation leading to the production of IgM antibodies (Dyevoich et al., 2020). The C3H/HeJ strain has been previously identified as susceptible to leptospirosis due to a spontaneous mutation in the *tlr4* gene (Pereira et al., 1998; Viriyakosol et al., 2006). On the other hand, wild-type C3H (functional *tlr4*) mice were described as resistant to leptospirosis (Viriyakosol et al., 2006). Additionally, mice crossbred between wild-type C3H (C3H/HeN) and C3H/HeJ mice resulted in a hybrid strain with functional *tlr4* gene that did not show any clinical signs of *Leptospira* infection, but these mice were infected and reported to have some lung hemorrhaging and kidney inflammation (Krangvichian et al., 2023). A very recent study reinvestigated the C3H/HeN mice model using *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 where significant weight loss, with no other clinical manifestations or death, was observed over the course of the study (Abil et al., 2025). Outside of these few studies, no other major information exists for the outcomes of *Leptospira* infection of C3H/HeJ mice compared to its wild type. In our study, we infected mice with *Leptospira interrogans* serovar Manilae strain L495 which is a serovar and strain that has not been previously investigated in this wild-type C3H mouse. The wild-type mice in these previous studies were infected *L. interrogans* serovar Pomona (unknown strain) at 10^3 or 10^6 cells, *L. interrogans* serovar Icterohaemorrhagiae strain HAI188 at 10^8 or 6×10^8 cells, or *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 at 10^8 cells, and no mortality was observed (Abil et al., 2025; Krangvichian et al., 2023; Viriyakosol et al., 2006). In our study, clinical signs developed in C3H/HeN mice resulting in endpoint criteria and euthanasia on days 3-4. The onset of clinical signs in the C3H/HeN mouse was quicker than the C3H/HeJ mouse in our study. However, the lethality of infection in the C3H/HeJ mice was more severe as more than half of the animals in

this group of mice went from exhibiting mild temperature decrease on day 3 to being found deceased on day 4. Previous studies have shown quick disease progression in three- to four-week-old mice infected with the serovar Icterohaemorrhagiae with strain HAI188 that lead to euthanasia (Viriyakosol et al., 2006). The inconsistencies observed in these models need further investigation. It is likely that the *Leptospira* strain serovar used in our study played a role in the disease of both mouse strains when compared to previous studies. The inconsistency observed in these models needs further investigation. Our findings of non-significant weight loss in our infected mice contradicts a previous study using the same serovar and strain of *Leptospira* (Papadopoulos et al., 2025). Further investigation into weight loss as an indicator of the progression of leptospirosis is needed.

Splenomegaly was observed in infected C3H/HeJ and C3H/HeN mice when compared to their controls (images shown in **Appendix 3**). The causes of splenomegaly can be attributed to a variety of issues including increased function of the spleen due to bacterial infection (McKenzie et al., 2018). Suspected lymphoid hyperplasia, increased congestion, and histiocyte infiltration were observed in the spleens of both infected groups during histopathological assessment. Additionally, there was a significant IgG and IgM antibody response after infection in both strains compared to the controls. These results in combination with the detection of significant levels of MCP-1 and KC, chemokines involved in the recruitment of immune cells, in both infected groups suggest hyperplasia might be the cause of splenomegaly (Abil et al., 2025). In previous studies where splenomegaly was observed in *L. interrogans* serovar Copenhageni infected mice, T-cell, myeloid cell, monocyte, and NK cell populations within the spleen were significantly higher compared to their controls (Richer et al., 2015; Shetty et al., 2021). Splenomegaly being observed in different serovar infections suggest that this clinical sign is a consistent outcome of leptospirosis and could be used potentially identify infection.

The liver is a significant organ in leptospirosis pathogenesis. Jaundice is a common presentation of severe leptospirosis in humans and animals (Ellis, 2015; Haake & Levett, 2015). Jaundice and inflammation of the liver has been observed in C3H/HeJ mice infected with pathogenic serovars of *Leptospira*, and the organism can be isolated from liver (Chen et al., 2017; Koizumi & Watanabe, 2003; Viriyakosol et al., 2006). Jaundice was only observed in one

C3H/HeJ mouse and two C3H/HeN mice. The finding of jaundice could be attributed to the dissociation of the hepatic cords or hepatocellular necrosis observed in the livers of both infected groups. C3H/HeJ mice were smaller in weight when compared to C3H/HeN mice from the beginning of the study. Therefore, organ weights were normalized across mouse strains by converting the weights into percent body weight to account for natural differences in body weight between the C3H/HeJ and C3H/HeN strains. No significant differences between the infected groups were found when weighing the organs. However, consistent with our visual observations, the livers of infected mice were significantly larger than their respective controls. Hyperplasia could be a potential cause of the enlarged livers in comparison to their controls due to the detection of MCP-1 in the liver that leads to the recruitment of immune cells into the organ, but further analysis such as immunohistochemistry should be used to make this conclusion.

No concrete conclusions about the leptospirosis-related clinical signs in the heart and lung could be made. For example, lung hemorrhaging is a major clinical sign of leptospirosis, but in this study, it was observed in both infected and uninfected animals. Cervical dislocation was used as a method of secondary euthanasia, but this could have caused the hemorrhaging as well. Therefore, the lung hemorrhages in the infected groups cannot be confirmed to be resulting from infection. Additionally, cardiac punctures were used to collect some blood during necropsy at euthanasia which can damage the heart and affect the histopathological evaluation. Also, the heart is not a significantly involved organ in *Leptospira* infection, so no major findings are expected.

ANTIBODY AND CYTOKINE RESPONSE

A significant antibody response was detected in C3H/HeJ and C3H/HeN mice only 3-4 days after challenge. The IgM response was much higher than the IgG response, as IgM is the first antibody released after exposure to pathogens until the more specific secondary IgG antibodies can be secreted by plasma cells. Additionally, leptospirosis infection is dominated by higher IgM antibody titers in the early stages of infections and then IgG antibody titers rise as detected in human patients (Adler & Faine, 1978). These antibody levels shift to become more IgG dominated as infection progresses. Serovar Copenhageni strain Fiocruz L1-130 infected 10-

to-14-week-old C3H/HeJ mice had a higher IgG levels compared to IgM measured 15 days after infection, and all mice survived infection (Richer et al., 2015). This study revealed higher levels of IgG antibody seem to correlate with better survival outcome, but age seems to heavily influence the immune response. Additionally, the mice in that study survived more than 10 days longer than the C3H/HeJ and C3H/HeN mice within this current study. The higher levels of IgG detected when compared to IgM could be related to the longer course of infection within those mice.

Microscopic Agglutination Test (MAT) is a reference serological test used for the diagnosis of leptospirosis. MAT detects the presence of *Leptospira*-specific agglutinating antibodies in the serum of infected animals but does not distinguish between IgM and IgG subclasses. Even though it cannot distinguish between antibodies from an active infection or a previously vaccinated animal, it does show whether the antibodies the animal produces is specifically against *Leptospira*. There were more agglutinating antibodies against LIM in the infected C3H/HeN mice compared to C3H/HeJ mice, but some non-specific agglutination was also seen in control C3H/HeN mice. The IgG response was significantly higher in infected C3H/HeN mice compared to C3H/HeJ mice, but each infected group had significantly higher IgG antibodies than their controls. These agglutinating antibodies are likely IgM-dominated because of the high response present in the infected mice even only a few days after infection. However, the agglutination seen in the control group could be due to human error when handling sera, so further investigation is needed.

Out of 23 different cytokines and chemokines tested, only the concentrations of nine (G-CSF, KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, IL-12p40, and eotaxin) were statistically significant when compared between groups. These cytokines and chemokines all appear to be pro-inflammatory which match with the typical presentation of leptospirosis (Murray, 2015). A variety of cytokines and chemokines of mice infected with *Leptospira* have been investigated before. Levels of RANTES, a T-cell chemokine, in the kidney of normal TLR4 BALB/c mouse is higher than C3H/HeJ mouse after infection with *L. interrogans* ATCC® BAA-1198™ which is reflected in our result of C3H/HeN having a significantly higher level of RANTES in the kidney when compared to infected C3H/HeN mice (Silva et al., 2020). Macrophage related

cytokines and chemokines (IL-12p40 and MCP-1) were found in statistically significant levels in the serum. MCP-1 was found to be upregulated in previous studies of C3H/HeJ mice infected with *L. interrogans* serovar Lai (Chen et al., 2017). IL-12p40 was only found to be significantly higher in C3H/HeN mice when compared to their controls. This cytokine is described to be involved in the regulation of macrophages, and macrophages can contain TLR4 on their outer membrane and can be induced by a TLR4-stimulated innate immune response (Jeong & Pahan, 2024; Mogensen, 2009). Therefore, it is suspected to see significant levels of these cytokines in TLR4 functional mice. MCP-1 was also found in statistically significant levels in the kidney, liver, and spleen. Notably, the levels of this cytokine were significantly higher in infected groups when compared to their control. Additionally, MCP-1 in infected C3H/HeN mice is significantly higher than infected C3H/HeJ mice in the kidney while the concentration for infected C3H/HeJ mice is higher in the liver compared to infected C3H/HeN mice. An increased level of macrophages has been observed in *Leptospira* infected C3H/HeJ mice which correlates with the significant increase of this MCP-1 in the liver (Nally, Fishbein, et al., 2005). However, MCP-1 concentrations were significantly higher in the liver and lower in the kidney of TLR4 normal mice (BALB/c) when compared to C3H/HeJ mice when infected with serovar Copenhageni strain 25179, and similar results are seen with a different strain of *Leptospira* (Silva et al., 2009; Silva et al., 2020). The opposite is observed in our study where MCP-1 was significantly higher in the kidneys and significantly lower in the liver for infected C3H/HeN mice compared to infected C3H/HeJ mice. The infectious doses used in these strains ranged between 10^6 to 10^7 , but the most recent study used three- to four-week-old mice (Silva et al., 2009; Silva et al., 2020). Therefore, it could be the age of the mice or the *Leptospira* strain that played a role in the differences of the MCP-1 levels.

***LEPTOSPIRA* COLONIZATION**

Colonization of all collected organs is typical of leptospirosis because the bacteria disseminate through the blood and colonize the kidney which is the organ *Leptospira* chronically colonizes (Murray, 2015; Surdel et al., 2022). No major conclusions can be drawn about the colonization of the mice after infection because the real-time PCR method we used only provides relative quantification of DNA. For absolute quantification, future studies should include

standard curve or digital PCR methods that will be helpful in investigating if differences of colonization exist between the C3H groups.

TRANSCRIPTOME

Both *Leptospira* and immune transcripts were identified in the mice. *Leptospira* transcripts were identified to understand which genes are being expressed during infection, and if there is a difference in the genes being expressed within C3H/HeJ and C3H/HeN mice. No significant differences of *Leptospira* transcripts were seen, so the copy number of both infected groups were averaged to identify highly expressed *Leptospira* transcripts. Some key housekeeping genes involved in the metabolism of *Leptospira* were identified. However, a unique set of genes that code for proteins of unknown function were also identified in the mice. A gene coding for a hypothetical protein had the highest expression among all identified transcripts. Even though the function of these proteins remains relatively unknown, they have potential to be further investigated for vaccine or diagnostic use. Additionally, a gene involved in membrane synthesis, murein biosynthesis integral membrane, was identified in both groups. This protein could be further studied to understand its role in the pathogenesis of *Leptospira*. *LipI32*, the target gene used in our study to amplify *Leptospira* DNA, was also expressed in the infected mice with lower copy numbers than the unknown genes. Additionally, the genes coding for LigA and LigB, proteins that have been previously investigated for vaccine use, were found to be expressed in lower amounts when compared to the unknown genes (Koizumi & Watanabe, 2004). These key yet lowly expressed transcripts show that there are potentially more useful proteins that can be investigated for their diagnostic and vaccine potential.

The immune transcripts identified in the liver infected groups were able to be compared to their respective controls. There were some similarities in the transcripts of infected C3H/HeJ and C3H/HeN mice. MCP-1 and RANTES were found to be upregulated in both infected groups, which is consistent with the results of the cytokine assay performed. However, the fold change of the MCP-1 gene was higher in infected C3H/HeN mice when compared to C3H/HeJ mice while MCP-1 in the liver was produced in higher levels in the C3H/HeJ mice when compared to C3H/HeN mice based on the cytokine assay. The other sample types in the assay reflect higher MCP-1 levels in C3H/HeN mice when compared to C3H/HeJ mice. There are a set of transcripts

found only to be upregulated in C3H/HeN mice that mainly have pro-inflammatory and macrophage related functions. MIP-1 α and KC are two chemokines that were found to be significantly increased in the cytokine assay. C3H/HeN concentrations of KC in the spleen and MIP-1 α in the kidney were significantly higher in the infected group versus its control while concentrations C3H/HeJ mice were lower, which reflects the upregulation of these genes in the transcriptomic analysis. However, C3H/HeJ concentrations of KC in the spleen were higher than C3H/HeN mice and significantly higher than the C3H/HeJ control. Interestingly, IFN- γ genes and genes related to this cytokine were also found to be upregulated only in the C3H/HeN group even though no significant increase in IFN- γ was seen on the cytokine assay.

The upregulation of more pro-inflammatory and macrophage related genes in the C3H/HeN mice compared to the C3H/HeJ mice could be related to the presence of a functional TLR4. A previous study using the same strain of *Leptospira* found that *Leptospira* LPS only appears to activate the MyD88 pathway after stimulating TLR4 and avoids the activation of the TRIF pathway (Bonhomme et al., 2020). In our study we saw an upregulation of MyD88-pathway genes like KC and RANTES, but there is also an upregulation of IFN- γ of the TRIF pathway. However, KC and RANTES were produced in significant levels as determined by the cytokine assay while no significant production of IFN- γ was detected which reflects the conclusion of Bonhomme et al. (2020).

These *Leptospira* and immune transcripts need to be further investigated to identify patterns in *Leptospira* pathogenesis and host-immune response on the genetic level. Even though these genes are identified as over expressed during infection, it does not mean that the proteins are actually being produced. The *Leptospira*-related genes will need to be expressed *in vitro* to assess their vaccine or diagnostic potential. The multiplex cytokine assay provided some insight into the protein level of the immune response, but multiplex assays are limited because of the number of cytokines that can be detected at a time in a sample. Additionally, these transcripts were only identified within liver tissue. Other organs will need to be analyzed to determine if the transcriptome is similar across sample types.

METABOLOME

Metabolomic data is relevant to identify biomarkers that could be used to indicate *Leptospira* infection. Preliminary data shows that the metabolite profile of the infected groups have minor differences. The volcano plots of each infected group compared to their control look similar with lots of upregulated and downregulated metabolites. However, there are little upregulated and downregulated metabolites when comparing each infected group to each other. This trend in the data is continued in the identified metabolite pathways, suggesting that the metabolome of C3H/HeJ and C3H/HeN mice are not majorly different after infection. Some metabolites have been identified to be relevant to liver and kidney function such as guanidoacetic acid and creatinine. The relevance of the currently identified metabolites and metabolic pathways need to be further investigated to understand their role in *Leptospira* infection. Other organs, especially the kidney, should be included in the metabolomic analysis to have a better understanding of the outcomes of *Leptospira* infection.

CONCLUSION AND FUTURE DIRECTIONS

In this study, we compared the outcomes of *Leptospira interrogans* serovar Manilae strain L495 (LIM) infection in a C3H/HeJ mice model that carries a mutation in the *tlr4* gene and its wild type, the C3H/HeN mice strain. LIM infection caused lethal disease in both mice strains, challenging the idea that wild-type C3H mice cannot develop lethal infection of leptospirosis (Abil et al., 2025; Krangvichian et al., 2023; Viriyakosol et al., 2006). Our mice succumbed to infection only three to four days after infection despite their adult age. Additionally, there were no major differences in the clinical signs between C3H/HeJ mice and C3H/HeN mice. However, there were differences in the immune response of the infected mice, supporting our hypothesis that the outcomes of infection would be different. The difference in immune transcripts of the mice also supports the hypothesis that C3H/HeJ mice would have a different outcome of infection when compared to C3H/HeN mice. On the other hand, preliminary metabolomic analysis shows little difference in the metabolite profile of infected C3H/HeJ and C3H/HeN mice.

For future directions, the highly expressed genes and metabolites of these mice need to be further analyzed to understand their usefulness in preventative measures or treatments.

Additionally, other pathogenic serovars of *Leptospira* should be investigated to better understand *Leptospira* infection in the C3H/HeJ mouse model and its wild type, the C3H/HeN. Other factors such as the dose of *Leptospira* infection as well as age of the mice should also be affected to understand their impact on disease outcomes. Overall, our study characterized the clinical manifestations, antibody and cytokine response, transcriptome, and metabolome of *L. interrogans* serovar Manilae strain L495 infection C3H/HeJ mice and its wild type, C3H/HeN mice. This data will provide better understanding of leptospirosis in mice and identifies potentially important genes and biomarkers potentially relevant for vaccine, diagnostic, and therapeutic use.

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APPENDICES

Appendix 1

Table 2: *Clinical Score Sheet.* Approved by University of Tennessee IACUC. Previously developed by Barbosa et al. (2024).

Clinical Signs	Score	Action
Normal behavior		
Well-conditioned (vertebrae and dorsal pelvis not prominent and palpable with slight pressure)	1	None required
None loss of weight		
Body temperature decreasing	2	Observe again in 2 hours
Body weight decreasing ($\leq 15\%$ weight loss)		Increase the frequency of observation
Hair loss; Piloerection; Ruffled or spiked fur/rough coat; Lusterless/dry/scaled tail		
Loss of motility; Hunched back		
Decreased activity: Listlessness, Lethargy or sleep		
Ocular and/or nasal discharge		
Very restless, Ataxia or tremor		
Cyanosis; Ears and paws presenting blue color		
Nasal bleeding	3	Euthanize
Jaundice		
Isolation		
Bulging or sunken eyes		
Heavily soiled anal orifice		
Hypothermia ($> 4-6^{\circ}\text{C}$ lost)		
$> 20\%$ weight loss		

Appendix 2

Table 3: Cytokine Assay Pools. Schematic of pools created for cytokine assay for serum, liver, kidney and spleen. Sera pools were based on the amount of this sample available. Sera from only three infected C3H/HeJ animals were collected due to the early deaths of five mice in this group.

Sample Type	Group	Number of Animals	Pool
Serum	Infected C3H/HeJ	2	1
		1	2
	Infected C3H/HeN	4	3
		4	4
	Control C3H/HeJ	2	5
		2	6
	Control C3H/HeN	2	7
		2	8
Liver	Infected C3H/HeJ	4	9
		4	10
	Infected C3H/HeN	4	11
		4	12
	Control C3H/HeJ	2	13
		2	14
	Control C3H/HeN	2	15
		2	16
Kidney	Infected C3H/HeJ	4	17
		4	18
	Infected C3H/HeN	4	19
		4	20
	Control C3H/HeJ	2	21
		2	22
	Control C3H/HeN	2	23
		2	24
Spleen	Infected C3H/HeJ	4	25
		4	26
	Infected C3H/HeN	4	27
		4	28
	Control C3H/HeJ	4	29
	Control C3H/HeN	4	30

Appendix 3

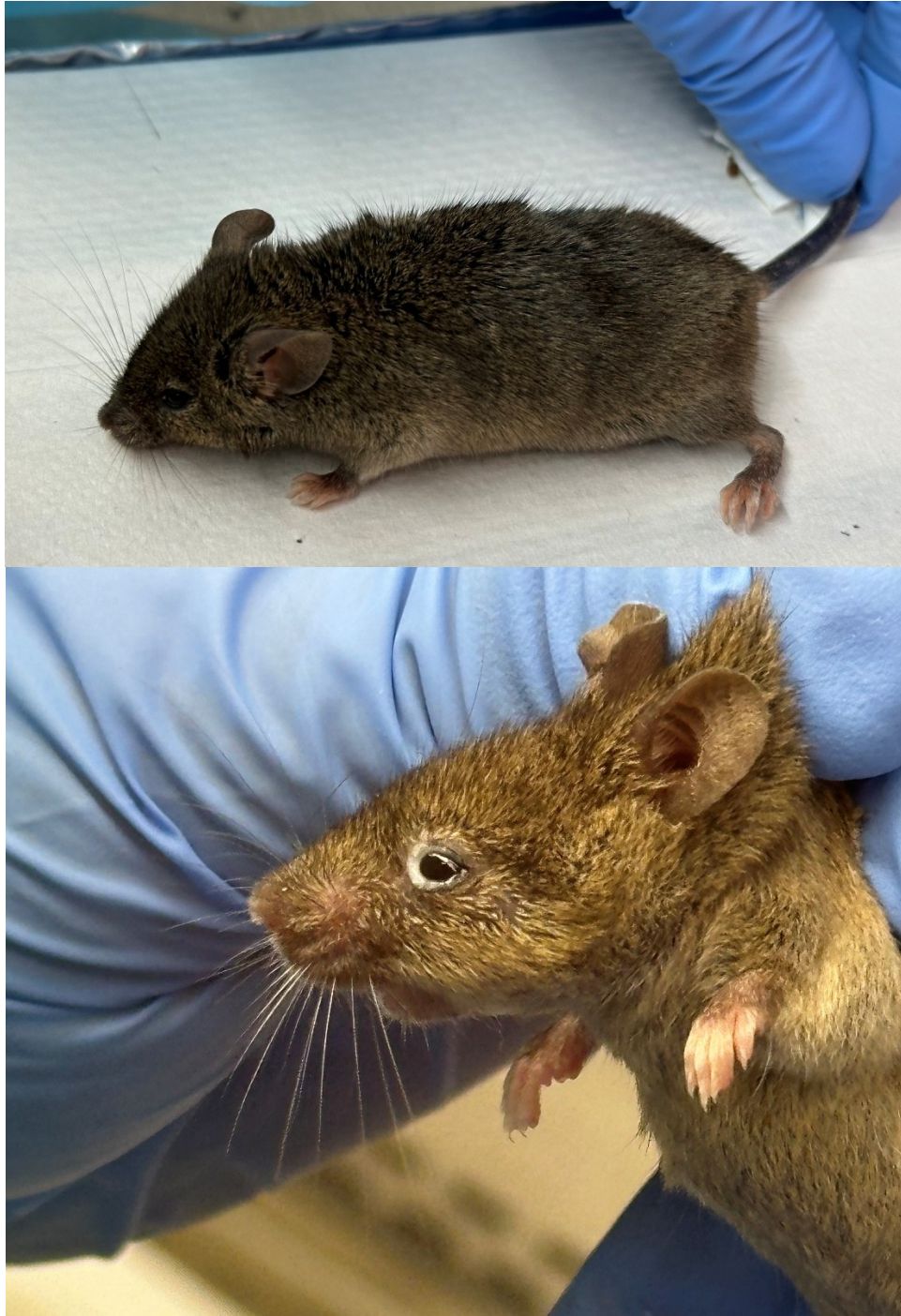


Figure 8: *Photos of Clinical Signs.* Piloerection (top) and conjunctivitis (bottom) observed in infected C3H/HeN mice.

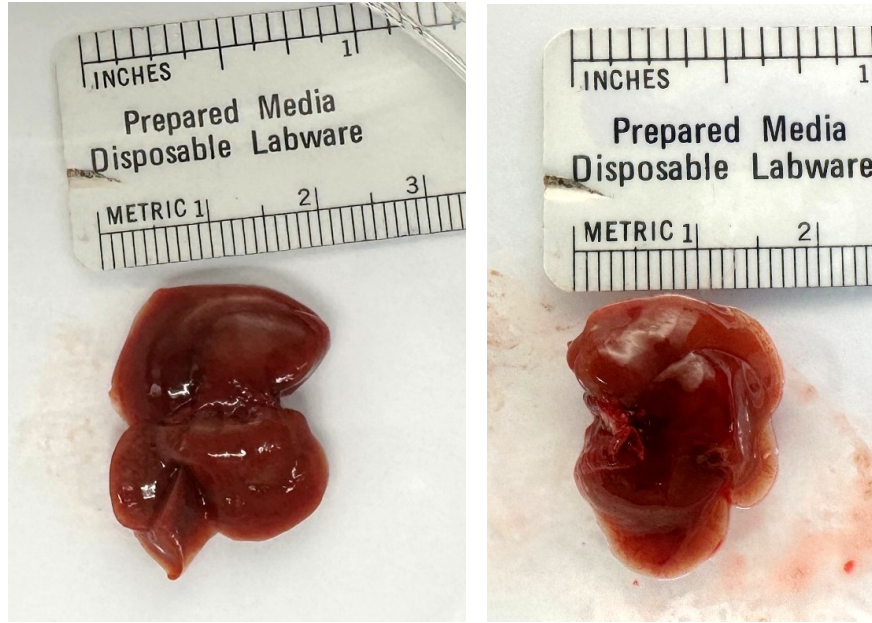


Figure 9: *HeJ* Livers. Mild jaundice shown in infected an C3H/HeJ mouse liver (left) compared to a control C3H/HeJ liver (right).



Figure 10: *HeN* Livers. Mild jaundice shown in infected C3H/HeN mouse liver (left) compared to a control liver (right).

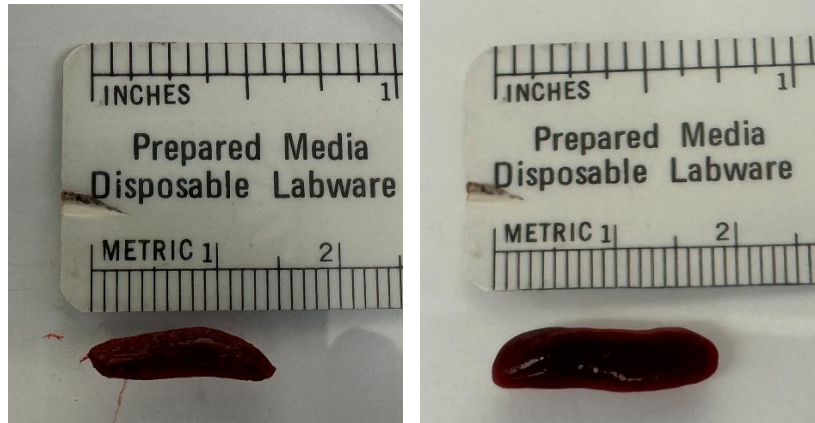


Figure 11: *HeJ Spleens*. Splenomegaly in infected C3H/HeJ mice. Control spleen (left) and infected spleen (right).

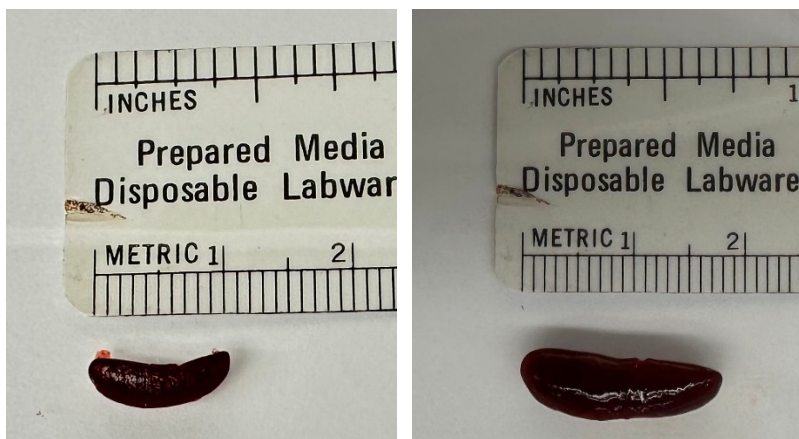


Figure 12: *HeN Spleens*. Splenomegaly in infected C3H/HeN mice. Control spleen (left) and infected spleen (right).

Appendix 4

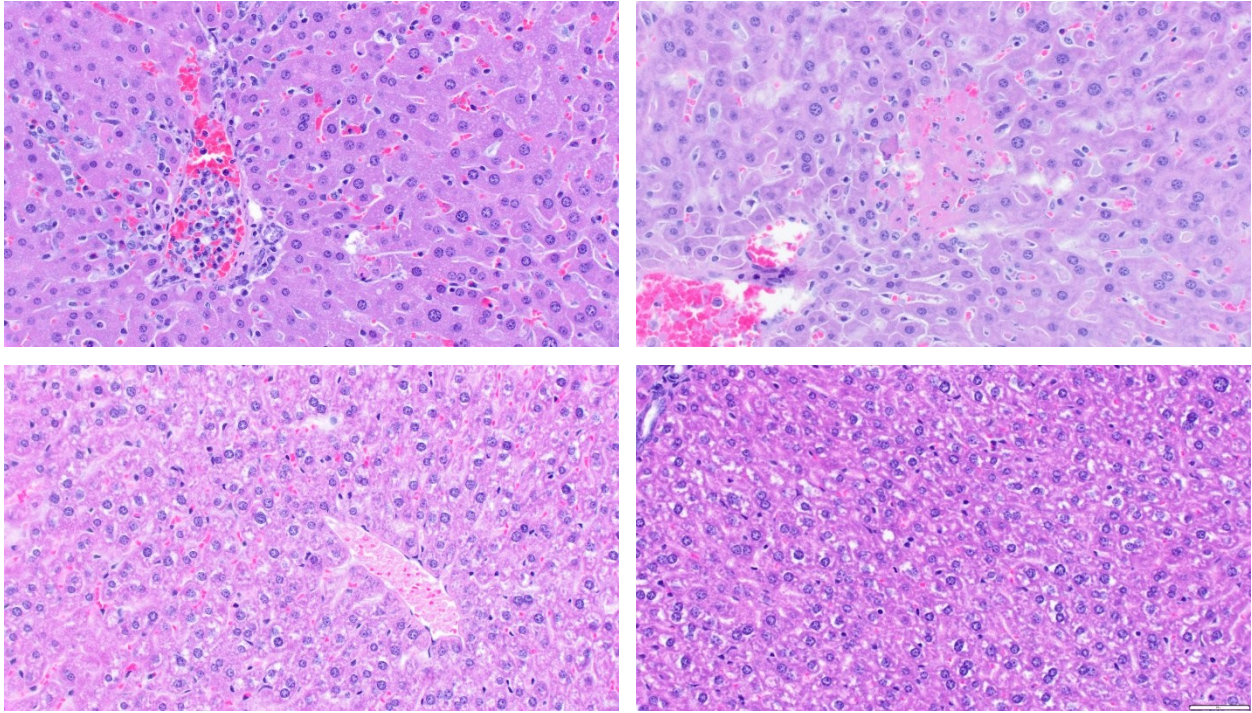


Figure 13: *Liver Histopathology.* 400x magnification of hematoxylin and eosin (H&E) stained liver tissue of infected C3H/HeJ (top left) and C3H/HeN (top right) compared to control C3H/HeJ (bottom left) and C3H/HeN (bottom right) mice. Hepatocellular necrosis and hepatic cord dissociation are shown in the infected livers.

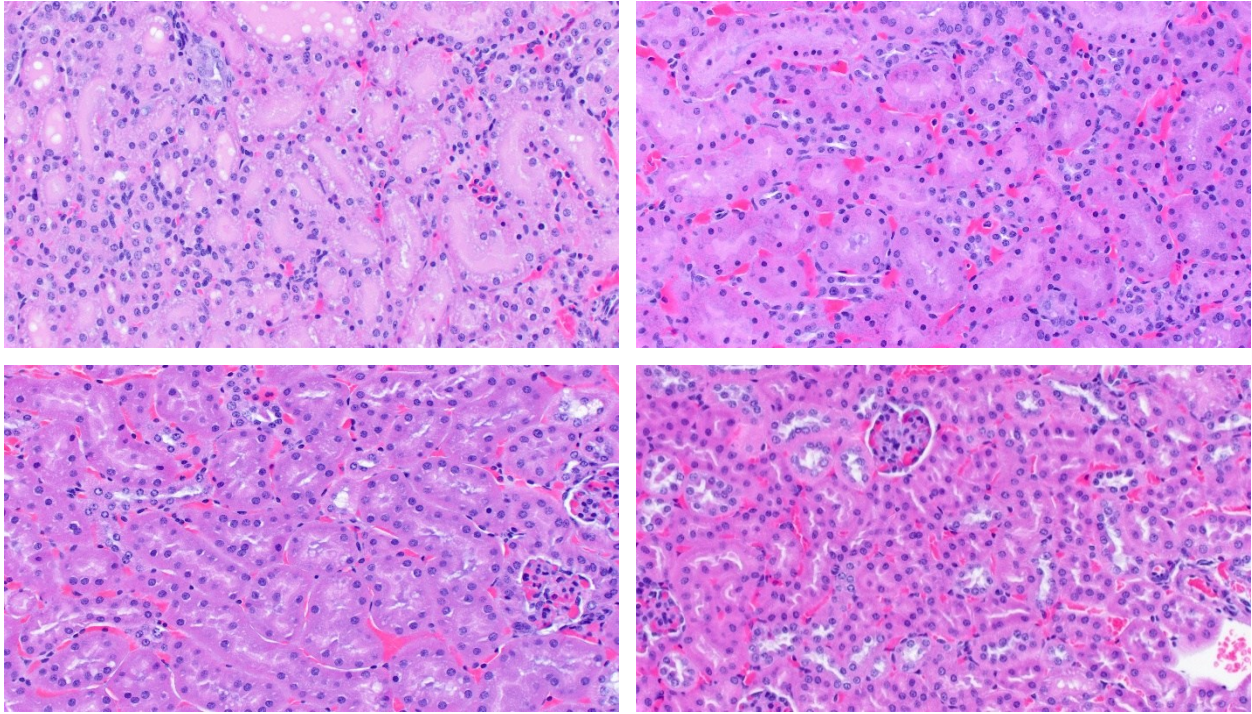


Figure 14: *Kidney Histopathology.* 400x magnification of hematoxylin and eosin (H&E) stained kidney tissue of infected C3H/HeJ (top left) and C3H/HeN (top right) compared to control C3H/HeJ (bottom left) and C3H/HeN (bottom right) mice. Tubular necrosis with cellular debris in the tubular lumen is seen in the infected C3H/HeJ kidney, and basophilia within the tubular lumens and surrounding the cell-cell borders is seen within the infected C3H/HeN kidney.

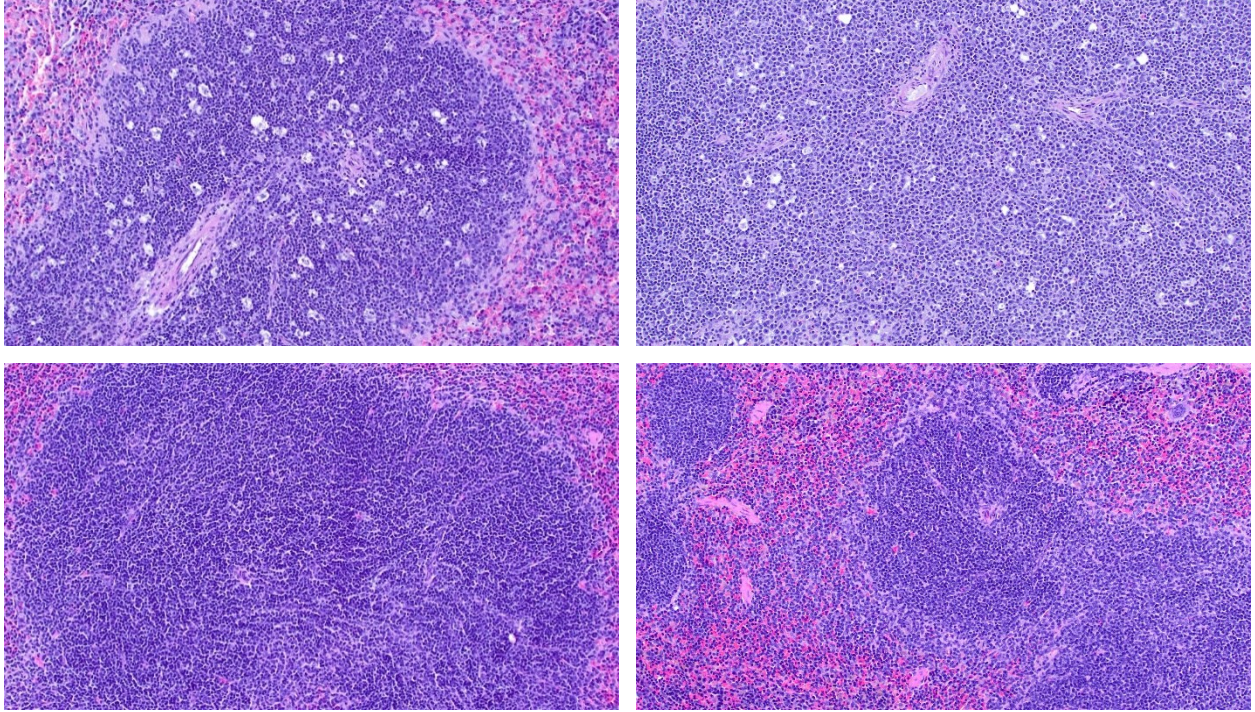


Figure 15: *Spleen Histopathology.* 200x magnification of hematoxylin and eosin (H&E) stained white pulp of spleen tissue of infected C3H/HeJ (top left) and C3H/HeN (top right) compared to control C3H/HeJ (bottom left) and C3H/HeN (bottom right) mice. Tingible body macrophages are shown in the infected spleens.

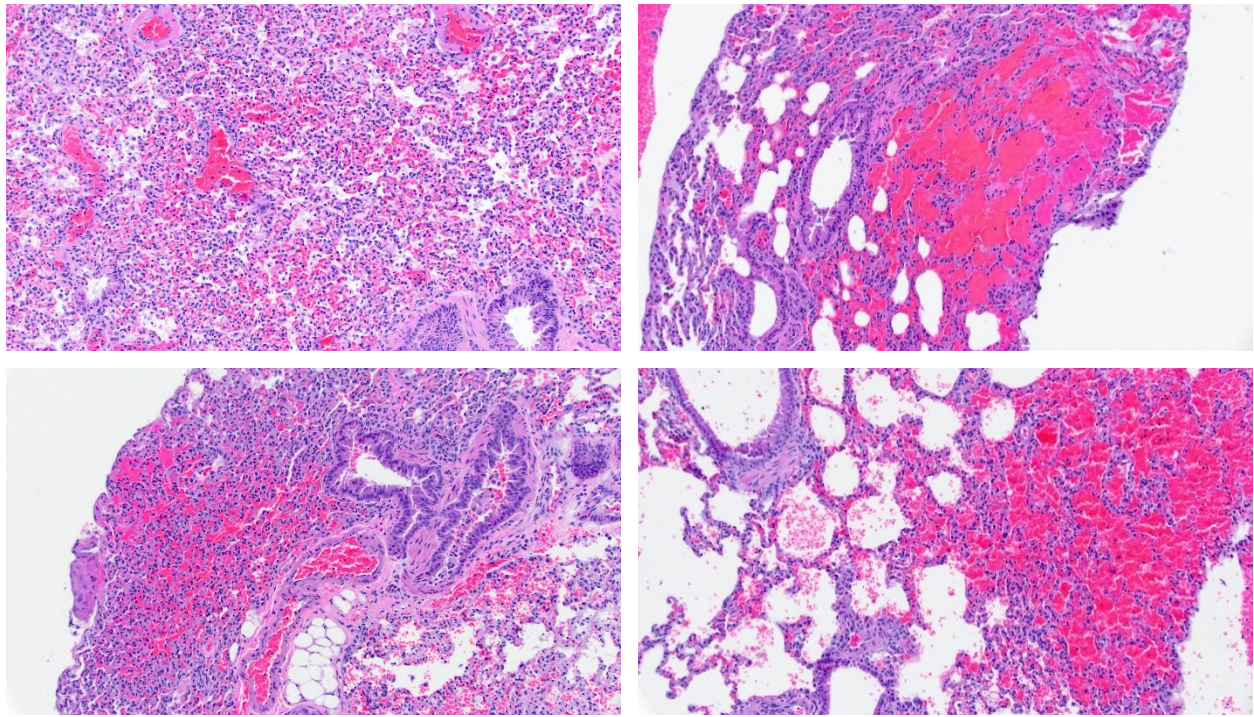


Figure 16: *Lung Histopathology.* 200x magnification of hematoxylin and eosin (H&E) stained lung tissue of infected C3H/HeJ (top left) and C3H/HeN (top right) compared to control C3H/HeJ (bottom left) and C3H/HeN (bottom right) mice. Alveolar hemorrhaging is observed in both infected and control lungs.

Appendix 5

Average cytokine concentrations in pg/ml from each group. Each group contains pooled samples from all animals within the group run in technical duplicates. Organs samples were lysed before testing. Sera was not further processed.

Table 4: Kidney Cytokines. Average cytokine concentrations for kidney (1.51 to 405.95 pg/ml).

Kidney				
Cytokines	Infected C3H/HeJ	Infected C3H/HeN	Control C3H/HeJ	Control C3H/HeN
IL-1 α	6.86	6.28	6.37	8.08
IL-1 β	12.37	14.55	7.68	9.77
IL-2	43.98	38.88	39.83	49.34
IL-3	2.27	1.95	1.51	2.05
IL-4	3.82	3.09	2.71	3.67
IL-5	6.78	6.69	4.88	6.00
IL-6	9.36	13.33	4.23	6.26
IL-9	21.28	19.40	17.47	21.78
IL-10	31.59	32.70	25.49	29.13
IL-12p40	16.15	32.53	7.73	9.26
IL-12p70	15.07	15.25	12.14	18.45
IL-13	57.68	53.61	50.40	60.76
IL-17A	5.46	4.76	4.41	5.30
Eotaxin	47.22	46.34	32.24	38.25
G-CSF	65.54	151.20	3.56	4.55
GM-CSF	15.92	16.13	13.30	14.94
IFN- γ	19.76	17.48	16.66	20.80
KC	240.90	136.15	15.98	19.47
MCP-1	290.16	405.95	83.86	93.97
MIP-1 α	12.71	42.16	1.57	1.98
MIP-1 β	106.25	143.05	44.58	54.82
RANTES	64.45	110.79	20.31	24.99
TNF- α	43.20	54.42	31.69	44.02

Table 5: Liver Cytokines. Average cytokine concentrations for liver (1.25 to 661.68 pg/ml).

Liver				
Cytokines	Infected C3H/HeJ	Infected C3H/HeN	Control C3H/HeJ	Control C3H/HeN
IL-1 α	8.89	8.65	5.77	4.54
IL-1 β	9.08	8.88	6.46	5.32
IL-2	32.95	28.98	30.14	22.58
IL-3	2.05	1.76	1.64	1.25
IL-4	3.43	2.77	2.65	1.99
IL-5	5.55	4.69	4.56	3.19
IL-6	12.52	14.22	6.83	6.07
IL-9	19.43	15.55	15.46	12.16
IL-10	22.59	22.36	22.52	15.79
IL-12p40	13.02	17.92	6.79	5.67
IL-12p70	13.76	13.90	14.86	12.80
IL-13	40.88	39.12	45.13	32.72
IL-17A	4.38	3.57	3.83	2.86
Eotaxin	39.61	41.48	37.43	60.28
G-CSF	46.44	63.04	3.60	3.18
GM-CSF	13.85	12.84	12.15	9.20
IFN- γ	17.05	13.72	13.36	10.81
KC	48.56	52.99	16.53	11.42
MCP-1	661.68	269.60	74.25	60.24
MIP-1 α	9.50	26.90	1.89	1.76
MIP-1 β	123.90	93.71	35.51	29.96
RANTES	77.88	85.64	20.78	49.95
TNF- α	224.63	232.15	159.70	135.36

Table 6: Spleen Cytokines. Average cytokine concentrations for spleen (0.00 to 172.93 pg/ml).

Spleen				
Cytokines	Infected C3H/HeJ	Infected C3H/HeN	Control C3H/HeJ	Control C3H/HeN
IL-1 α	2.80	6.58	1.35	1.11
IL-1 β	4.96	6.27	1.46	1.35
IL-2	4.93	6.27	9.08	6.86
IL-3	0.15	0.47	0.18	0.12
IL-4	0.45	0.46	0.45	0.41
IL-5	0.00	0.87	0.00	0.00
IL-6	0.00	1.26	0.00	0.00
IL-9	1.73	2.54	2.32	2.18
IL-10	2.89	6.58	4.17	2.70
IL-12p40	11.45	10.33	12.88	8.61
IL-12p70	0.43	0.97	0.70	0.45
IL-13	6.57	13.08	8.31	7.88
IL-17A	0.71	1.32	1.06	0.87
Eotaxin	19.35	33.45	35.07	30.52
G-CSF	6.01	12.65	0.75	0.29
GM-CSF	2.62	8.89	2.56	2.89
IFN- γ	1.22	1.78	1.62	1.57
KC	33.66	63.05	9.85	2.91
MCP-1	76.08	109.68	28.30	16.76
MIP-1 α	13.44	43.13	7.94	3.48
MIP-1 β	35.39	56.29	24.21	15.76
RANTES	102.81	101.99	172.93	123.40
TNF- α	0.01	14.51	0.63	0.00

Table 7: Serum Cytokines. Average cytokine concentrations for the serum (0.00 to 1324.14 pg/ml).

Serum				
Cytokines	Infected C3H/HeJ	Infected C3H/HeN	Control C3H/HeJ	Control C3H/HeN
IL-1 α	1.26	3.65	1.82	1.64
IL-1 β	2.24	1.88	1.25	1.15
IL-2	7.90	27.07	3.37	2.44
IL-3	1.25	0.46	0.22	0.42
IL-4	0.47	0.39	0.29	0.19
IL-5	2.74	2.06	1.55	1.30
IL-6	3.47	6.55	0.00	0.00
IL-9	3.98	3.18	2.24	2.21
IL-10	16.12	25.50	3.85	2.68
IL-12p40	224.15	273.02	100.96	86.73
IL-12p70	5.09	4.00	1.82	2.27
IL-13	9.30	25.10	2.13	4.10
IL-17A	4.55	3.02	0.61	1.65
Eotaxin	149.16	306.66	436.28	739.75
G-CSF	198.08	1324.14	8.78	8.98
GM-CSF	8.13	21.37	3.97	4.29
IFN- γ	1.59	4.88	1.79	1.50
KC	16.62	55.29	20.41	21.61
MCP-1	102.76	225.88	40.80	31.92
MIP-1 α	0.84	1.93	1.84	0.48
MIP-1 β	46.49	81.99	12.38	12.28
RANTES	20.70	43.34	15.41	32.49
TNF- α	9.04	9.45	11.51	6.06

Appendix 6

Table 8: PCR Summary. Summary of organs found positive for *Leptospira* DNA and cycle threshold (Ct) values per group. "N/A" means no Ct value detected for the sample.

Group	Organ	Positive	Average Ct Value
Infected C3H/HeJ	Liver	8/8	24.36
	Kidney	8/8	24.56
	Spleen	8/8	28.28
	Lungs	8/8	25.02
	Heart	8/8	29.17
Infected C3H/HeN	Liver	8/8	25.40
	Kidney	8/8	26.17
	Spleen	8/8	29.91
	Lungs	8/8	26.60
	Heart	8/8	29.17
Control C3H/HeJ	Liver	0/4	N/A
	Kidney	0/4	N/A
	Spleen	0/4	N/A
	Lungs	0/4	N/A
	Heart	0/4	N/A
Control C3H/HeN	Liver	0/4	N/A
	Kidney	0/4	N/A
	Spleen	0/4	N/A
	Lungs	0/4	N/A
	Heart	0/4	N/A

Appendix 7

Table 9: Housekeeping and Membrane Genes. Key *Leptospira* housekeeping and membrane synthesis gene transcripts found highly expressed in infected mice. The locus tag is the unique identifier for the gene. Mean copy number is the average copies found between the infected C3H/HeJ and C3H/HeN groups.

Locus Tag	Mean Copy #	Description
LIC_RS02125	6293	HEAT repeat domain-containing protein
LIC_RS13095	2517	nitrilase-related carbon-nitrogen
LIC_RS02390	1564	adenosine deaminase
LIC_RS12445	1469	sulfatase-like hydrolase/transferase
LIC_RS14110	995	NADH-quinone oxidoreductase subunit
LIC_RS12610	946	glutamine-hydrolyzing carbamoyl-phosphate synthase small subunit
LIC_RS08935	628	glycosyltransferase family 9 protein
LIC_RS10570	568	murein biosynthesis integral membrane
LIC_RS15925	556	5'/3'-nucleotidase SurE
LIC_RS12750	516	MBOAT family O-acyltransferase

Table 10: Unknown Genes. Key *Leptospira* gene transcripts for membrane synthesis or proteins of unknown function found highly expressed in infected mice. The locus tag is the unique identifier for the gene. Mean copy number is the average copies found between the infected C3H/HeJ and C3H/HeN groups.

Locus Tag	Mean Copy #	Description
LIC_RS19390	16171	hypothetical protein
LIC_RS09875	1357	outer membrane beta-barrel protein
LIC_RS15830	856	DUF1566 domain-containing protein
LIC_RS17440	838	DUF1566 domain-containing protein
LIC_RS02395	657	VOC family protein

Table 11: Known *Leptospira* Genes. Previously researched *Leptospira* gene transcripts. The locus tag is the unique identifier for the gene. Mean copy number is the average copies found between the infected C3H/HeJ and C3H/HeN groups.

Locus Tag	Mean Copy #	Description
LIC_RS06940	418	major surface lipoprotein LipL32
LIC_RS02420	71	lipoprotein adhesin LigA
LIC_RS02415	212	lipoprotein adhesin LigB

Table 12: Immune Transcripts in Both Groups. Upregulated immune transcripts of infected mice in comparison to their controls. Log² FC is the fold change in copy number in comparison to the controls.

Gene Symbol	Protein	Log ² FC C3H/HeJ	Log ² FC C3H/HeN	Function
<i>Ccl12</i>	MCP-5	3.30	4.97	Coordinates cell movement during immune responses
<i>Ccl2</i>	JE/MCP-1	3.75	4.50	Chemotactic factor that recruits macrophages during immune responses
<i>Ccl5</i>	RANTES	3.20	3.47	Proinflammatory chemokine recruiting various cells to the site of inflammation
<i>Ccl6</i>	CCL6	2.14	1.60	Rodent-specific chemokine produced by macrophages
<i>Ccl7</i>	MCP-3	3.36	4.76	Recruits various cells to infected tissues to mediate the immune response
<i>Ccr1</i>	CD191	3.70	3.22	Membrane receptor that interacts with several proinflammatory cytokines
<i>Cxcl13</i>	BLC	6.09	6.92	Chemotactic factor that specifically recruits B cells
<i>Cxcl2</i>	MIP-2	5.24	6.69	Chemotactic factor with a preference for neutrophils
<i>Cxcl3</i>	MIP-2beta	6.57	5.42	Macrophage inflammatory protein
<i>Cxcl5</i>	ENA-78/SCYB5	5.62	3.25	Chemotactic factor with a preference for neutrophils
<i>Cxcr2</i>	IL8RB	3.36	1.84	Multiple cellular functions
<i>Il10</i>	IL-10	5.73	5.68	Major anti-inflammatory cytokine

Table 13: Immune Transcripts in HeN Mice. Upregulated immune transcripts of infected C3H/HeN mice in comparison to their controls. Log² FC is the fold change in copy number in comparison to the controls. N/A means no difference in fold change in comparison to the controls.

Gene Symbol	Protein	Log ² FC C3H/HeJ	Log ² FC C3H/HeN	Function
<i>Ccl19</i>	CCL19	N/A	4.05	Macrophage proinflammatory chemokine strongly regulated by IL-10
<i>Ccl3</i>	MIP-1 α	N/A	4.75	Macrophage inflammatory protein stimulated by bacterial endotoxins
<i>Ccl8</i>	MCP-2	N/A	4.63	Recruits various cells during immune responses
<i>Ccr12</i>	CCRL2	N/A	2.65	Function currently not well understood
<i>Cxcl1</i>	KC	N/A	3.06	Recruits various cells to infected tissues to mediate the immune response
<i>Cxcl10</i>	IP10	N/A	4.32	Secreted in response to IFN-gamma and recruits various cells
<i>Cxcl11</i>	I-TAC	N/A	1.56	Secreted in response to interferons and recruits various cells
<i>Cxcl9</i>	MIG	N/A	3.83	Secreted in response to IFN-gamma and recruits various cells
<i>Ifng</i>	IFN- γ	N/A	3.83	Major cytokine with a generally proinflammatory effect
<i>Ccl19</i>	CCL19	N/A	4.05	Macrophage proinflammatory chemokine strongly regulated by IL-10
<i>Ccl3</i>	MIP-1 α	N/A	4.75	Macrophage inflammatory protein stimulated by bacterial endotoxins
<i>Ccl8</i>	MCP-2	N/A	4.63	Recruits various cells during immune responses

Table 14: Downregulated Immune Transcripts. Downregulated immune transcripts of infected mice in comparison to their controls. Log² FC is the fold change in copy number in comparison to the controls. N/A means no difference in fold change in comparison to the controls.

Gene Symbol	Protein	Log ² FC C3H/HeJ	Log ² FC C3H/HeN	Function
<i>Ccl24</i>	Eotaxin-2	-7.59	-7.23	Interacts with CCR3 to recruit immune cells
<i>Ccr3</i>	CCR3	-2.36	N/A	Membrane receptor that interacts with several pro-inflammatory cytokines

Appendix 8

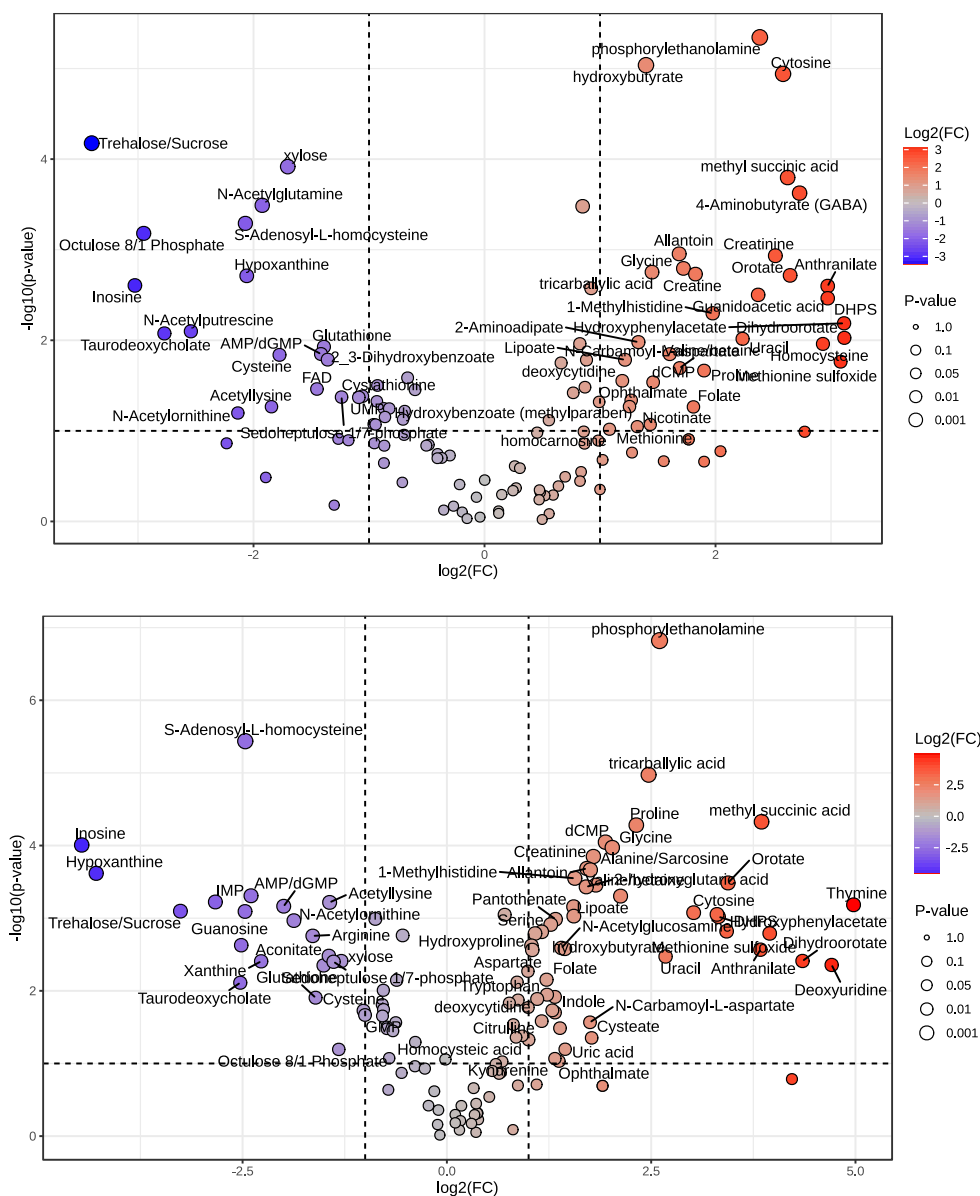


Figure 17: Metabolite Volcano Plots. Volcano plots where the y-axis represents the $-\log(\text{p-value})$ and the x-axis represents the $\log^2$ fold change (fc). Metabolites with a $\text{fc} > 2$ and $p < 0.1$ are shown. For the top graph, blue represents metabolites that are upregulated in the control C3H/HeJ group while metabolites in red are upregulated in the infected C3H/HeJ group. For the middle graph, blue represents metabolites that are upregulated in the control C3H/HeN group while metabolites in red are upregulated in the infected C3H/HeN group. For the bottom graph, blue represents metabolites that are upregulated in the infected C3H/HeN group while metabolites in red are upregulated in the infected C3H/HeJ group.

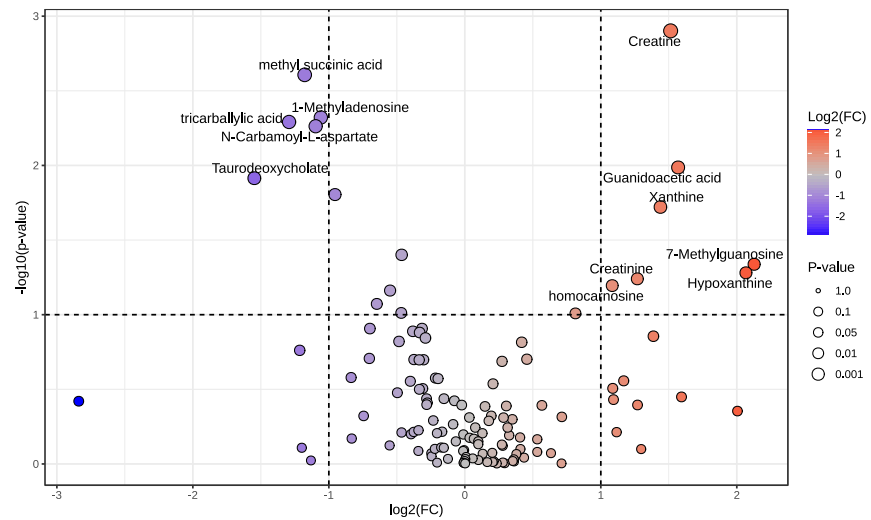


Figure 17 (Continued): *Metabolite Volcano Plots.*

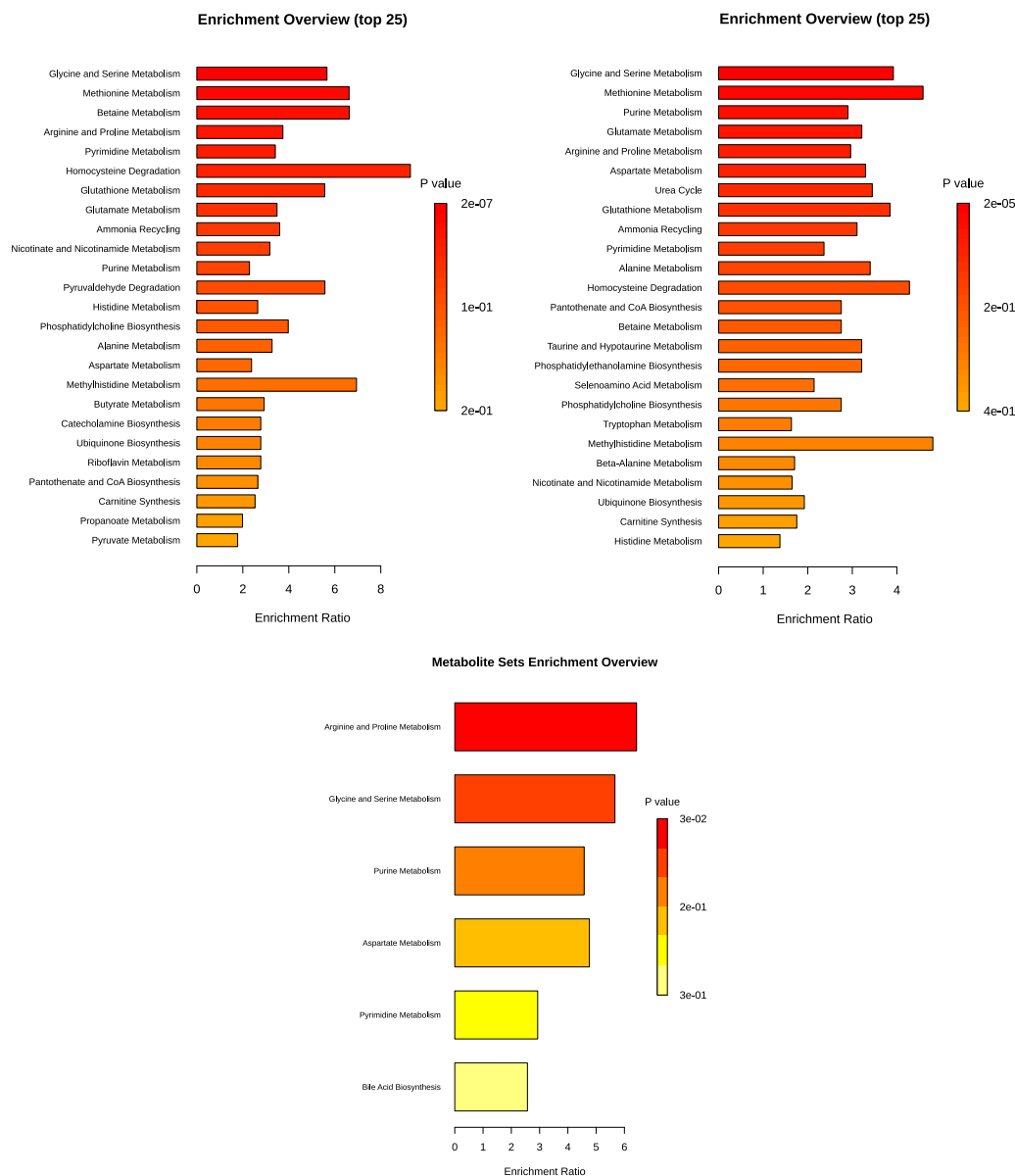


Figure 18: Top Metabolic Pathways. The differentially abundant (DA) metabolites from the infected and control C3H/HeJ mice (top left), infected and control C3H/HeN mice (top right), and infected C3H/HeJ and infected C3H/HeN mice (bottom) comparison were used to perform an enrichment analysis to determine pathways impacted. Metabolites were DA if $FC > 2$ ($fc < 0.5$) with $p < 0.1$. Enrichment Ratio is computed by Hits / Expected, where hits = observed hits; expected = expected hits.

VITA

Bryanna Fayne was born and raised in Memphis, Tennessee. After high school, she attended the University of Tennessee, Knoxville where she graduated summa cum laude with a Bachelor of Animal Science. Her passion for research guided her to pursue a Master of Science at her alma mater in the Comparative and Experimental Medicine program. Her research interests focus on zoonotic diseases and animal models with the goal of improving human and animal health. After graduation, she will attend the University of Tennessee College of Veterinary Medicine to pursue her DVM degree. She is grateful for the support she received through her graduate studies from her graduate mentor, lab mates, family, and friends.