Maternal nematode infection alters neonatal brain gene expression and maternal and neonatal microbiomes

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Abstract

The objectives of the thesis were to study the consequences of maternal nematode infection and protein deficiency on fetal and neonatal brain gene expression and on maternal and neonatal microbiome using timed pregnant mice and its intestinal nematode, *Heligmosomoides bakeri,* as the experimental model.

The first study showed that repeated maternal nematode infection during pregnancy caused differential expression of several genes in the fetal brain at gestation day 18, including genes involved in metabolic, developmental and immune system processes. Up-regulated gene expression was noted for *Gdf15* and *Ing4* that are involved in the neuro-development and synaptic plasticity, *miRNA let-7* that is involved in neural differentiation, *S100A8* and *S100A9* that are associated with neuro-inflammation and *Tnnt1* and *Atf3* that are involved in glucose metabolism. Maternal protein deficiency up-regulated only *Prss22* gene whereas the combination of protein deficiency and maternal infection down-regulated only *Dynlt1a* gene. The study suggested that the homeostasis of fetal brain development is maintained during maternal protein deficiency but not during maternal nematode infection. Hence, subsequent studies focused only on the impact of maternal infection.

The second study explored the consequence of maternal nematode infection on postpartum brain gene expression (postnatal day 2 and 7) of male pups. The results showed that the postnatal day 2 brain was not influenced by maternal nematode infection however, on postnatal day 7 I recorded differential expression of 5736 genes ($P<0.05$; 2751 up-regulated and 2985 down-regulated). KEGG pathway analysis demonstrated that the differentially expressed genes were involved in 99 pathways. Of particular interest were five key up-regulated pathways responsible for conferring synaptic plasticity and long-term potentiation in the neonatal brain. The evidence of limited neuro-inflammation aligns with the anti-inflammatory responses to nematode infections. Thus, these data suggested that the maternal nematode infection was positively associated with post-natal synaptogenesis in the brain in uninfected 7-day old male pup.

As gut-brain cross-talk plays an important role in brain development, and as this cross-talk is influenced by the gut microbiome, the third study investigated the effect of maternal nematode infection on colonization of the neonatal microbiome by 16s RNA analysis of maternal intestinal, vaginal and milk microbiomes and the pup stomach microbiome. Although there was no absolute difference in bacterial abundance in any of the maternal microbiomes, maternal nematode infection increased the relative abundance of *Lactobacillus* in both intestinal and vaginal microbiomes. The pup stomach microbiome was similar to that of the maternal vaginal tract, indicating that this was the primary source of initial microbial colonization. Furthermore, predictive metabolic pathway analysis of the pup stomach microbiome showed up-regulation of the acetate production pathway. This short-chain fatty acid is a mediator of gut-brain communication, and up-regulation of this pathway may provide a mechanism through which maternal infection influences pup brain gene expression. Additionally, maternal infection up-regulated amino acid biosynthesis, carbohydrate/ energy metabolism and vitamin-B biosynthesis pathways in the pup stomach microbiome, suggesting possible benefits of maternal nematode infection on neonatal functional microbiome.

In conclusion, this study provides evidence that maternal infection may be beneficial to the uninfected pups both through improved neuro-development and heighted gastrointestinal nutrient synthesis pathways. Possible underlying mechanisms may involve reduced neuro-inflammation and altered microbe-mediated communication between the pup gastrointestinal tract and the brain.

Abstrait

Les objectifs de cette thèse étaient d'étudier les conséquences d'une infection maternelle par nématodes et d'une carence maternelle en protéines sur l'expression génétique neuronale fœtal et néonatale et le microbiome maternel et néonatal en utilisant des souris femelles enceintes et leur nématode intestinal, *Heligmosomoides bakeri,* en tant que model expérimental.

La première étude a démontré qu'une infection par nématodes répétée lors de la grossesse engendre l'expression différentielle de multiples gènes dans le cerveau fœtal au 18ième jour de gestation, incluant des gènes impliqués dans des procédés métaboliques, développementaux et immunitaires. Une expression génétique régulée à la hausse a été notée pour *Gdf15* et *Ing4* qui sont impliqués dans le développement neuronal et la plasticité synaptique, *miRNA let-7* qui est impliqué dans la différentiation neuronale, *S100A8* et *S100A9* qui sont associés à la neuro-inflammation et *Tnnt1* et *Atf3* qui sont impliqués dans le métabolisme du glucose. La carence maternelle en protéines n'a seulement régulé à la hausse que le gène *Prss22*, alors que la combinaison de carence en protéines et l'infection maternelle n'a seulement régulé à la baisse que le gène *Dynltla*. Cette étude suggère que l'homéostasie du développement du cerveau fœtal est maintenue lors de la carence maternelle en protéines, mais non lors d'une infection maternelle par nématodes. Par conséquent, les études suivantes furent seulement concentrées sur l'impact de l'infection maternelle.

La deuxième étude a exploré les conséquences d'une infection maternelle par nématodes sur l'expression génétique cérébrale postpartum (jour postnatal 2 et 7) des souriceaux males. Les résultats ont démontré que le cerveau au second jour postnatal n'était pas affecté par l'infection maternelle alors qu'au septième jour postnatal, j'ai enregistré l'expression différentielle de 5736 gènes ($P < 0.05$; 2751 régulés à la hausse et 2985 régulés à la baisse). L'analyse contre la base de données KEGG a démontré que les gènes exprimés de façon différentielle étaient impliqués dans 99 mécanismes biologiques. Un intérêt particulier a été porté aux cinq principaux mécanismes régulés à la hausse responsables de conférer la plasticité synaptique et la potentialisation à long terme au cerveau néonatal. La preuve de la présence d'une neuro-inflammation limitée correspond avec les réponses anti-inflammatoires contre les infections par nématodes. De ce fait,

ces données suggèrent que l'infection maternelle par nématodes serait positivement associée avec la synaptogenèse postnatale dans les cerveaux de souriceaux males non-infectés et âgés de sept jours.

Étant donné que la diaphonie intestinale-cérébrale joue un rôle important dans le développement cérébral et que cette diaphonie est influencée par le microbiome intestinal, la troisième étude a investigué les effets d'une infection maternelle par nématodes sur la colonisation du microbiome néonatal. Ceci a été accompli grâce à l'analyse 16s RNA des microbiomes maternels intestinaux, vaginaux et laitier ainsi que le microbiome de l'estomac des souriceaux. Malgré qu'aucune différence absolue concernant l'abondance bactériale dans tous les microbiomes maternels ne fût présente, l'infection maternelle par nématodes a augmenté l'abondance relative de *Lactobacillus* dans les microbiomes vaginaux et intestinaux. Le microbiome présent dans l'estomac des souriceaux était similaire à celui de la voie vaginale, ce qui indique que ce dernier était la source primaire de colonisation microbactérienne initiale. Par ailleurs, l'analyse prédictive des mécanismes métaboliques du microbiome de l'estomac des souriceaux démontre une régulation à la hausse du mécanisme de production d'acétate. Cet acide gras à courte chaine est un médiateur des communications entre les intestins et le cerveau et la régulation à la hausse de cette voie biologique peut fournir un mécanisme au travers lequel une infection maternelle par nématodes pourrait influencer l'expression génétique des cerveaux des souriceaux. De plus, l'infection maternelle a régulé à la hausse la biosynthèse d'acides aminés, le métabolisme énergétique et d'hydrate de carbone et le mécanisme de biosynthèse de vitamine B dans le microbiome de l'estomac des souriceaux. Ceci suggère de possibles bénéfices pour les microbiomes néonatals fonctionnels qui seraient attribués à l'infection maternelle par nématodes.

En conclusion, cette étude démontre évidences qu'une infection maternelle peut possiblement être bénéfique pour les souriceaux non-infectés en améliorant le développement neurologique et en haussant les voies de la synthèse de nutriments gastro-intestinaux. De possible mécanismes sous-jacents peuvent inclure une réduction de l'inflammation neurologique et une altération de la communication microbienne entre le système gastro-intestinal et le cerveau du souriceau.

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Contributions of Authors

The thesis is written by Manjurul Haque as manuscript-based style as per the guidelines of McGill University. It contains three manuscripts, co-authored with my academic supervisors Dr. Marilyn E. Scott and Dr. Kristine G. Koski. Additionally, the first manuscript (Chapter-III) is co-authored with Dr. Lisa M. Starr.

For the first (Chapter-III) study, Dr. Starr had collected the fetal brain samples from her previous study. I extracted RNA from the fetal brain and Illumina Hi-seq RNA sequencing was performed in the McGill University and Génome Québec Innovation Centre. After obtaining the raw sequencing data, I filtered, and processed the sequencing reads, analyzed and interpreted the results, performed qPCR confirmation and wrote the manuscript. My supervisors, Dr. Scott and Dr. Koski, provided input on the study design and data interpretation and provided critical suggestions that have been incorporated into the manuscript.

For the second (Chapter-IV) study, I designed, conducted the experiment, and collected the pup liver and the brain samples and extracted DNA from the pup liver and RNA from the pup brain. I determined the sex of pups by PCR. Illumina Hi-seq RNA sequencing was performed in the McGill University and Génome Québec Innovation Centre. After obtaining the raw sequencing data from the sequencing centre, I processed and analyzed the data. I conducted all the time-series, KEGG pathway, and ontology analysis, interpreted the results and wrote the manuscript. My supervisors, Dr. Scott and Dr. Koski, provided input on the study design and data interpretation and provided critical suggestions that have been incorporated into the manuscript.

I designed the third (Chapter-V) study and collected the microbiome samples from my second experiment. I extracted DNA from all the microbiome samples and the illumine Mi-seq sequencing was done in McGill University and Génome Québec Innovation Centre. After obtaining 16S rRNA sequence data, I processed the data using bioinformatic tools, interpreted the results, and wrote the manuscript. My supervisors, Dr. Scott and Dr. Koski, provided input

on the study design and data interpretation and provided critical suggestions that have been incorporated into the manuscript.

Dr. Scott obtained funding for all three studies. First (Chapter-III) and second (Chapter-IV) manuscripts are published and are reproduced with permission of the publishers, and the third manuscript (Chapter-V) will be submitted in the near future.

Statement of Originality

Using an *in vivo* model where pregnant and lactating mice were infected with an intestinal nematode that remains in the maternal intestine and does not directly infect the fetus or neonate, I have made the following novel contributions to research.

1. This is the first study to explore the impact of maternal nematode infection on fetal (GD18) and postnatal (P2, P7) brain gene expression.

2. By comparing the consequences of maternal infection and/or maternal protein deficiency on gene expression at GD18, I demonstrated that maternal infection increased expression of 88 genes including several important for neurodevelopment and neural differentiation whereas moderate maternal protein deficiency had minimal impact on fetal brain gene expression, only influencing expression of three genes.

3. An unappreciated effect of maternal nematode infection on the temporal pattern of neonatal brain gene expression was found. Between GD18 and P2, gene expression was up-regulated for the vast majority of genes, regardless of maternal infection. However, between P2 and P7, expression of about 35% of these genes remained elevated in pups of infected dams but not in control pups.

4. In contrast to the perception that nematode infections are harmful, I reported a beneficial effect of maternal nematode infection in pregnancy on the key gene expression pathways controlling synaptogenesis and long-term potentiation in the P7 pup brain.

5. Furthermore, several pathways related to autoimmune diseases (autoimmune thyroid disease, systemic lupus erythematosus and type I diabetes mellitus) and immune activation related (asthma) disease pathways were down-regulated in response to maternal infection. Also, maternal infection down-regulated genes for pro-inflammatory cytokines such as IL-1β, IL-6 and up-regulated genes related to anti-inflammatory

response such as IL-4, TGF-β and FOXP3. Thus, the improved synaptogenesis and long-term potentiation may be the result of nematode-induced anti-inflammatory responses.

6. However, pathways related to purine and pyrimidine metabolism were down-regulated in the P7 pup brain of infected mothers.

7. The impact of nematode infection of the maternal microbiome was expanded to report region-specific shifts in the intestinal tract early during lactation, and to include information on the vaginal and milk microbiome. Of note, the relative abundance of beneficial *Lactobacillus* was higher in the vaginal tract of infected dams.

8. I also showed for the first time that a maternal nematode infection altered the relative abundance and diversity of the neonatal stomach microbiome and enhanced the relative abundance of *Lactobacillus* in the P7 pup stomach.

9. Maternal nematode infection up-regulated functional pathways related to *de novo* amino acid biosynthesis including essential amino acids, carbohydrate and energy metabolism, vitamin biosynthesis and other co-factors in the P2 microbiome. This may have beneficial consequences to the growing pup.

10. Furthermore, the functional alteration of neonatal stomach microbiome included up-regulation of microbial pathways involved in gut-brain cross-talk that may have enhanced gut-brain communication in the pups of infected dams, contributing to altered brain gene expression.

11. Taken together, this study provides evidence that maternal nematode infection during pregnancy and early lactation may provide benefits to early postpartum growth and development.

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List of Abbreviations

AMPA	α-amino-3-hydrozy-5-methylisoxazole-4-propionic acid
ANCOM	Analysis of composition of microbiomes
ANOVA	Analysis of variance
BAM	Binary alignment map
cDNA	Complementary DNA
CFU	Colony-forming unit
Cq	Quantification cycle
DAVID	Database for annotation, visualization and integrated discovery
DNA	Deoxyribonucleic acid
E	Embryonic day
EdgeR	Empirical analysis of digital gene expression data in R
GBA	Gut-brain axis
GD	Gestation day
GEO	Gene expression omnibus
GI	Gastrointestinal
GO	Gene ontology
IL	Interleukin
IMEX	International molecular exchange
KEGG	Kyoto encyclopedia of genes and genomes
L_n	n stage of larvae
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEBA	Multivariate empirical bayes time-series analysis
MIQE	Minimum information for publication of quantitative real-time PCR experiments
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartic acid
NSERC	Natural sciences and engineering research council of Canada
OTU	Operational taxonomic unit

P	Postnatal day
PANTHER	Protein analysis through evolutionary relationships
PCoA	Principal coordinate analyses
PD	Protein deficient
PERMANOVA	Permutational multivariate analysis of variance
PI	Post-infection
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PS	Protein sufficient
QIIME	Quantitative insights into microbial ecology
qPCR	quantitative PCR
REVIGO	Reduce visualize gene ontology
RNA	Ribonucleic acid
SAM	Sequence alignment map
Th	T helper
TNF- α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
WNT	Wingless-related integration site

Chapter I

Introduction

Globally more than 1.5 billion people (24% of the world's population) are infected with soil transmitted gastrointestinal (GI) helminth parasites namely, *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (WHO, 2017). Although the intensity and prevalence of *A. lumbricoides* and *T. trichiura* decrease in adulthood (Bundy et al, 1987), the prevalence of hookworm tends to increase with age, and pregnant women living in endemic settings are likely to have a high burden of infection (Pullan et al, 2010; Mpairwe et al, 2014). Studies in animal and human populations suggest that pregnancy may predispose females to helminth infections (Pelletier et al, 2005; Adegnika et al, 2007, 2010; Hernández-Bello et al, 2010).

Pregnancy induces hormonal changes which generate an immunotolerant environment to accommodate the immunologically foreign fetus. Immunotolerance is driven by a high concentration of progesterone which suppresses the pro-inflammatory Th1 response and increases the anti-inflammatory Th2 response in the pregnant female (Szekeres-Bartho & Polgar, 2010). Furthermore, helminth infection produces a strong Th2 response with the elevation of Th2 cytokines such as IL-4, IL-10 and TGF-β (Reynolds et al, 2012). In concurrent pregnancy and nematode infection, Odiere et al (2010a) showed that nematode infection exacerbates the Th2 response by further increasing IL-4 concentrations in pregnant mice. Additionally, like the developing fetus, helminths rely on their host for nutrients (Blackwell 2016), reducing nutrient availability for the host. Furthermore, GI helminths also impair nutrient absorption in the gut (Solomons 1993).

Helminth infection and malnutrition generally co-occur and are widely prevalent in developing countries (Stephenson 1994; Hailegebriel 2018). Due to the synergistic nutritional and energetic demand by helminth infection and fetal development, helminth infection in malnourished pregnant females imposes a huge nutritional demand which, if not met optimally, affects intrauterine growth (Steketee 2003). One of the key nutrients

is protein and the demand for protein increases in pregnancy in order to maintain maternal homeostasis and support fetal growth (Mousa et al, 2019). In addition to acting as a building block of maternal uterine and fetal tissue, protein is also required for producing immunoglobulins and cytokines in helminth infections (McGaha et al, 2012). Although the placenta protects the developing fetus from obnoxious cues thus ensuring normal growth and development (Broad and Keverne, 2011), recent work from my lab using a mouse model showed that maternal helminth infection and protein deficiency in pregnancy not only altered the gene expression in the placenta but also changed the concentration of cytokines and growth factors in the amniotic fluid and in the fetal circulation (Starr et al, 2015; Starr et al, 2016). Despite the critical role of protein and the frequent occurrence of helminth infections in pregnancy, the individual and combined effects of protein deficiency and helminth infection on neonatal growth and development is only recently beginning to be understood.

The intrauterine environment plays a crucial role on an organism's later growth and functioning through a mechanism known as "developmental programming" by which a number of external environmental factors negatively influence the structure and function of organs (Seckl and Meaney, 2004). Maternal protein deficiency and nematode infection have been shown to be associated with alterations in fetal and subsequent postnatal development. Odiere et al (2010a) showed that maternal protein deficiency reduced fetal mass and elevated stress hormone concentrations whereas maternal nematode infection reduced fetal length and elevated the pro-inflammatory cytokine IL-1β in pup circulation. Furthermore, Starr et al (2015) demonstrated alterations of stress hormones, growth factors, and cytokines in the maternal and fetal compartments in response to maternal protein deficiency and nematode infection. The intrauterine effects of maternal protein deficiency and nematode infection persist in postnatal life as Odiere et al (2010b) reported stunting among pups from both nematode-infected and protein-deficient mothers. Furthermore, Kristan (2002) reported larger liver and stomach, longer small intestine and heavier serosa in pups from nematode-infected mothers. Despite these effects of maternal nematode infection and protein deficiency on neonatal organogenesis, no information is available on the impact of these factors on neonatal brain development.

Development of the mammalian brain begins with the differentiation of neural progenitor cells early in pregnancy and continues postpartum through neural differentiation, migration and synaptogenesis at least until early adulthood (Stiles and Jernigan, 2010). During this developmental period, maternal (e.g., infection, nutritional deficiencies) and environmental factors (e.g., toxins, stress) may influence the perinatal brain (Seckl and Meaney, 2004). Maternal protein deficiency has been associated with a reduction in growth rate (Marín et al, 1995), alteration of brain architecture (Gressens et al, 1997), and fewer neural stem cells (Gould et al, 2018) in the neonatal brain. Maternal nematode infection has been associated with poor early brain executive function and language (Nampijja et al, 2012) and poor motor and cognitive development among infants (Mireku et al, 2015).

Additionally, at the time of birth, the neonatal gut is colonized by the maternal microbiome, and the gut microbiome plays a critical role in postnatal brain maturation (Heijtz et al, 2011; Jašarević et al, 2015). Neonatal colonization takes place at the time of delivery, and the maternal birth canal and vaginal microbiomes are the major source for the neonatal microbiome (Jašarević et al, 2015). Dysbiosis of the neonatal microbiome has been associated with impaired sensorimotor behaviors (Hsiao et al, 2013), a defective hypothalamic-pituitary-adrenal (HPA) axis (Sudo et al, 2004), faulty neuronal circuits (Heijtz et al, 2011), and an imperfect blood-brain barrier (Braniste et al, 2014). Maternal stress alters the vaginal microbiome leading to dysbiosis in the neonatal stomach (Jašarević et al, 2015). Moreover, helminths have been shown to alter the gut microbiome in the host (Walk et al, 2010).

Rationale and Research Objectives

Given that maternal protein deficiency and nematode infection alter neonatal organogenesis, that maternal protein deficiency and nematode infection may affect neonatal brain development, that neonatal microbiome contributes significantly to the postnatal brain maturation, that maternal stress alters maternal vaginal microbiome, and that nematodes alter the host microbiome, my thesis addresses the following objectives:

Study 1: To identify the changes in mouse fetal brain gene expression induced by maternal protein deficiency, GI nematode infection and their interaction;

Study 2: To evaluate the impact of maternal nematode infection on neonatal brain gene expression; and

Study 3: To examine the effect of maternal nematode infection on the maternal and neonatal microbiomes and on neonatal microbial colonization.

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Chapter II

Literature Review

2. 1 Pregnancy

Pregnancy induces a variety of physiological alterations in the body to accommodate the immunologically distinct fetus. In 1953, the fetus was first proposed as a semi-allograft (Medawar, 1953) that requires hormonal and immunological modifications to ensure successful implantation and growth over the entire duration of gestation which in mice is typically 20-21 days (Jørgensen et al, 2019).

2.1.1 Hormonal changes and immunomodulation in pregnancy

Prolongation of the corpus luteum (CL) and suppression of ovulation are requirements for pregnancy (Mesen and Young, 2015). Survival of the CL is initially supported by prolactin and follicle stimulating hormone and later maintained by the luteotropic hormone complex in mice (Barkley et al, 1979). Maintenance of the CL is essential for the continuous secretion of progesterone, which is crucial for a successful gestation (Schindler, 2015). In addition to progesterone, the initial stage CL also produces estrogen which is required to support pregnancy. By mid-gestation (in mice around day 11), the placenta starts producing the needed hormones (Barkley et al, 1979). The requirement of estrogen starts on day 11 and last for 3-4 days (Milligan and Finn, 1997). From gestation day 15 onwards, pregnancy is maintained by only progesterone whose concentration also declines (Milligan and Finn, 1997) especially from gestation day 19 onwards to facilitate the parturition process (Virgo and Bellward, 1974).

High concentrations of progesterone during pregnancy induce a tolerogenic effect in the maternal immune system to accommodate the fetus and favor its growth (Szekeres-Bartho and Polgar, 2010). Lymphocytes from pregnant mouse dams are 100-fold more sensitive to progesterone compared to those from non-pregnant dams suggesting

enhanced progesterone binding in the lymphocytes of pregnant dam (Szekeres-Bartho et al, 1985). The effect of progesterone is mediated by lymphocyte-derived protein known as progesterone-induced blocking factor (PIBF) (Laskarin et al, 2002). PIBF stimulates the production of Th2 cytokines via interleukin-4-receptor mediated activation of Jak/Stat signaling (Szekeres-Bartho and Polgar, 2010) which suppresses proinflammatory Th1-cytokines *viz.* interferon (IFN)-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)-α production and promotes anti-inflammatory Th2-cytokines *viz.* IL-4, IL-5, IL-9, IL-10 and IL-13 production providing a immunotolerant environment in the body (Veenstra van Nieuwenhoven et al, 2003).

2.2 *Heligmosomoides bakeri* - mouse model

Heligmosomoides bakeri (also known as *H. polygyrus* and previously known as *Nematospiroides dubius*), a naturally occurring GI nematode of mice, is in the family Trichostrongylidae. It has been adopted as an excellent laboratory model of chronic GI nematode infection including animal and human hookworms (Behnke et al, 2009; Behnke and Harris, 2010).

2.2.1 *H. bakeri* life cycle

The life cycle of *H. bakeri* (Figure 1) is direct, involving three free-living larval stages (L_1, L_2 and L_3), and parasitic L_4 and adult worms. The adult parasite resides predominantly in the anterior part of the small intestine and female worms lay eggs that are excreted out via faeces into the environment (Lewis and Bryant, 1976). Eggs are typically 70-84 μm in length and 37-53 μm in width and contain 8-16 cells (Bryant, 1973). Within 36 hours of release, eggs hatch into the L_1, which subsequently undergo two moults into a non-feeding L_3. The L_3 is the infective stage and infection occurs when mice ingest the L_3 along with food and water or experimentally by oral gavage. During the first 24 hours, the larvae invade the small intestine and infiltrate the mucosa and submucosa where they undergo two further moults and subsequently emerge back into the intestinal lumen as adult worms 7-9 days post-infection (PI). The adult worms do not

migrate out of the intestinal lumen; they secure themselves by coiling around the intestinal villi where they mature, mate and produce eggs (Reynolds et al, 2012). By day 9 PI, the infected mouse starts passing eggs in faeces. The maximum egg production was documented to occur between 20 to 40 days PI when approximately 1200-1500 eggs per female worm per day were recorded from mice infected with 100 infective L_3 (Bryant, 1973; Kerboeuf, 1982).

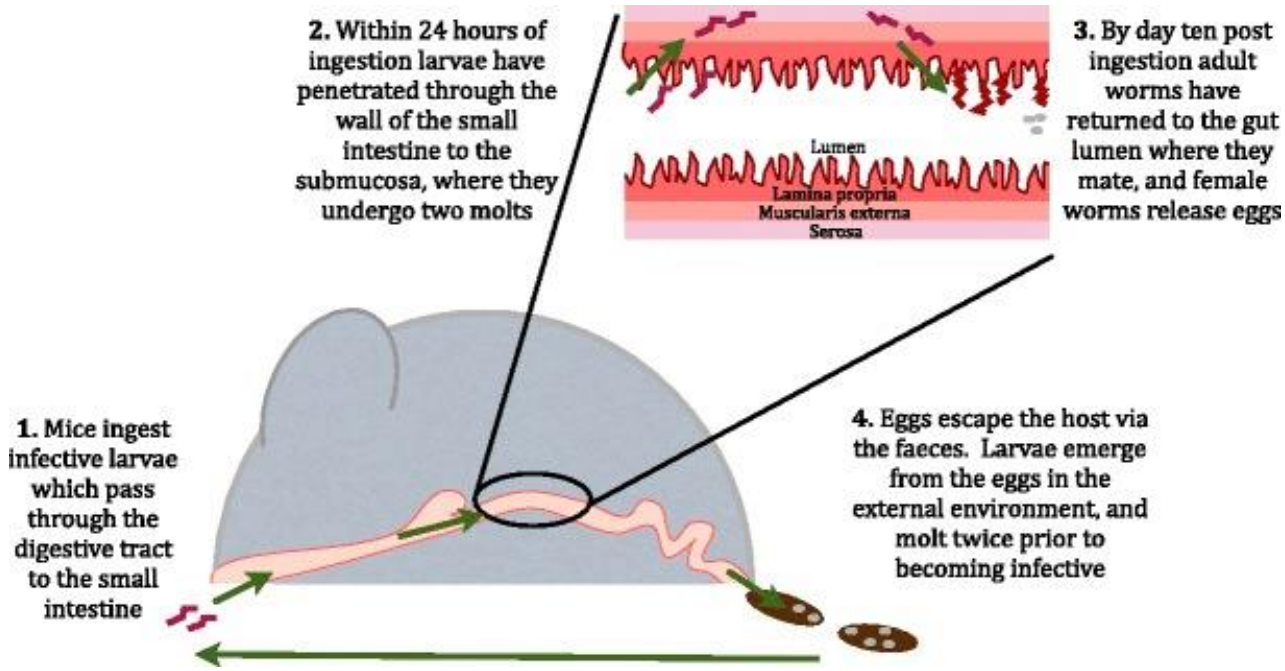

Figure 1. Life cycle of *Heligmosomoides polygyrus* in mice (Reynolds et al, 2012). Permission to use content in the thesis was granted by Copyright Clearance Centre's RightsLink® service on behalf of Springer Nature on May 9, 2019.

2.2.2 Immunity to *H. bakeri*

H. bakeri is a widely used nematode model to understand the immune interaction between host and parasite (Behnke et al, 2009). The mouse immune response against this nematode is CD4+ Th2-cell mediated, and its effectiveness varies based on the frequency of the infection (Maizels et al, 2012). A primary infection is generally non-resolving

(Maizels et al, 2012) and becomes chronic (Valanparambil et al, 2017) because adult *H. bakeri* release immunosuppressive factors that minimize the protective Th-2 response (Filbey et al, 2014) whereas a secondary, or challenge, infection is typically cleared within a month. A repeated trickle infection persists longer than a challenge infection but not as long as a primary infection (Maizels et al, 2012).

During a primary infection, following penetration of the intestinal mucosa by *H. bakeri* L_3, the infection provokes expression of IL-3, IL-4, IL-5 and IL-9 genes in the mesenteric lymph nodes and Peyer's patches and stimulates release of IL-4, IL-5, IL-9, IL-10 and IL-13 cytokines (Reynolds et al, 2012). Among these, IL-4 has been shown to be the most important for conferring protective immunity against the nematode (Liang et al, 2011). Exogenous supplementation of IL-4 in IL-4 deficient mice induced a Th2 response against invading nematode larvae (Urban et al, 1995) whereas depletion of IL-4 abolished the immune response (Urban et al, 1991).

During repeated or challenge infections, an extraordinary surge of nonspecific IgG1 was also recorded (Pritchard et al, 1983). Compared with uninfected mice, this IgG1 concentration increases as high as 30-fold following trickle infection over 4 weeks (Maizels et al, 2012). Passive transfer of IgG1 obtained from mice with repeated infection can confer protection in naïve mice from *H. bakeri* infection as evidenced by reduction in worm length and fecundity (McCoy et al, 2008). The nematode-induced IL-4 and IL-13 stimulate the production of goblet cells and mucin and also the release of RELM-β which has inhibitory activity against adult worms (Herbert et al, 2009). The cytokines induce Th2 effectors including serum IgE, eosinophils, mucosal and mast cells leading to enhanced mucous secretion from goblet cells, ion and fluid influx and increased intestinal smooth muscle contraction that subsequently leads to expulsion of worms from the intestine (Tu et al, 2008; Hashimoto et al, 2009).

The challenge infection is characterized by the development of granulomatous lesions at the site of larval encystment in the intestine (Maizels et al, 2012). These granulomas contain IL-4 and/or IL-13- dependent IL-4R induced alternatively activated macrophages and granulocytes that are associated with either killing or immune-mediated

damage of the nematode larvae (Prowse et al, 1979). Although the lesions persist, parasites may leave the granuloma and enter the intestinal lumen (Cywinska et al, 2004). Because the adaptive response to the second infection occurs much more rapidly than in a primary infection, immunosuppression by the adult worms is less important during challenge infection, and worm expulsion is more efficient.

2.2.3 Immune regulation by *H. bakeri*

The primary immune response against *H. bakeri* is mediated by a Th2 response which also activates proliferation of regulatory T (Tregs) cells (Dubey et al, 2019). The proliferation of Foxp3+ CD4+ Tregs suppresses host immunity and induces tolerance (Finney et al, 2007). The mechanism of immune suppression is suggested to be mediated by cytokines specially IL-10 and TGF-β (Walk et al, 2010) but it was demonstrated that in absence of IL-10, *H. bakeri* alone can minimise experimentally induced colitis (Elliott et al, 2004) suggesting that IL-10 does not play a principle role in immune suppression. Subsequent studies proved that IL-10 along with IL-4 actually promote the Th2 response by suppressing Th1 cells (Finney et al, 2007). Another important cytokine is TGF-β which rises three-fold over 30 days of infection and the parasite also secretes a TGF-β mimic into circulation (Ince et al, 2009). The demonstration that *H. bakeri* infection fails to improve colitis in TGF-β receptor knockdown mice suggests that TGF-β plays a very important role in infection-mediated immunomodulation (Ince et al, 2009). A recent study demonstrated that *H. bakeri* down-regulates the expression of *Smad7* gene in CD4+ T cells leading to induction of TGF-β mediated immune regulation which blocks colitis (Hang et al, 2019).

2.2.4 Trickle infection protocol for *H. bakeri*

Under natural conditions, mice are exposed to *H. bakeri* infection in frequent intervals throughout their lives. In contrast, most experiments designed to understand the nature of host-parasite interactions use a single dose infection (a primary infection) which

does not reflect the natural transmission (Brailsford and Behnke, 1992). Hence, trickle infection protocols were introduced to simulate natural transmission in laboratory settings (Brailsford and Behnke, 1992). During trickle infections, animals are repeatedly infected with infective larvae which continuously stimulates host immune system. Infected hosts therefore harbor both larval and adult stages simultaneously (Berding et al, 1986). It has been demonstrated that as few as 10 *H. bakeri* larvae can induce strong immunological resistance to challenge infection following trickle infection in NIH mice (Brailsford & Behnke, 1992). The immune reaction is mediated by Th2 cytokines (IL-4, IL-5, and IL-13) (Brailsford and Behnke 1992) which increasingly delay larval development and increase expulsion of adult worms from the intestine as transmission continues (Scott 1991). The adult nematode and its larval stages remain in the intestine and there is no evidence indicating migration of the nematode by transplacental or intramammary route. Therefore, infected pregnant dams do not transfer *H. bakeri* to the fetus or to suckling pups.

2.2.5 Interactions between GI nematodes and host nutrition

The complex interplay of malnutrition and GI nematode infection has received considerable attention since the 1980's. Underlying mechanisms have been explored through lab models including *H. bakeri* and *Nippostrongylus brasiliensis*, through experiments in livestock, and through epidemiological studies in human populations. The results have been reported in a number of review articles (Stephenson 1994; Stephenson et al, 2000; Scott and Koski 2000; Koski and Scott 2001; Brooker et al, 2008; Athanasiadou 2012; Papier et al, 2014; Yap et al, 2014; Clough et al, 2016; Hoste et al, 2016; Ibrahim et al, 2017). GI nematodes respond to a variety of nutrient deficiencies but also contribute to host malnutrition leading to a negative spiral whereby infection exacerbates malnutrition and malnutrition exacerbates infection.

Nutrients play an integrative role in the course of GI nematode infection starting from establishment in hosts. For example, penetration of hookworm larvae was found to be positively correlated with high concentrations of saturated fatty acids such as lauric,

myristic and palmitic acids in skin (Haas et al, 2005) and infective *Strongyloides* larvae were shown to be attracted to urocanic acid which is produced in higher concentrations in the skin of the protein deficient host (Hug et al, 1998; Safer et al, 2007).

Many nutrient deficiencies impair host immunity to GI nematode infections. The immunogenicity of fourth stage larvae invokes a Th2 immune response that triggers mucus production, water efflux and increase peristalsis in the GI tract and thus generates an unfavorable milieu for survival of adult nematodes (Strandmark et al, 2016), but host protein deficiency and energy restriction reduce the Th2 immune response and thus favor the establishment and propagation of *Heligmosomoides* (Koski et al, 1999; Ing et al, 2000). Protein deficiency has also been shown to reduce B and T cell proliferation in *Nippostrongylus* infection in rats (Athanasiadou et al, 2011). Additionally, vitamin D, zinc and selenium deficiencies were also shown to impair the Th2 immune response against *Heligmosomoides* infection (Scott and Koski, 2000; Smith et al, 2005; Yamshchikov et al, 2009).

Additionally, macronutrients play an essential role in nematode egg production. Dubinsky et al (1986) showed that glucose is critical for chitin formation in the eggshell of *Ascaris suum*, energy restriction reduced the per capita egg production in *H. bakeri* in mice (Shi et al, 1995) and protein deficiency increased per worm egg production following a challenge infection with *H. bakeri* in mice (Tu et al, 2007). In goats infected with *Haemonchus contortus*, and a high protein diet significantly decreased fetal egg output perhaps because a high protein diet was able to maintain the integrity of the mucosal layer of the GI tract thus delaying nematode establishment of *Haemonchus* (Nnadi et al, 2007). The form of dietary fiber was also shown to be associated with the nematode egg production in a study by Sun et al (2002) that showed that high dietary pectin increased egg production in *H. bakeri* compared to the mice fed with cellulose.

Micronutrient deficiencies including zinc and selenium deficiency have been shown to favor survival, maturation, and egg production in *H. bakeri* (Shi et al, 1995; Smith et al, 2005). However, dietary boron deficiency decreased larval establishment and

accelerated worm expulsion following a challenge infection (Bourgeois et al, 2007). Additionally, vitamin A lowered the fecundity of *Trichuris* in pigs (Pedersen et al, 2001), whereas vitamin E deficiency increased egg production in *H. bakeri* (Smith et al, 2005; Au Yeung et al, 2005).

GI nematode infections also contribute to host malnutrition primarily by reducing food intake, impairing absorption through their negative impact on food intake and damage to the intestinal brush border that impairs absorption leading to vitamin A and B_{12} deficiency and trace mineral deficiencies depending on the type of infection (Stephenson et al, 2000). In human studies, *Ascaris* infection was associated with vitamin A malabsorption among preschool children (Haque et al, 2010) and with lower serum iron, zinc, and selenium among pregnant women (Arinola et al, 2015). Similarly, higher worm burden of Trichuris was associated with lower serum zinc concentration among preschool children (Kongsbak et al, 2006). High intensity *Trichuris* and hookworm infection can cause extensive damage to the enterocytes leading to loss of blood, serum albumin and total serum protein (Brooker et al, 2008; Khuroo et al, 2010; Thayer et al, 2017), and *Trichuris*, hookworms and *Strongyloides* have been shown to cause iron deficiency leading to hypohemoglobinemia and anaemia (de Gier et al, 2016; Loukas et al 2016; Echazú et al, 2017).

Evidence of the impact of *H. bakeri* on host nutrient concentrations is limited to minerals in liver and spleen that respond differently between a primary and challenge infection. *H. bakeri* was shown to increase copper concentration and decrease iron concentration in the spleen of infected mice but only during a challenge infection (Minkus et al, 1992). Furthermore, challenge infection reduced liver concentrations of boron, iron and zinc but increased concentrations of chromium, molybdenum, potassium, sodium and sulfur relative to a primary infection (Bourgeois et al, 2007). Finally, Tu et al (2009) observed lower calcium and iron concentrations and a lower calcium/zinc ratio in the spleen of *H. bakeri* challenged mice compared to a primary infection that had been resolved by drug treatment, perhaps due to the Th2 response to a challenge infection.

2.3 Helminth infection, pregnancy and immunity

GI helminths and the developing fetus are both immunologically foreign to the maternal body and they both induce an immunomodulatory effect. Animal studies indicate that pregnant animals are predisposed to subsequent helminth infection more so than non-pregnant animals (Pelletier et al, 2005; Hernández-Bello et al, 2010). As discussed above, pregnancy is associated with polarization towards a Th2 immune response; similarly, GI helminths suppress Th1 response and induce Th2 response (van Riet et al, 2007; Wammes et al, 2010). Moreover, *H.bakeri* infection during pregnancy further heightens concentrations of Th2 cytokines (Odiere et al, 2013). The resemblance of immune responses by helminths to immunomodulation during pregnancy suggests that helminths may have evolved to utilize parallel mechanisms for avoiding maternal immunity (Blackwell, 2016).

2.3.1 Effect of maternal nematode infection on pregnancy outcomes

A previous study showed that pregnancy induced favorable physiological changes in the host for helminths (van Eijk et al, 2009). Furthermore, *H. bakeri* infection was shown to increase IL-4, IL-5 and IL-13 production in the serum and IL-1β in the amniotic fluid of infected pregnant mice (Odiere et al, 2010a). In addition to these alterations in the cytokine environment, *H. bakeri* lowered maternal femur bone density and increased IL-6, IFN-γ and IL-1β in the serum of pregnant mice (Odiere et al, 2010a). The high concentrations of nematode-induced IFN-γ and IL-1β were further linked with fetal stunting as the fetal crown-rump length was lower in the nematode-infected dams (Odiere et al, 2010a). In another study, Odiere et al, (2010b) also recorded stunting among neonatal pups of nematode-infected dams that had elevated IL-1β and IL-6 in serum.

The impaired perinatal growth was associated with nematode-induced alteration of cytokines and growth factors in both maternal and fetal components as well as changes in the placenta. *H. bakeri* infection increased concentrations of Th2 cytokines (IL-4, IL-5, IL-13, IL-2, IL-10) and eotaxin in the serum of dams and eotaxin in the pup serum

(Odiere et al, 2013). Furthermore, maternal infection lowered f-prolactin which was positively associated with fetal length (Starr et al, 2015). Infection also increased placental mass, and a microarray-based study of placental gene expression recorded down-regulation of 109 genes related to oxidative phosphorylation, and up-regulation of 214 genes related to ATP binding and hemopoiesis in the *H. bakeri* infected dam (Starr et al, 2016). All these results clearly demonstrate an impact of this GI nematode infection in pregnancy on neonatal development of uninfected offspring.

2.4 Mouse brain development

Brain development is an extremely complex process and in mice approximately 75 million neurons and 25 million glial cells are formed, transferred and integrated into numerous neuronal circuits during the course of intrauterine and postnatal development, involving expression of approximately 40,000 genes (Sutcliffe, 1988). Among these, almost 5000 genes are involved in cellular metabolism and their highly coordinated expression leads to formation of different brain regions (Usui et al, 1994; Gautvik et al, 1996). The brain mass is formed by the extensive proliferation of progenitor cells through a process known as neurogenesis, which includes the initial cellular expansion phase and the neurogenic phase in which functional neurons are generated (Götz & Huttner, 2005; Zhong and Chia, 2008). Neural stem cell precursors undergo symmetric proliferation that increases the number of nerve cells followed by an asymmetric differentiation to generate functional neural units (Hardwick et al, 2015). These processes are controlled by precise expression of a variety of genes. A key regulatory factor of nerve cell proliferation and differentiation is microRNAs (miRNAs), especially miRNA let-7b, which can modulate the function of the cell cycle through cell cycle regulator cyclin-D1 and TLX receptors, expressed in the forebrain. Overexpression of miRNA let-7b may lead to reduced neural differentiation and increased neural proliferation (Zhao et al, 2010).

Peak neurogenesis in mice begins at gestation day 9 (E-9) (Finlay and Darlington, 1995). At embryonic day (E)-12, the pre-plate of the brain appears and at E-14, the

intermediate zone is traversed by migrating neurons that form the base of the cortical plate (CP). Due to extensive neuronal migration, the normal CP increases in thickness at E-16 and by parturition (normally on day E-21), development of the internal pyramidal layer is completed. Synaptogenesis begins on postnatal day 5 (P-5) and 20% of fast-spiking (FS) neurons are electrically coupled by P-6. Between P-15 and P-18, 42% of the FS have developed electrical synapses and the brain has become fully functional (Pressler and Auvin, 2013).

2.4.1 Stressors and mouse brain development

A number of maternal and environmental factors may influence the perinatal development and maturation of brain. Among these factors, the three relevant to my thesis are maternal infection, nutrient deficiency and microbiome.

2.4.1.1 Maternal infection and perinatal brain development

Infection in pregnancy leads to maternal immune activation which imposes a high risk for normal brain development. It has been seen that maternal infections (generally bacterial and viral) which induce strong pro-inflammatory response cause neurodevelopmental disorders and cognitive damage via pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and INF-γ (Golan et al, 2005). During maternal infections, cytokines are generally present in both in maternal and fetal compartments (maternal serum and amniotic fluid and fetal serum) and cytokines also cross the placental and blood-brain barriers (Fidel et al, 1994).

Among all the inflammatory cytokines, IL-6 has been identified to have the most potent influence on fetal brain development in response to maternal immune activation (Smith et al, 2007). IL-6 is believed to interact directly on the fetal brain and plays an important role in brain development, learning and memory (Balschun et al, 2004). During the developmental stage, IL-6 signals through the STAT (signal transducers and activators of transcription) pathway and regulates neurogenesis and gliogenesis. It also

triggers brain endothelial cell division and migration in normal physiological conditions (Yao et al, 2006). However, in response to maternal inflammation, high levels of IL-6 may cause neuronal degeneration by an unknown mechanism (Harry et al, 2006). IL-6 can regulate the expression of brain-derived neurotrophic factor (BDNF) that is responsible for growth and development of neurons and an IL-6-mediated low level of BDNF was found in embryos and in placentas 24 hours after induction of inflammation in pregnant mice (Gilmore et al, 2005). A microarray-based study in mice showed that altered gene expression in the fetal brain due to maternal inflammation was normalized by eliminating maternal IL-6 (Smith et al, 2007). In addition to IL-6, IL-1β and TNF-α were also shown to cause damage in the neonatal brain (Dammann and Leviton, 1997; Girard et al, 2010; Burd et al, 2012).

On the other hand, helminth infection in pregnancy induces an anti-inflammatory response with upregulation of anti-inflammatory cytokines such as IL-4, IL-10, TGF-β (Reynolds et al, 2012). However, the consequences of maternal helminth infection on neonatal brain development have not been studied explicitly. One study showed that maternal helminth infection was associated with poor cognitive function among infants (Mireku et al, 2015) indicating a potential effect of helminth infection in pregnancy on neonatal brain development, but the causal relationship was unknown (Mireku et al, 2015). However, in the past we showed that the concentration of certain pro-inflammatory cytokines in maternal serum were directly proportional to their concentration in the fetal circulation (Starr et al, 2015), consistent with the intriguing possibility that maternal inflammation might influence the neonatal neurodevelopment through inflammatory mediators (Golan et al, 2005).

2.4.1.2 Effect of maternal nutrition on brain development

Nutritional deficits during prenatal and early postnatal life have been shown to impair neurodevelopment, which may lead to neurodevelopmental disorders in postnatal life (de Souza et al, 2011). The volume of grey matter and white matter increases significantly with the highest growth rate in the cerebellum and cortical grey matter, and

this dynamic phase is highly vulnerable to malnutrition (Dubois et al, 2007). Intrauterine nutrient deficiency leads to reduction in the number of brain cells, myelin production, and the number of synapses as well as alteration of neurotransmitters (Dubois et al, 2007). The cerebellum and hippocampus have been shown to be the most vulnerable to early postnatal malnutrition (Levitsky and Strupp, 1995). Research has also demonstrated that prenatal malnutrition is associated with cognitive abnormalities and neurodevelopmental disorders (Roseboom et al, 2011).

Although most nutrients are important for neuronal growth and differentiation, protein, iron, zinc, selenium, iodine, vitamin A, folate, choline and long-chain polyunsaturated fatty acids (LC-PUFAs) (specifically docosahexaenoic acid and arachidonic acid) are considered the most important during late fetal and early neonatal times (Georgieff, 2007). Focussing on protein, maternal protein restriction in early pregnancy in rats was associated with smaller neonatal brains (de Kieviet et al, 2012) and dietary supplementation with the amino acid, glutamine, was associated with greater brain microstructural integrity and larger volume of the white matter, hippocampus and brain stem (de Kieviet et al, 2012), clearly emphasizing the important role of protein in neonatal brain development. Maternal protein deficiency both in the prenatal and postnatal period was associated with a reduction in the synaptic density and numbers of vesicals in rats (Jones and Dyson, 1981; Wiggins et al, 1984) which may be due to a reduction in the level of BDNF and insulin like growth factor-1 (Antonow-Schlorke et al, 2011). Also, we previously demonstrated that maternal protein deficiency altered concentrations of several growth factors that may affect the neonatal brain (Odiere et al, 2010a; Starr et al, 2015). Together, these findings establish that prenatal or postnatal maternal protein restriction may result in adverse consequences on perinatal brain development.

2.4.1.3 Role of microbiota on perinatal brain development and programming

As mice give birth to immature pups, much of the neuroendocrine development occurs in the postnatal period concurrent with the colonization of GI microbiota. There is

emerging evidence that the gut microbiota influences the HPA axis and synaptogenesis, two key processes that regulate perinatal brain development (Clarke et al, 2013). Much of this evidence emerged by comparing the stress response between neonatal germ-free mice and mice with conventionally colonized GI microbiota. Although the exact mechanism is not clear, the elevation in ACTH in young germ-free mouse compared to normally colonized mice suggests that the absence of GI microbiota increased activity of the HPA axis (Sudo et al, 2004). Also, in the absence of GI microbiota, the decline in the expression of synaptic junction proteins (synaptophysin and PSD-95) provides further evidence that GI microbiota are required for normal HPA axis functioning (Heijtz et al. 2011). Synaptophysin, a synaptic vesicle glycoprotein expressed in most neurons of central nervous system, is considered as a marker for synaptic vesicle maturation and synaptogenesis in the developing brain (Ulfig et al, 2000) whereas PSD-95 is responsible for maturation of excitatory synapses. Moreover, it was recorded that exposure to a higher concentration of glucocorticoids reduced the expression of synaptophysin in the fetal brain (Antonow-Schlorke et al, 2003). Together these findings indicate that a high level of stress hormones (ACTH and corticosterone) due to impaired programming of HPA axis in absence of neonatal GI microbiota may interfere with normal brain development. Furthermore, microbiota depletion in neonatal mice was also demonstrated to be associated with cognitive deficits and reduction of brain oxytocin and vasopressin mRNA expression (Desbonnet et al, 2015). A recent study demonstrated that altered neonatal microbial colonization reduces the concentrations of tryptophan, which is the precursor of serotonin in developing brain (Jašarević et al, 2015b). Moreover, it has been experimentally shown that the neonatal microbiome plays a critical role in the development of the neonatal blood-brain barrier (Braniste et al, 2014). Thus, the role of the microbiome on neonatal brain maturation is now well acknowledged.

2.5 Microbiome mediated gut brain cross-talk

The GI tract is the habitat of trillions of microbes belonging to seven phyla (*viz.* Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria). The most abundant bacterial species belong to the phyla Firmicutes

(gram positive) and Bacteroidetes (gram negative) (Backhed et al, 2005; Tap et al, 2009). The microbiome plays a crucial role in providing nutrients, controlling epithelial cell proliferation, and in development of innate immunity and the neonatal and perinatal brain (Eckburg et al, 2005; Heijtz et al, 2011).

The microbiome has been shown to play a crucial role in the bi-directional communication between the gut and brain including brain development in the neonatal period (De Palma et al, 2015). Although the pathways are not well established, it is assumed that the communication may involve the autonomic nervous system (ANS) mediated by the vagus nerve, the enteric nervous system (ENS) mediated by neuroactive substances, and neurotransmitters including monoamines (noradrenaline, serotonin and dopamine) and metabolites (5-hyroxyindole acetic acid (5-HIAA), dihydroxyphenylacetic acid, and L-3, 4-dihydroxyphenylalanine) (Foster and Neufeld, 2013).

The ANS mediated pathway of gut-brain communication involves the vagus nerve (Foster and Neufeld, 2013) and the main ligand for the vagus nerve is acetylecholine (Ach). It has been reported that GI microbiota secretes Ach, which minimizes the host immune response by down-regulating proinflammatory cytokines (TNF, IL-1β, IL-6 and IL-18) and up-regulating the anti-inflammatory cytokine (IL-10) which provides a suitable environment for the microbiome (Borovikova et al, 2000; Walk et al, 2010). This microbiome-derived Ach may influence the neonatal brain development (Al-Asmakh et al, 2012).

ENS mediated signalling involves an interaction between bacterial metabolites such as short-chain fatty acids (SCFAs), butyric acid, propionic acid and acetic acid and nerve endings of the sympathetic nervous system. It was recorded that the SCFAs, by directly binding with the G-protein coupled receptor of the intestinal sympathetic nerve, transmit signals to the brain (Kimura et al, 2011) but whether this is relevant during early post-partum brain development is not known.

Modulation of the neurotransmitters (noradrenaline, serotonin and dopamine) and their metabolites (5-hyroxyindole acetic acid (5-HIAA), dihydroxyphenylacetic acid, and L-3, 4-dihydroxyphenylalanine) is another potential path of communication. Parallel alteration of concentrations of the serum serotonin metabolite, 5-HIAA in the hippocampus and its precursor (tryptophan) in serum of germfree mice support the hypothesis that serotonin might mediate microbiota-gut-brain communication (Desbonnet et al, 2015).

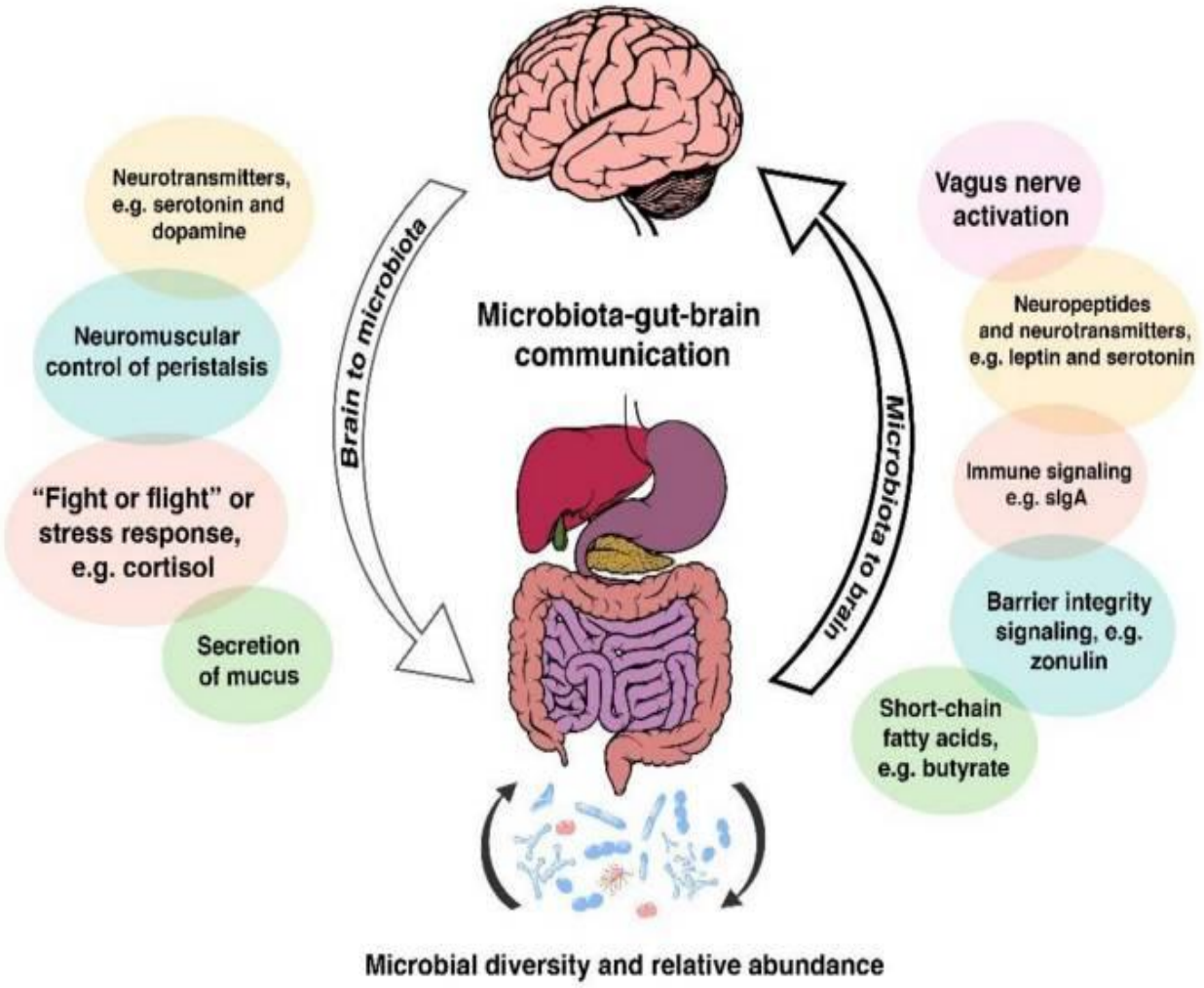

Figure 2. Bidirectional communication pathways between the gut microbiota and the brain, featuring a range of molecules originating in the gut that are involved in the upstream part of the communication system (Toribio-Mateas, 2018). Permission of reuse of the figure is licensed with "Open Access" under Creative Commons Attribution (CC-BY).

2.5.1 Role of maternal stress on fetal microbial colonization

The intestine of offspring is first colonized with the maternal vaginal microbiota as the offspring emerge fom the birth canal and dysbiosis of the vaginal microbiome due to maternal inflammation or stress may lead to altered neonatal colonization (Jakobsson et al, 2014). The microenvironment of the female reproductive tract, especially pH, determines the survival and abundance of specific microbes (Jasarevic et al, 2015a). Normally during pregnancy, estrogen and progesterone induce deposition of high amounts of glycogen which is metabolized to glucose by bacterial enzymes in vaginal epithelial cells (Linhares et al, 2011). Further, *Lactobacillus* metabolizes the glucose into lactic acid and hydrogen peroxide (H_2O_2), which lowers the vaginal pH. The lower pH favours the growth of healthy *Lactobacillus* population and safeguards the female reproductive from the invasion of opportunistic pathogens (Mijac et al, 2006; Tomas et al, 2003). It has been shown that physical and psychological stress during pregnancy (gestation day 1 to 7) changes the vaginal microbiome and alters microbial colonization of the GI in the offspring (Jašarević et al, 2015b).

Another source of microbiome for neonates is the maternal milk and maternal factors affect the composition and diversity of the milk microbiota (Cabrera-Rubio et al, 2012). Human breast milk contains approximately 1,000 CFUs/mL bacterial species (Jeurink et al, 2013) and a breastfed infant consumes up to 800,000 bacteria/day (Heikkila and Saris, 2003), thus making maternal milk an important source of microbes in the neonatal gut. The predominant bacterial species in human milk are *Bifidobacterium*, *Lactobacillus, Enterococcus* and *Staphylococcus* (Le Doare et al, 2018). They have been shown to competitively exclude the growth of other pathogenic bacteria by producing or secreting antimicrobial substances. For instance, *Lactobacillus*, one of the most important commensal bacteria present both in neonatal stomach microbiome and milk microbiome, inhibits adhesion and growth of *Escherichia coli*, *Shigella* spp, *Pseudomonas* spp, and *Salmonella* spp (Olivares et al, 2006; Jara et al, 2011). A recent study demonstrated that maternal milk is an important factor in shaping neonatal microbial community and may even introduce new bacterial species (Matsuyama et al, 2019). Again, as with the vaginal

microbiome, maternal factors such as maternal diet, cytokines (infection and inflammation), and antibiotic treatment significantly influence the milk microbiome (Moossavi et al, 2019).

2.5.2 Interaction between gastrointestinal nematodes and gut microbiota

A number of important GI nematodes coexist with the GI microbiota and studies have shown that nematode infection significantly alters the abundance and composition of GI microbiota (Berrilli et al, 2012). Walk et al (2010) demonstrated that *H. bakeri* significantly increased the relative abundance of bacterial species belonging to the family Lactobacillaceae in the ileum of infected mice at two weeks PI. They suggested that the localization of the effect to the ileum was associated with reduced inflammation at the site of penetration of parasite larvae. *H. bakeri* L_3 invade the intestinal mucosa where they activate a number of immunoregulatory signals by inducing Th2 responses that decrease inflammatory responses and favour the proliferation of *Lactobacillus* species (Walk et al, 2010). Furthermore, Rausch et al (2013) reported significantly higher numbers of gram-negative bacteria γ-*Proteobacteria* / *Enterobacteriaceae* and species of the *Bacteroides* /*Prevotella* group in the cecum of *H. bakeri* infected mice, but their study did not show any significant alteration in the abundance of gram positive bacteria such as Lactobacilli and Clostridia species or in the total bacterial load. The contradictory results between Walk et al (2010) and Rausch et al (2013) regarding the abundance of *Lactobacillus* species might be due to the differences in methodology. Walk and coworkers (2010) examined the bacterial species by generating 16s rRNA clone library from terminal ileum tissue after removing the intestinal content whereas Rausch and colleagues (2013) used DGGE derived DNA based sequencing for the detection of bacterial DNA from the intestinal contents. Differences in the composition of gut-wall-associated versus gut-content microbes might also explain the different results. Nevertheless, these two studies clearly established that *H. bakeri* alters the GI microbiome in the host.

Apart from *H. bakeri,* chronic infection of *Trichuris muris* also causes significant reduction in the abundance of the members of phylum Bacteroidetes, specifically Prevotella and Parabacteroides (Houlden et al, 2015) and a significant increase in the relative abundance of *Lactobacillus* spp. (Holm et al, 2015) in mice. Interestingly, these changes in the microbiota were transitory as the removal of helminths normalised the microbiota (Houlden et al, 2015). These laboratory studies have used one type of parasitic larvae in their infection protocol, however, in the environment mice mostly get infected with multiple helminths. The interaction of multiple helminths (*H. bakeri, Syphacia* spp. and *Hymenolepis* spp.) and gut microbiota in wild rodents also revealed that variation in abundance and composition of microbiota was helminth specific (Kreisinger et al, 2015).

Porcine-nematode models also indicate significant interactions between helminths and the microbiota. Li et al (2012) reported that *Trichuris suis* infection induced significant alteration in the composition and abundance of the microbiota in the proximal colon of infected pigs. The most affected bacterial species were *Proteobacteri* and *Deferribacteres* and approximately 13% of the total genera were significantly altered. In a similar study, a significant decrease in the abundance of *Fibrobacter* and *Ruminococcus* was observed in the proximal colon of pigs infected with *T. suis* (Wu et al, 2012).

2.6 Summary

In our previous studies using a pregnant mouse model, we showed that maternal protein deficiency and *H. bakeri* infection independently reduced neonatal crown-rump length and altered concentrations of several growth factors and cytokines in both maternal and neonatal circulations (Odiere et al, 2010a; Starr et al, 2015). Although the maternal placenta normally protects the developing fetus from obnoxious cues from the maternal and external environment, both maternal protein deficiency and nematode infection altered gene expression in the maternal placental (Starr et al, 2016) raising the possibility that these maternal stresses may pass the placental barrier and affect development of vital organs such as the neonatal brain. In light of altered placental gene

expression (Starr et al, 2016), in light of the critical murine brain development that is initiated in the intrauterine phase and continues postpartum (Pressler and Auvin, 2013), and in light of the important role of stress-mediated alteration of maternal microbiome (Jašarević et al, 2015a), I hypothesized that neonatal brain development might be adversely affected by maternal protein deficiency and nematode infection.

Therefore, in the subsequent chapters of my thesis, I examine the consequences of maternal nematode infection and protein deficiency on gene expression in the fetal brain, and the effects of maternal nematode infection both on postnatal brain gene expression and microbial colonization.

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Chapter III

Differential expression of genes in fetal brain as a consequence of maternal protein deficiency and nematode infection

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Abstract

Maternal dietary protein deficiency and gastrointestinal (GI) nematode infection during early pregnancy have negative impacts on both maternal placental gene expression and fetal growth in the mouse. Here we used next-generation RNA sequencing (RNA-Seq) to test our hypothesis that maternal protein deficiency and/or nematode infection also alter the expression of genes in the developing fetal brain. Outbred pregnant CD1 mice were used in a 2x2 design with two levels of dietary protein (24% versus 6%) and two levels of infection (repeated sham versus *Heligmosomoides bakeri* beginning at gestation day 5). Pregnant dams were euthanized on gestation day 18 to harvest the whole fetal brain. Four fetal brains from each treatment group were analyzed using RNA Hi-Seq sequencing and the differential expression of genes was determined by the *edgeR* package using NetworkAnalyst. In response to maternal *H. bakeri* infection, 96 genes (88 up-regulated and eight down-regulated) were differentially expressed in the fetal brain. Differentially expressed genes were involved in metabolic processes, developmental processes and the immune system according to the PANTHER classification system. Among the important biological functions identified, several up-regulated genes have known neurological functions including neuro-development (*Gdf15, Ing4*), neural differentiation (*miRNA let-7*), synaptic plasticity (via suppression of NF-κβ), neuro-inflammation (*S100A8*, *S100A9*) and glucose metabolism (*Tnnt1*, *Atf3*). However, in response to maternal protein deficiency, brain-specific serine protease (*Prss22*) was the only up-regulated gene and only one gene (*Dynlt1a*) responded to the interaction of maternal nematode infection and protein deficieincy. In conclusion, maternal exposure to GI nematode infection from day 5 to 18 of pregnancy may influence developmental programming of the fetal brain.

***Keywords*:** Fetal brain gene expression, Maternal nematode infection, Maternal protein deficiency, Mouse, *Heligmosomoides bakeri*, Neuro-development

1. Introduction

The fetal brain has been shown to be sensitive to a variety of maternal stresses during pregnancy. Fetal head size and lateral ventricular brain volume were reduced in response to vitamin D deficiency (Hawes et al., 2015), the amino acid profile was altered in response to noise, odor and handling (Jarasevic et al., 2015), expression of genes that control gliogenesis and cell proliferation was down-regulated by alcoholism (Mandal et al., 2015), and fetal brain gene expression responded to hypoxia (Trollmann et al., 2010) and vitamin D deficiency (Hawes et al., 2015). With regard to maternal infections, the maternal immune responses to influenza and *Streptococcus* infections have been associated with neuro-inflammation in the developing brain (Madore et al., 2016), and increased expression of brain-derived neuro-trophic factor and nerve growth factor has been reported following injection with lipopolysaccharide that simulates a bacterial infection (Gilmore et al., 2003).

Recent evidence suggests that the placenta plays an important role in brain development. The placenta may lessen the impact of maternal stress (Bronson and Bale, 2016) on the fetal brain during the interval before the blood-brain barrier is fully functional (Brandiste et al., 2014). Short-term food deprivation of pregnant mice has been shown to disrupt the normal co-regulation of placental and fetal brain development, resulting in placental autophagy of ribosomal proteins that provide a nutrient source for the fetal brain (Broad and Keverne, 2011). In response to maternal inflammation, impaired placental function was associated with impaired fetal brain development (Goeden et al., 2016). In response to hypoxia, parallel shifts in gene expression were seen in the placenta and fetal brain not only for hypoxia-related genes but also for genes related to angiogenesis, cell differentiation, mRNA processing and embryonic development (Trollman et al., 2010).

Malnutrition and gastrointestinal (GI) nematode infection are widely prevalent in different parts of the world and they commonly co-occur during pregnancy (Crompton and Nesheim, 2002). Our laboratory has had an interest in the independent and combined effects of the GI nematode, *Heligmosomoides bakeri*, and protein deficiency on growth

and development of the mouse fetus. We have demonstrated that maternal infection increased placental mass (Starr et al., 2015) and reduced linear growth of the fetus (Odiere et al., 2010a) whereas protein deficiency reduced fetal mass but had no effect on linear growth or placental mass (Starr et al., 2015). Concentrations of stress hormones, growth factors and cytokines in amniotic fluid and fetal serum responded differently to the two maternal stressors (Starr et al., 2015). We also found that combined *H. bakeri* infection and protein deficiency altered placental gene expression including genes associated with fetal growth (Starr et al., 2016). Given these findings, and in light of evidence that the placenta plays an important role in fetal brain development (Broad and Keverne, 2011), this study was designed to determine whether fetal brain genes were differentially expressed in response to the main effects of maternal *H. bakeri* infection and maternal protein deficiency, and their interaction in our mouse model. We were particularly interested in genes involved in developmental processes, immune system processes and metabolic processes.

2. Materials and methods

2.1. Experimental design

The design was a 2 × 2 factorial with two levels of dietary protein (24% versus 6%) and two levels of infection (sham versus repeated trickle infection), resulting in four experimental groups: (i) protein sufficient (PS) uninfected; (ii) PS infected; (iii) protein deficient (PD) uninfected; and (iv) PD infected. Fetal brain samples were collected during our previous study (Starr et al., 2015) using 8 to 9-week-old primiparous timed pregnant outbred CD1 mice and the murine GI nematode *Heligmosomoides bakeri* (*Heligmosomoides polygyrus* or *Nematospiroides dubius*; see Behnke and Harris, 2010) as the experimental model. The protocol (# 2000-4601) was approved by the Animal Care Committee McGill University, Canada, according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Pregnant mice were received from Charles River Laboratory (Senneville, QC, Canada) on gestation day (GD) 4 and housed individually at 22-25$_{o}$ C and a 14 h light: 10 h dark cycle in Nalgene cages (Fisher Scientific, Canada) with stainless steel racks to prevent coprophagia. Food and water were supplied ad libitum. On GD 5, mice were randomly assigned to one of the four treatment groups. Mice were fed either a PS (24% protein, TD. 90017) diet or a PD (6% protein, TD. 90016) isoenergetic diet from Harlan Teklad, USA (Odiere et al., 2010b). The uninfected groups were intubated with sham doses of distilled water on GD 5, 10 and 15 whereas the infected groups were intubated with three doses of 100 ± 3 L_3 of *H. bakeri* on GD 5, 10 and 15. Thus, each infected mouse received a cumulative dose of 300 L_3 which had been shown earlier to have no impact on the normal reproductive physiology of mice (Scott, 1991). Establishment of *H. bakeri* infection in infected dams was confirmed at necropsy by counting the number of worms in the GI tract.

2.2. Fetal brain tissue collection

On GD 18, dams were euthanized by isoflurane anaesthesia followed by cardiac puncture and exsanguination. Following caesarean section, fetal brains were excised, snap frozen in liquid nitrogen, and stored at -80^{0}C. All samples were collected between 10:00 and 14:00.

2.3. RNA extraction, cDNA library preparation and transcriptome sequencing

Total RNA from the fetal brain was isolated using a commercial kit (NucleoSpin® RNA Plus, Macherey-Nagel, Germany) following the manufacturer's protocol and quantified spectrophotometrically using NanoDrop. Total fetal brain RNA (four samples/ experimental group) was sent to the Functional Genomics Platform of the McGill University and Génome Québec Innovation Centre, Canada for cDNA library preparation and subsequent sequencing in the Illumina HiSeq2000 sequencing system using four lanes (four samples/ lane).

2.4. Initial processing of raw reads and mapping to reference genome

The initial processing of raw data (FASTQ) including filtering of low tags, trimming, adaptor removal and alignment to the mouse reference genome (GRCm38) was done by the Génome Québec Innovation Centre who provided us with the paired-end BAM files. The BAM files have been deposited into the Gene Expression Omnibus (GEO) repository (GSE96103).

2.5. Counting reads and analysis of differential gene expression

The paired end BAM files were sorted using samtools with the parameters *–n*: name; -*o*: bam (Li et al., 2009). The sorted files were used to count the reads for expressed exons under each gene by HTSeq-count (v0.6) with the following parameters: -*f*, bam; -*s*, no; -*r*, name (Anders et al., 2015). The raw counts were uploaded to NetworkAnalyst (http://www.networkanalyst.ca), a web-based tool for comprehensive gene expression profiling (Xia et al., 2015). The *edgeR* method was selected with adjusted P value < 0.05 and fold change > 1.5 to identify genes differentially expressed by our main effects. The interactive effect of protein deficiency and nematode infection was determined by creating a data matrix with diet as the primary variable and nematode infection as the secondary variable, and then performing a nested comparison between these two variables to identify those genes that responded differently using the "interaction only" option. Results were confirmed by using infection as the primary variable.

2.6. Identification of genes of interest

Those genes that were differentially expressed were analysed in the PANTHER classification software (Mi et al., 2013) to examine their involvement in different biological systems. Also, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang et al., 2009) was used for functional annotation clustering and enrichment score representation of Gene Ontology (GO) classes. In addition, the full list of differentially regulated genes was reviewed for genes

known to be involved in energy metabolism, neuro-development and neuro-inflammation.

2.7. Validation of expression data by real-time quantitative (q)PCR

To validate the results obtained from the next-generation sequencing, real-time qPCR was performed on 15 genes associated with either developmental (*Ing4, Gdf15, Adad2*), immune system (*Camp, Reg3g, CD1d2, Klk-7, S100A8, S100A9, H2-Eb1*) or metabolic process (*Tnnt1, Atf3, Cav3, Hist1h1c, Atp6ap1l*), together with four randomly selected genes (*Pomc, Rab17, Mael, Tpsab1*) and *Prss22*, the only gene that responded to the main effect of protein deficiency.

The qPCR was carried out according to the MIQE guidelines (Bustin et al., 2009). Eight fetal brains were selected from each group, including the four used for RNA-Seq. The total RNA was extracted as described in Section 2.3. Initially, 2.5 µg of total RNA from each sample was reverse transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Canada). Subsequently, the cDNAs were diluted (1:10) and used in the qPCR. The primer sequences were obtained from PrimerBank (Wang et al., 2012) and the amplification efficacy for each pair was determined (Table 1). The qPCR was carried out with the following protocol: initial denaturation at 95◦ C for 3 min followed by 39 cycles at 95◦ C for 15 s and 56◦ C for 45 s for annealing, and finally 95◦ C for 10 s. The expression data were normalized to the geometric mean of Cq (quantification cycle) values of three reference genes, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *Hprt* (hypoxanthine guanine phosphoribosyl transferase) and *Actb* (β-actin). The relative gene expression and fold changes in the *H. bakeri* –infected compared with uninfected samples and PD compared with PS samples were calculated using the Standard Curve method to reflect the main effects of maternal infection and protein deficiency. Reactions were considered efficacious within the range of 90-110. For efficacies ≥ 100, the fold change of $2_{\Delta\Delta Cq,}$ was used; for efficacies < 100, the fold change of $1.9_{\Delta\Delta Cq}$ was used.

3. Results

3.1. Maternal H. bakeri infection

In response to the main effect of maternal *H. bakeri* infection (infected versus uninfected dams), 96 genes were differentially expressed in the fetal brain (Supplementary Table S1). Among them, 88 genes were up-regulated and eight genes were down-regulated (Table 2). Only 10 of the 96 are included in the Allen Developing Mouse Brain Atlas (http://developingmouse.brain-map.org/) of genes expressed in the fetal brain on GD 18.5 (Supplementary Table S1).

The differentially expressed genes were associated with 11 PANTHER classes (Fig. 1) and several genes were represented in more than one class. Of the 26.8% of genes associated with metabolic processes (Fig. 1), 22 have an identified PANTHER protein class (Table 3). Of the 8.1% of genes associated with developmental processes, six have a known protein class and two of these are related to neurological development (*Gdf15, Ing4*). Of the 5.7% of genes involved in immune system processes, protein classes are known for all seven genes and one is involved in neuro-inflammation (*S100A8*). In addition to those listed above, *S100A9* was associated with inflammation in the more detailed DAVID listing of GO terms (Supplementary Table S2). Finally, review of the full list of differential regulated genes also revealed up-regulation of *miRNA let7* which is involved in brain development, and of *Tnnt1* and *Atf3* which are associated with energy metabolism.

3.2. Maternal protein deficiency

In response to the main effect of maternal protein deficiency (PS versus PD dams), brain-specific serine protease (*Prss22*) was the only up-regulated gene and no gene was down-regulated (Table 2). Analysis was also carried out without employing the fold change cut-off but no additional genes were differentially expressed in PD mice.

3.3. Interaction between infection and protein deficiency

Only one gene responded to the interaction of maternal nematode infection and protein deficiency. Expression of dynein light chain a (*Dynlt1a*) was reduced by infection but only in the PD group (Fig. 2).

3.4. Real-time qPCR verification

Among the total 20 genes used to verify RNA-Seq, qPCR confirmed up-regulation by infection of 13 genes related to energy metabolism, neuro-development or neuro-inflammation (*Ing4, Adad2, Gdf15, Klk7, S100A8, S100A9, Tnnt1, Atf3, Atp6ap1l, Camp, Reg3g, Cd1d2,* and *H2-Eb1*) and of three genes with non-specific function in the brain (*Rab17, Pomc* and *Mael*). Up-regulation of *Prss22* in response to protein deficiency was also confirmed (Fig. 3). We were unable to estimate the fold change for three genes (*Cav3, Slamf6 and Tpsab1*) whose expression level was below the lower detection level of qPCR. This low expression was also confirmed from the RNA-Seq data by counting the number of expressed transcripts.

4. Discussion

An important novel finding of this exploratory study was that maternal infection with an GI nematode differentially regulated expression of 96 fetal brain genes on GD 18, the majority (86) of which had not been reported to respond to other maternal stressors according to the October 2016 Allen Developing Mouse Brain Atlas (http://developingmouse.brain-map.org/) of genes expressed in the developing mouse brain on GD18.5. Genes that were up-regulated by maternal nematode infection had implications for several aspects of brain development and function: (i) less proliferation of neurons and smaller brain size (*miRNA let7*); (ii) altered neuro-development (*Gdf15*), neural differentiation, synaptic plasticity and neuro-inflammation (*S100A8, S100A10*), and neuro-development, synaptic plasticity and signal transduction (*Ing4*); (iii) impaired innate immunity (*Ing4)*; (iv) and increased need for energy (*Tnnt1* and Atf3). In contrast to maternal nematode infection, the fetal brain was largely protected from maternal

protein deficiency as only serine protease 22 (*Prss22*) was differentially expressed in response to protein deficiency, and only dynein light chain a (*Dynlt1a*) responded to the interaction between protein deficiency and nematode infection.

The developing brain has enormous metabolic turnover and anabolic needs (Cheng et al., 2000) and uses glucose as the principal source of energy (Mergenthaler et al., 2013). Troponin T1 (*Tnnt1*) together with forkhead box-containing protein, O subfamily 1 (*FoxO1*), have been shown to interact with the insulin-signalling pathway, glucose transportation and energy homeostasis in the CNS (Lowe et al., 2007). Thus, the higher expression of *Tnnt1* in response to maternal nematode infection might be due to a higher energy demand in the developing brain in the infected group. Moreover, maternal nematode infection up-regulated the expression of hypothalamic activating transcription factor 3 (*Atf3*) which also interacts with *FoxO1* and controls glucose and energy metabolism. *Atf3* expression was previously demonstrated to increase under energy-deprived conditions (Lee et al., 2013). The higher expression of *Tnnt1* and *Atf3* in response to maternal nematode infection is consistent with the higher placental expression of insulin-like growth factor-1 receptor (*Igf-1r*) in nematode-infected PD dams (Starr et al., 2016). Together, these findings indicate that maternal infection, but not protein deficiency, increases the need for energy in the fetal brain.

Maternal nematode infection was associated with up-regulation of *Ing-4* that is likely to suppress innate immunity by inhibiting the p65 subunit of the NF-κβ heterodimer (Meffert et al., 2003). Interestingly, in vitro expression of p65 in mouse mesenteric lymph node cells has been shown to increase in response to *H. bakeri* antigens (Doligalska et al., 2012). In addition to controlling DNA transcription and cytokine production, NF-κβ is expressed in the developing brain where it is activated at the synaptic junction by basal synaptic stimulation and where it exerts synapse-to-nucleus transcriptional control (Meffert et al., 2003). Our finding of up-regulation of *Ing-4* in the fetal brain of infected dams therefore indicates that NF- κβ induced synaptic plasticity and signal transduction may be impaired by maternal nematode infection with implications for learning and memory (Meffert et al., 2003; Kaltschmidt and Kaltschmidt, 2009).

Maternal nematode infection also up-regulated expression of *S100A8* and *S100A9* that encode for inflammatory cytokine indicators of stress and tissue damage (Vogl et al., 2014) and that are also involved in antimicrobial activity (Raquil et al., 2008). Although the functions of *S100A8* and *S100A9* in the developing brain are not known, these two proteins are associated with neuro-inflammatory and neuro-degenerative diseases (Kim et al., 2014; Lu et al., 2015). *S100A8* has been shown to induce microgliosis in the hippocampus by activating mononuclear macrophages, resulting in neuro-inflammation (Lu et al., 2015). Similarly, the higher expression of *S100A9* may have been induced by the pro-inflammatory cytokine, TNF-α (Kim et al., 2014), which was present at high concentrations in the fetal circulation of these nematode-infected dams (Starr et al., 2015). Up-regulation of *S100A8* and *S100A9* in this study is thus consistent with the observation that maternal nematode infection during pregnancy was associated with poor cognitive and motor development in infants (Mireku et al., 2015).

The size of the developing brain mainly depends on transcription factors which regulate mitosis, the cell cycle and neural proliferation (Krichevsky et al., 2003). MicroRNAs (miRNAs) have been shown to play an important role in these processes (Krichevsky et al., 2003). Our study showed higher expression of *miRNA let-7* in the fetal brain in response to maternal nematode infection. As an important regulator of neural cell proliferation and differentiation (Zhao et al., 2010), *miRNA let-7* interacts with the stem cell regulator TLX and the cell cycle regulator cyclin D1, and higher expression leads to less neural proliferation but increased neural differentiation (Zhao et al., 2010, 2013). Thus, higher expression of *miRNA let-7* in response to maternal nematode infection may contribute to smaller pup brain size and to less proliferation of neurons. This also supports our unpublished observation that neonates born from nematode-infected dams had smaller brains than the control group.

Brain expression of growth/differential factor 15 (*Gdf15*) was also higher in fetuses of nematode-infected dams. *Gdf15* is a neuro-trophic factor for the dopaminergic neurons and is normally expressed at low levels in the choroid plexus and ventricular and sub-ventricular layers of the striatum in the developing brain (Schober et al., 2001).

However, in the case of brain injury, it acts as a lesion-induced factor and significantly higher expression has been recorded (Schober et al., 2001). As a distant member of the transforming growth factor beta (TGF-β) superfamily, it has been hypothesized that *Gdf15* may play a dual role in lesioned neurons where higher expression orchestrates either survival or apoptotic pathways (Schindowski et al., 2011). The higher expression of *Gdf15* in response to maternal nematode infection indicates that the fetal brain had received a harmful stimulus, perhaps an excretory/secretory molecule released into maternal circulation (Valanparambil et al., 2014) or a parasite-specific antibody that crossed the placenta. The observed higher expression of ATPase, H+ transporting, lysosomal accessory protein 1-like (*Atp6ap1l*) in the fetal brain might result in transfer of stress through altered programming of the hypothalamic–pituitary–adrenal axis (Logue et al., 2015). Further study is needed to identify the underlying mechanism(s).

The placenta is known to act as a 'nutrient sensor', as placental nutrient transporters normally adjust nutrient supply to the fetus in a way that ensures that the rate of fetal growth matches nutrient availability (Jansson and Powell, 2007). In the present study, only brain-specific serine protease (*Prss22*) was differentially expressed in the fetal brain in response to maternal protein deficiency. This is consistent with our previous report that GD18 fetal mass was lower but relative brain mass was higher in response to maternal protein deficiency (Starr et al., 2015) and with our finding that only *Prss22* was up-regulated in response to maternal protein deficiency. However, we previously reported that GD18 expression of placental transporters was not affected by maternal protein deficiency, perhaps because differential expression occurred earlier than GD 18 (Starr et al., 2016). The relative protection of the fetal brain from maternal protein deficiency contrasts with previous reports that maternal vitamin D deficiency alters expression of fetal brain genes involved in neuro-development (Hawes et al., 2015), and that short-term food deprivation elevated fetal brain expression of the paternally expressed gene 3 (*Peg3*) (Broad and Keverne, 2011).

To our knowledge, this is the first study to show the impact of maternal GI nematode infection on fetal brain gene expression. However, we acknowledge a few

limitations. First, neither RNA-Seq nor qPCR indicate whether the differential expression reflects changes in translation to protein. Second, we may have detected differential expression of some genes which may have minimal biological impact at a fold change of 1.5. This limitation was, in part, overcome by the PANTHER and GO analyses that provided important information regarding the involvement of these genes in different pathways and processes, and by qPCR confirmation of up-regulation of 14 genes of particular interest.

In conclusion, maternal GI nematode infection from day 5 to 18 of pregnancy altered the expression profile of fetal brain genes, particularly those related to neural development, neuro-inflammation and energy metabolism. This may affect intrauterine and postnatal development, and developmental programming of the mouse brain, as previously we reported that the effect of maternal protein deficiency and GI nematode infection persist even in postnatal day 21 pups (Odiere et al., 2010b). Further study is needed to explore underlying mechanisms and implications for the growing pup.

Supplementary data

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Table 1. Primers used in quantitative real-time PCR for verification of differential expression of selected genes in the mouse fetal brain

Primer Bank ID	Gene Name	Forward Primer (5'- 3')	Reverse Primer (5′- 3′)	Amplification Efficiency (%)
145966868c1	***Actb*** (Actin, beta)	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC	99.40
254675157c1	***Adad2*** (Adenosine deaminase domain containing 2)	TGCCCTCCGAGATACCCAC	GGGATCAGCTAGAACAAGGCT	97.6
160333688c1	***Atf3*** (Activating transcription factor 3)	TTTGCTAACCTGACACCCTTTG	AGAGGACATCCGATGGCAGA	95.1
225007584c1	***Atp6ap1l*** (ATPase, H+ transporting, lysosomal accessory protein 1-like)	TCAGTTTGGATCAGATACTTGCC	CCATTCCGTACAACCACTTCTTT	104.2
226423894c1	***Camp*** (cathelicidin antimicrobial peptide)	GGCTGTGGCGGTCACTATC	GTCTAGGGACTGCTGGTTGAA	109
31982475c1	***Cav3*** (Caveolin 3)	TCTGGAAGCTCGGATCATCAA	TCCGCAATCACGTCTTCAAAAT	-
6671706a1	***Cd1d2*** (CD1d2 antigen)	GTCCCAGGGCAAGTTGAGTAA	CCCAGGGTACATTTCACAGCC	92.7
126012538c1	***Gapdh*** (Glyceraldehyde-3-phosphate dehydrogenase)	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	99.70
6753968a1	***Gdf15*** (Growth differentiation factor 15)	CTGGCAATGCCTGAACAACG	GGTCGGGACTTGGTTCTGAG	94.3
114431227c1	***H2-Eb1*** (Histocompatibility 2, class II antigen E beta)	GCGGAGAGTTGAGCCTACG	AGGCCCGTGGACACAATTC	94.0
7305155a1	***Hprt*** (Hypoxanthine guanine phosphoribosyl transferase)	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	96.70
142387601c1	***Ing4*** (Inhibitor of growth family, member 4)	AAGCTGGCCCTTCTCAGACA	CAGCCGCCGAATGTGTTTG	98.5

18777665a1	***Klk-7*** (Kallikrein related-peptidase 7)	CTCCTTTCCCTGATAACTGTGC	GGGTGCGAGCCTTCTTTACAT	92.6
118129866c1	***Mael*** (Maelstrom spermatogenic transposon silencer)	CCTCCCTTGTGAAATTGGCTG	AATGGAATCGAAATCCTCGTGG	104.0
142346465c1	***Pomc*** (Pro-opiomelanocortin-alpha)	ATGCCGAGATTCTGCTACAGT	CCACACATCTATGGAGGTCTGAA	102.6
63079716c1	***Prss22*** (Protease serine 22)	CTGAACCGGATTGTGGGAGG	AGCCATTCTTGAGGATGCTAAC	102.40
228480276c1	***Rab17*** (RAB17, member RAS oncogene family)	GGCCCTCCGGTACATGAAG	GTGGCAGACGCTTTGGTACT	107.6
6755309c1	***Reg3g*** (Regenerating islet-derived 3 gamma)	ATGCTTCCCCGTATAACCATCA	ACTTCACCTTGCACCTGAGAA	100
7305453a1	***S100a8*** (S100 calcium binding protein A8)	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA	92
133893069c1	***S100a9*** (S100 calcium binding protein A9)	GCACAGTTGGCAACCTTTATG	TGATTGTCCTGGTTTGTGTCC	92.6
133892889c1	***Slamf6*** (SLAM family member 6)	ACTCCGCCTGTCAGAGGAT	AACGCCATTCATTAGCTGGGG	-
6755840c1	***Tnnt1*** (Troponin T1 skeletal)	AAGGGGAGCGTGTGGATTTTG	TCCTCCTTTTTCCGCTGTTCA	98.9
11493900a1	***Tpsab1*** (Tryptase alpha/beta 1)	GCCAATGACACCTACTGGATG	GCTTACGGAGCTGTACTCTGA	-

Table 2. Overview of differentially expressed genes in the mouse fetal brain at gestation day 18 in response to maternal infection with *Heligmosomoides bakeri* and/or protein deficiency.

Treatment Group	Expression	Number of genes	Genes of Interest[a]
Maternal *H. bakeri* infection[b]	Up-regulated	88	*Tnnt1, Atf3, Ing4, S100A8, S100A9, Gdf15, Atp6ap1, miRNAlet-7*
	Down-regulated	8	-
Maternal protein deficiency[b]	Up-regulated	1	*Prss22*
	Down-regulated	0	-
Interaction[c]	Down-regulated	1	*Dynlt1a*

[a] The full list of differentially expressed genes is provided in Supplementary Table S1.

[b] Fold change >1.5 and $P < 0.05$.

[c] No fold cut-off was applied; see Fig. 2.

Table 3. Differentially expressed genes included in three PANTHER categories of interest

Gene Symbol	PANTHER Family/Subfamily	PANTHER Protein Class[a]	Categories
Adad2	Adenosine deaminase domain-containing protein 2	DNA binding protein; RNA binding protein; deaminase; defense/immunity protein; kinase activator	M, I
Agr2	Anterior gradient protein 2	reductase; surfactant	M
Asgr1	Asialoglycoprotein receptor 1	cell adhesion molecule; immunoglobulin receptor superfamily	I
Atp6ap1l	V-type proton ATPase subunit s1-like protein	ATP synthase; hydrolase	M
Bpifa1	Bpi fold-containing family a member 1	antibacterial response protein; transfer/carrier protein	M, I
Cav3	Caveolin-3	G-protein modulator; membrane traffic protein; structural protein; transmembrane receptor regulatory/adaptor protein	M
Ccnb1ip1	E3 ubiquitin-protein ligase ccnb1ip1	-	M
Cd1d2	Antigen-presenting glycoprotein CD1d2;	immunoglobulin receptor superfamily; major histocompatibility complex antigen	I
Dlx3	Homeobox protein Dlx-3	DNA binding protein; homeobox transcription factor	M, D
E030002O03Rik	Protein E030002O03RIK	receptor; structural protein	M, D
Echdc2	Enoyl-coa hydratase domain-containing protein 2, mitochondrial	acetyltransferase; acyltransferase; dehydrogenase; epimerase/racemasez; hydratase; ligase	M
Entpd8	Ectonucleoside triphosphate diphosphohydrolase 8	nucleotide phosphatase	M
Ftcd	Formimidoyltransferase-cyclodeaminase	deaminase; transferase	M
Gdf15	Growth/differentiation factor 15	growth factor	M, D

Gm14434	MCG112779-related	-	M
Gm4787	A disintegrin and metallopeptidase domain 4-related	-	D
Hist1h1c	Histone H1.4	histone	M
Hsd17b13	17-beta-hydroxysteroid dehydrogenase 13	dehydrogenase; reductase	M
Hus1b	Checkpoint protein hus1b	-	M
Ing4	Inhibitor of growth protein 4	chromatin/chromatin-binding protein; transcription cofactor; zinc finger transcription factor	M, D
Il13ra2	IL-13 receptor subunit alpha-2	Cytokine defense/immunity protein	D, I
Mael	Protein maelstrom	-	M
Mgst2	Microsomal glutathione s-transferase 2	transferase	M
Ovol2	Transcription factor ovo-like 2	-	M
Rpl35a	60S-Ribosomal protein l35a	ribosomal protein	M
Rp1l1	Retinitis pigmentosa 1-like 1 protein	receptor	D
S100-A9	Protein S100-A9	calmodulin; signaling molecule	M
Slamf6	Slam family member 6	cell adhesion molecule; immunoglobulin receptor superfamily; membrane-bound signaling molecule; tyrosine protein kinase receptor	M, I
S100A8	Protein S100A8	calmodulin; signaling molecule	M, I
Spo11	Meiotic recombination protein spo11	DNA binding protein	M
Stfa2l1	MCG130173-related	cysteine protease inhibitor	M
Wfdc18	Activated macrophage/microglia wap domain protein-related	serine protease inhibitor	M
Xlr4b	Protein xlr4a-related	-	D
Xlr3b	X-linked lymphocyte-regulated protein 3a-related	-	D

Xlr3a	X-linked lymphocyte-regulated protein 3a-related	-	D

[a]Dash indicates no known PANTHER protein class.

M, metabolic process; D, developmental process; I, immune system process.

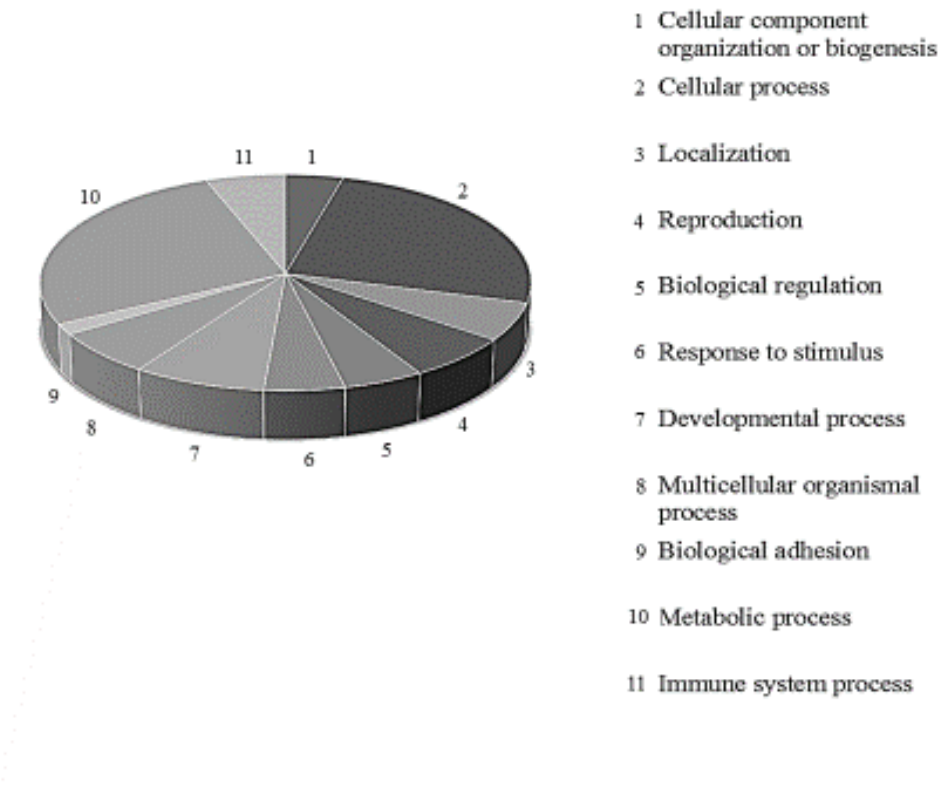

Figure 1. Pie-chart of PANTHER GO-Slim analysis showing proportions of differentially expressed genes in the fetal mouse brain in response to maternal *Heligmosomoides bakeri* nematode infection involved in 11 biological processes.

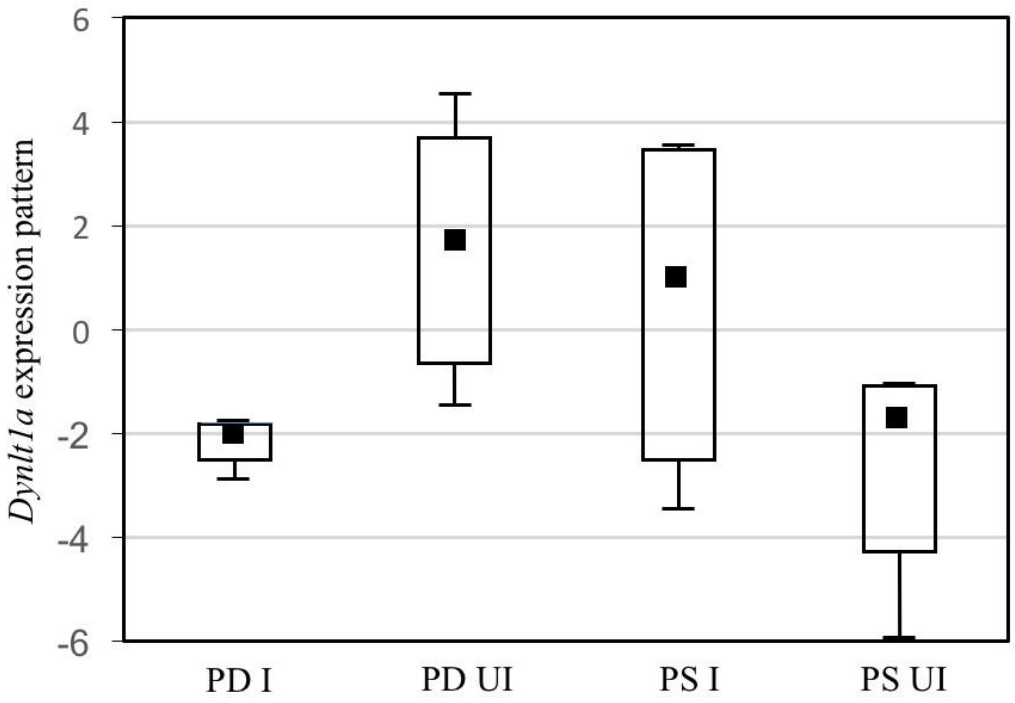

Figure 2. Box and whisker plot showing the interaction of maternal diet (PS, protein sufficient; PD, protein deficient) and maternal *Heligmosomoides bakeri* nematode infection (UI, uninfected; I, infected) on expression of the *Dynlt1a* gene in the fetal mouse brain. The dots represent the median, the box represents the first to third quartile of data, and the whiskers represent the data range.

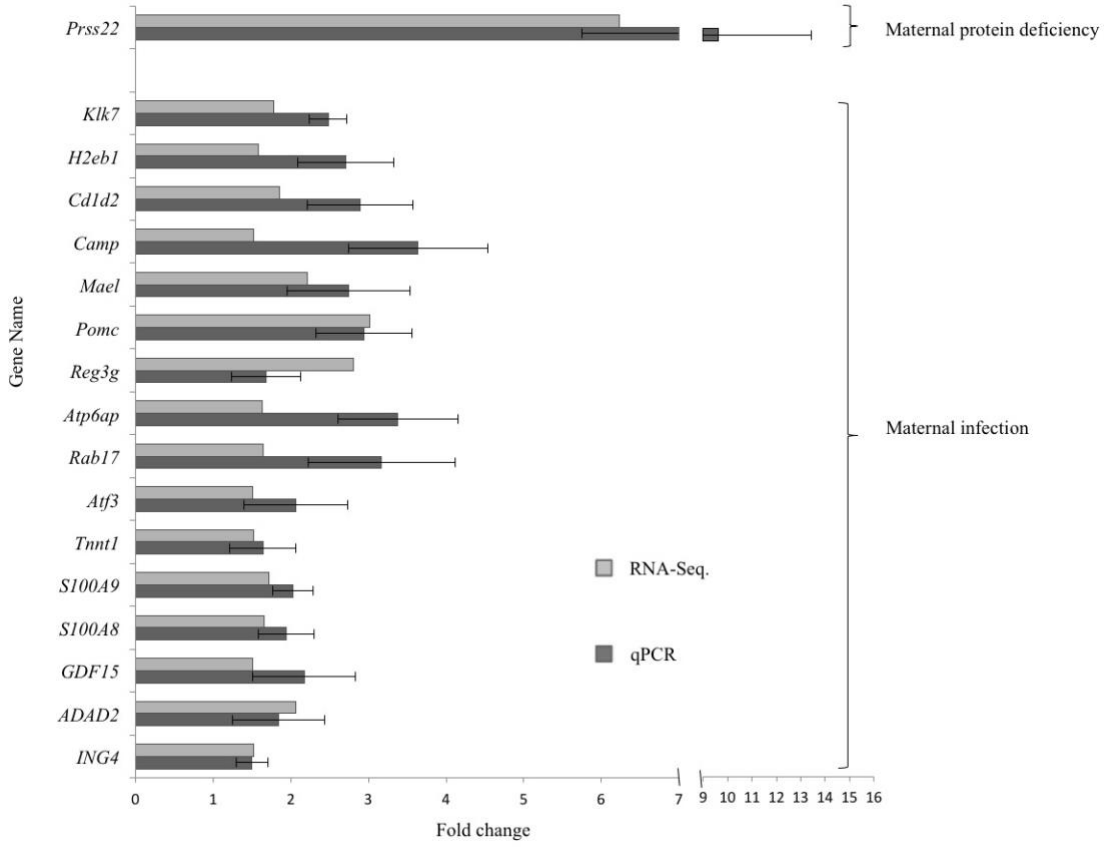

Figure 3. Quantitative PCR (qPCR) confirmation (light grey bar) of RNA-Seq up-regulation (dark grey bar) of one fetal brain gene by maternal protein deficiency and of 16 fetal brain genes by maternal *Heligmosomoides bakeri* infection. The error bars represent S.E.M.

Connecting Statement I

In chapter-III, using a 2 x 2 factorial design I examined the main effect and interactive effect of maternal protein deficiency and nematode infection on gestation day 18 fetal brain gene expression. The results showed three distinct patterns. The interactive effect of maternal protein deficiency and nematode infection resulted in down-regulation of only one gene in the fetal brain and likewise, maternal protein deficiency was associated with up-regulation of only one gene in the fetal brain. In contrast, maternal nematode infection up-regulated 88 genes and down-regulated of 8 genes in the fetal brain. These 96 differentially expressed genes are known to have an important function in various important biological processes such as developmental, metabolic, and immune system processes.

Given that significant brain growth and maturation in mice occurs after birth, I hypothesized that the effect of these alterations in fetal brain gene would persist in the postnatal brain. Therefore, in chapter-IV, I examined the effect of maternal nematode infection on brain gene expression in 2- and 7-day old pups. Due to the negligible effect of maternal protein deficiency on fetal brain gene expression, I dropped protein deficiency as an experimental intervention for the next chapter.

Chapter IV

Maternal Gastrointestinal Nematode Infection Up-regulates Expression of Genes Associated with Long-Term Potentiation in Perinatal Brains of Uninfected Developing Pups

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Abstract

Establishment of neural networks critical for memory and cognition begins during the perinatal period but studies on the impact of maternal infection are limited. Using a nematode parasite that remains in the maternal intestine, we tested our hypothesis that maternal infection during pregnancy and early lactation would alter perinatal brain gene expression, and that the anti-inflammatory nature of this parasite would promote synaptic plasticity and long-term potentiation. Brain gene expression was largely unaffected two days after birth, but in seven-day old pups, long-term potentiation and four related pathways essential for the development of synaptic plasticity, cognition and memory were up-regulated in pups of infected dams. Interestingly, our data suggest that a lowering of Th1 inflammatory processes may underscore the apparent beneficial impact of maternal intestinal infection on long-term potentiation.

Introduction

Processes that underscore cognition and memory begin early in development through induction of five key neurological pathways that prime long-lasting plasticity in synaptic strength[1,2,3]. This phenomenon is referred to as long-term potentiation and relies on early, efficient transport of calcium and glutamate across the glutamatergic synapse. A key early step is the transition from "silent" glutamatergic synapses where only a few glutamate receptors are present on the post-synaptic membrane to the persistent activation of the N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydrozy-5-methylisoxazole-4-propionic acid (AMPA) receptors for calcium and glutamate[4]. This relies on up-regulation of Wnt, glutamatergic, calcium and MAPK signaling pathways as well as the long-term potentiation pathway (Fig 1) [1,2,3]. When these five pathways are established during early brain development, they are more likely to become "hard-wired" leading to long-term synaptic plasticity that enhances cognition and memory[5]. In mice, synaptic connections begin to form just before birth and most neurons have established synaptic connections within a week of birth[6]. Thus, the early postpartum period provides a developmental window where maternal factors may not only influence pup brain gene expression but also influence long-term potentiation.

Maternal factors including *in utero* exposure to glucocorticoids6 and toxins[7], neonatal separation[8], grooming and licking of pups[9] and immune activation[10] have been shown to impair development of long-term potentiation during the perinatal period. In addition, systemic and intrauterine viral and bacterial infections that induce strong pro-inflammatory (Th1) responses during pregnancy[11] have been linked to perinatal brain damage in response to resulting immune activation and neuro-inflammation[12]. Also, intrauterine infection with *Escherichia coli* and the associated increase in the pro-inflammatory cytokine, IL1-β, has been associated with a reduction long-term potentiation and impaired responses to motor and cognitive tests[13]. In contrast, we recently explored the impact of low dose infection of pregnant mice with a nematode that is restricted to the maternal intestine and that induces anti-inflammatory Th2 responses[14]. We observed up-regulated expression of genes related to neurodevelopment and synaptic plasticity in response to maternal infection, in the fetal brain at embryonic day 18. Together, these studies show that maternal conditions that induce Th1 responses may have

harmful effects on perinatal brain development but that conditions that promote Th2 responses may have beneficial effects.

The present study was conducted using the mouse intestinal nematode, *Heligmosomoides bakeri*, a common model for the intestinal nematodes that infect over 24% of the world's population[15]. After infective larvae are ingested, the parasite remains in the intestine of the host, first in the submucosa as a larval stage, and then in the lumen of the small intestine where adult worms mate and females release eggs in the faeces[16]. The strong anti-inflammatory nature of this parasite is evident in Th2 responses both in the local tissue and in circulation of infected mice[17], and in the enhancement of the Th2 response over that normally observed in pregnancy[18]. Furthermore, this infection reduces symptoms of auto-immune diseases that induce a strong inflammatory response[16]. Thus, this model is suitable for testing the intriguing possibility that an infection that remains restricted to the maternal intestine and that is known to induce a Th2 response may have a positive impact on pathways associated with long-term potentiation (Fig. 1) in the uninfected pups.

The study had three objectives. First, we documented differential brain gene expression at postnatal day 2 (P2) and P7 and compared it with the data from embryonic day 18 (E18) from our previous experiment[14], in order to record the impact of maternal infection on the developmental progression of brain gene expression in the uninfected pups. Second, we used gene ontology and pathway analyses to determine whether maternal nematode infection altered expression of individual genes and pathways associated with long-term potentiation in the post-partum brain. Finally, we used pathway analysis and interrogated the gene expression database to determine if maternal infection altered neuro-inflammation or the cytokine environment in the pup brain on P7.

Results

Maternal nematode infection changed developmental progression of brain gene expression.

Time series analysis provided a heatmap of the full developmental profile of brain gene expression from E18 to P2 to P7 in control and infected groups (Supplementary Fig 1). When only the differentially expressed genes were visualized, three developmental patterns emerged

(Fig 2). In pattern 1, gene expression was higher on E18 than on P2 and P7 in both control and infected groups, reflecting developmental down-regulation of pattern 1 genes post-partum. In pattern 2, gene expression increased from E18 to P2 in both control and infected groups but remained elevated at P7 only in the infected group. In pattern 3, gene expression increased from E18 to P2 to P7 irrespective of the infection status of the dams. Expression of the 100 most highly ranked genes in the time series analysis differed between the infected and control groups only on P7 and was up-regulated in 90 these 100 genes (Supplementary Dataset 2), a pattern consistent with pattern 2 genes (Fig 2).

Analysis of the P2 and P7 datasets revealed that only three genes were differentially expressed in the P2 pup brain: one gene (high mobility group AT-hook 1B [*Hmga1b*]) was up-regulated and two genes (MORN repeat containing 2 [*Morn2*] and Gm38402 predicted gene [*Gm38402*]) were down-regulated. In contrast, at P7, 2751 brain genes were up-regulated and 2985 were down-regulated in the infected compared with the control group (Supplementary Dataset 1), and the majority had a log2 fold change between 1 and 2 (Supplementary Fig 2). Several genes known to be critical for postnatal brain development were up-regulated on P7 including thrombospondin 1 (*Tsp1*) which promotes synaptogenesis[19], orthodenticle homeobox 1 (*Otx1*) which is essential for dendritic growth of neurons[20] and bromodomain PHD finger transcription factor (*Bptf*) which helps in neurodevelopment[21]. In addition, expression of transcription factors, receptors for glutamate, GABA, glycine, cholinergic, dopamine and serotonin, several ion channels (calcium, potassium, sodium, chloride, and potassium/chloride co-transporter), synaptic proteins, cell adhesion molecules, and cell signalling pathways (Wnt, Bmps and hedgehogs) was up-regulated in the infected group at P7 (Supplementary Dataset 3).

Maternal nematode infection altered P7 brain gene ontologies, pathways and protein-protein interaction networks.

Gene ontology analysis: Maternal nematode infection significantly enriched 868 PANTHER GO terms (Supplementary Dataset 4 and 5). When reanalyzed to remove redundancy and ensure semantic similarity, infection upregulated 34 REVIGO GO terms at P7, 19 of which were involved in biological processes central to postnatal mouse brain development, namely central

nervous system development, 9 were in regulation of small GTPase-mediated signal transduction, and 4 were in synapse organization (Fig. 3).

KEGG pathway analysis: Differential expression was detected for 99 pathways (Supplementary Dataset 6). Among the 37 pathways with at least 15 differentially expressed genes (Fig 4), maternal nematode infection down-regulated three pathways: pyrimidine metabolism, purine metabolism, and cytokine-cytokine receptor interaction. The insulin signaling pathway was both down-regulated and up-regulated. Maternal infection up-regulated 30 pathways including three pathways directly involved in synaptogenesis (axon guidance, cholinergic synapse, and dopaminergic synapse) and five specifically associated with glutamatergic long-term potentiation (glutamatergic synapse, Wnt signaling, MAPK signaling, calcium signaling, and long-term potentiation).

Protein-protein interaction network: The IMEx protein-protein interaction network confirmed that the five pathways associated with long-term potentiation interacted with one another and that maternal nematode infection altered expression of a very large number of the nodes (Fig. 5). In addition, several pathways that promote cell division and growth were up-regulated including ErbB signaling and Jak-STAT signaling which presumably indicates more favorable conditions for cellular proliferation in the pup brains of the infected compared with the control group.

Maternal nematode infection up-regulated five pathways associated with long-term potentiation. We interrogated the pathways and heatmaps for specific critical genes involved with Wnt signalling (Fig 6), glutamatergic signalling (Fig 7), MAPK signalling (Fig 8), calcium signalling (Fig 9) and long-term potentiation (Fig 10) at P7. In addition, the P7 database of differentially expressed genes was examined for specific genes known to be associated with long-term potentiation. The fold-change values for all differentially expressed genes at P7 is presented in the Supplemental Dataset 1.

Wnt signalling of the pre-synaptic cell is a first step in the cascade leading to long-term potentiation. Among the genes in the Wnt signalling pathway (Fig 6), the important up-regulated genes for canonical Wnt signalling were frizzled family receptors (*Fzd*), catenin cadherin-associated proteins (*Ctnnd1* and *Ctnnd2*), dishevelled segment polarity protein 3 (*Dvl3*), glycogen synthase kinase 3 beta

(*GSK3b*), and low-density lipoprotein receptor-related proteins (*LRP1, 2, 3, 4, 5*, 6, and *8*). In addition, up-regulation of non-canonical Wnt signalling was evidenced by up-regulation of Wnt family members including *Wnt1, 2b,* and 5a, associated receptor tyrosine kinase-like orphan receptors (*RoRa* and *b*), several phospholipase Cs (*PLCs*), several potassium and calcium voltage-gated channels (*KCNQs* and *VGCCs*, respectively), lymphoid enhancer binding factor 1 (*Lef1*), transcription factor 7 like 1 and 2 (*Tcf7l 1* and *Tcf7l 2*), Creb binding protein (*Crebbp*), and several G-protein coupled receptors (*GPCRs*). Furthermore, we also recorded up-regulation of the abnormal spindle microtubule assembly (*Aspm*) gene that positively regulates Wnt signaling in the brain[22].

The presence of excitatory glutamatergic receptors (NMDARs and AMPARs) on the post-synaptic cell is critical not only for the functioning of the glutamatergic synapse (Fig 7), but also for long-term potentiation (Fig. 10). Two sub-units of NMDARS, *NR2A* and *NR2B* (also known as *Grin2A* and *Grin2B*) as well as several AMPARs including the metabotropic glutamate receptors 1 and 2 (*mGlu-R1* and *mGlu-R2*), *Gria3*, *Grm2*, *Grm4*, *Grik3* and *Grik5* were up-regulated in the pup brain on P7 in response to maternal nematode infection.

Up-regulation of MAPK signalling (Fig. 8) is also required for long-term potentiation (Fig 10). Braf transforming gene (*Braf*) was up-regulated and it is involved in both MAPK signalling and long-term potentiation. Other up-regulated genes in the MAPK pathway included 11 MAPKs, guanine-nucleotide exchange factor (*Rapgef2*) and genes for several GTPase-activating proteins (*ras-GAPs)* including guanine-nucleotide exchange factors (*ras-GEF*). A number of calcium channel genes were also up-regulated including several subunits of *Cacna 1* and *Cacna 2.*

Calcium signalling (Fig 9) is also required for MAPK (Fig 8), glutamatergic signalling (Fig 7) and long-term potentiation (Fig 10). Expression of calcium/calmodulin dependent protein kinase II (*CaMK* 2) was up-regulated and it is an important mediator in the Ca^{2+} signaling pathway.

Finally, long-term potentiation is negatively associated with protein phosphatase kinases[23]. Consistent with our finding that the long-term potentiation pathway was up-regulated, expression of several protein phosphatase kinases (*Ppp1ca*, *Ppp1cc*, and *Ppp1cb*), was down-regulated in the long-term potentiation pathway (Fig 10). Taken together, these gene expression and pathway data provide

compelling evidence that long-term potentiation was up-regulated in the P7 brain of uninfected offspring of mothers that were infected with a gastrointestinal nematode throughout pregnancy and the first week of lactation.

Maternal nematode infection up-regulated TH2 responses and down-regulated Th1 responses. Consistent with the Th2-mediated immune tolerance induced by *H. bakeri,* we observed up-regulated expression of the Th2 cytokine, IL-4, a hallmark of *H. bakeri* infection[16] and of forkhead box protein 3 (*Foxp3*) and transforming growth factor beta (*TGFβ*) genes which exert immune tolerance effects in response to helminth infection[24], as well as interleukin 6 signal transducer (Il6st), interleukin 6 receptor (*Il6ra*), signal transducer and activator of transcription 3 (*Stat3*), and interleukin 17 receptor (*Il17rd*) (Supplementary Dataset 1). In contrast, we observed down-regulation of several Th1-associated responses. Expression of the pro-inflammatory cytokine gene, *IL-1β*, was down-regulated, the cytokine-cytokine receptor interaction pathway was down-regulated (Supplementary Dataset 6), and several autoimmune disease pathways associated with Th1 immune activation were down-regulated (autoimmune thyroid disease, systemic lupus erythematosus, type 1 diabetes mellitus, asthma) (Supplementary Dataset 6). Together these observations showed that maternal infection was associated with up-regulation of Th2 responses and down-regulation of Th1 inflammatory responses in the postnatal (P7) brain.

Discussion

This study extends evidence of maternal effects on early brain development by revealing changes at P7 but not P2 in pup brain gene expression in response to infection with a nematode parasite that is restricted to the maternal small intestine. Maternal nematode infection up-regulated five highly integrated pathways that are required for synaptogenesis, synaptic plasticity and long-term potentiation. Maternal infection also shifted expression brain cytokines in favour of a Th2 response indicating an anti-inflammatory environment in the P7 pup brain. Together, these findings raise the intriguing possibility that this maternal nematode infection may have a beneficial impact on pup brain development.

Considerable neurodevelopment occurs during the perinatal period in mice. By E18 synaptic connections between neural axons and dendrons have started to form and astrocyte production increases in the first week after birth[6]. Astrocytes secrete thrombospondin (TSPs) proteins which are crucial for synaptogenesis[19]. Under normal conditions, most neurons have established synaptic connections forming neural circuits by P7[6]. This early brain development is controlled by precise expression of genes related to transcription factors, receptors, ion channels, synaptic proteins, cell adhesion molecules, cell signaling pathways and other associated factors[6] with higher expression at P7 compared to E18[6]. Our results extend these findings in that maternal infection further increased expression of these genes at P7. Moreover, our observation of up-regulation of essential genes for brain development including *Tsp1*[19], *Otx1*[20] and *Bptf*[21] suggests that maternal nematode infection may have a positive impact on the developmental progression of gene expression from E18 to P7.

Glutamatergic synaptic receptors are a type of excitatory receptor in the brain that play a crucial role in neuronal development, synaptic plasticity and long-term potentiation[25]. NMDARs are one of the main ionotropic glutamatergic receptors and consist of NR2A and NR2B subunits whose differential expression pattern depends on the stage of brain developmental[26]. In mice, expression of *NR2B* is up-regulated from birth and throughout the postnatal development whereas expression of *NR2A* begins around P7 and increases until 2 or 3 weeks before adulthood[27]. In response to maternal nematode infection, expression of both *NR2A* and *NR2B* (also known as *Grin2A* and *Grin2B*) genes was higher in the P7 brain. Such early up-regulation of NMDAR sub-unit expression in response to prenatal exposure to valproic acid was shown to result in synaptic plasticity and long-term potentiation in neocortical pyramidal neurons[28] whereas lower NMDAR expression in response to maternal stress resulted in learning deficits in offspring[29]. Based on our findings, pups of dams infected with an intestinal nematode during pregnancy and early lactation may have improved NMDAR-mediated synaptic plasticity and long-term potentiation.

Ca^{2+} signalling is required for NMDAR-mediated synaptic plasticity and long-term potentiation[30], and the Ca^{2+} signalling pathway was up-regulated in pups of nematode-infected dams. The activation of NMDARs accelerates the intracellular influx of Ca^{2+} ions which activate CaMK*2* which subsequently auto-phosphorylates the synaptic AMPARs, enhancing the conductance of the receptor leading to long-term potentiation[31,32]. Furthermore, Ca^{2+} signaling activates the transcription

factor cAMP response element binding protein (*Crebbp*) which enhances transcription of genes involved in neural growth and survival as well as synaptic connectivity[33]. The higher expression of both *CaMK2* and *Crebbp* as well as the Ca^{2+}signaling pathway itself in the brain of P7 pups of nematode-infected dams indicates up-regulated Ca^{2+} signalling, and furthermore, down-regulation of protein phosphatase kinases which suppress long-term memory[23] supports the observation that maternal nematode infection exerts a positive influence on the developing brain of the pups.

Maternal nematode infection also up-regulated the Braf-MAPK signaling pathway that is essential for synaptic transmission and long-term potentiation[34]. Braf-MAPK signalling provides the phosphorylation link between activated CaMK2 receptors and AMPARs[35]. Small GTPases initiate a phosphorylation reaction cascade through *ras-GEF* and *ras-GAP* that were both up-regulated. Phosphorylation of AMPARs, including the up-regulated metabotropic glutamate receptors 1 and 2 (*mGlu-R1* and *mGlu-R2*), leads to their expression on the post-synaptic membrane resulting in synaptic plasticity and long-term potentiation[36]. Deficiency of *mGlu-R1* has been shown to impair motor function and synaptic plasticity[37]. Hence, up-regulation of the Braf-MAPK pathway together with enhanced expression of key genes including the Braf transforming gene (*Braf*), several *MAPK*s, *Ras-GEF,* and *Ras-GAP* in the pup brain would also contribute to synaptic plasticity and long-term potentiation.

Wnt signaling is needed for transcription of genes involved in synaptic plasticity and long-term potentiation[38], and this pathway was up-regulated in our study. Canonical Wnt signalling through β-catenin mediates neural differentiation from the neural precursor cells[39]. Several genes associated with canonical Wnt signaling were up-regulated in the P7 brain of nematode-infected dams including *Fzd3, catenin* and several *LRPs*. Non-canonical Wnt signalling through Ca^{2+}[40] leads to higher expression of NMDARs in the synapses, release of intracellular Ca^{2+} *via* voltage-gated Ca^{2+} channels, and synaptic responses including long-term potentiation[3]. We also have evidence for up-regulation of non-canonical Wnt signalling as seen in higher pup brain expression of *Wnt5a*, its associated receptors (*RoRa* and *b, PLC*) and ion channels (*KCNQs* and *VGCC*) in response to maternal infection. Our findings differ from the observation that maternal exposure to toxic chemicals down-regulates non-canonical Wnt signaling and impairs growth of a range of tissues in offspring[41,42]. Both higher canonical and non-canonical Wnt signaling observed in our study further strengthen our

conclusion that *in utero* and postpartum exposure to a nematode restricted to the maternal intestine may improve synaptic plasticity in the next generation.

The exact mechanism whereby a nematode living in the lumen of the maternal intestine would augment the signalling pathways associated with synaptic plasticity and long-term potentiation in the P7 pup brain is not clear, but our data raise two possibilities. First, maternal nematode infection reduced neuro-inflammation in the pup brain as evidenced by the down-regulated expression of the inflammatory cytokine *IL-1*β which impairs neurogenesis and cognition[43] and up-regulated expression of the anti-inflammatory cytokine *IL-4* which has been previously shown to promote long-term potentiation[44]. Moreover, up-regulation of *Foxp3*, *TGF*β, *Il6st*, *Il6ra*, *Stat3* and *Il17rd* strongly suggest that maternal nematode infection induced an immune tolerant or anti-inflammatory environment which is known to promote long-term potentiation in the pup brain[45]. IL17 has also been shown to have anti-inflammatory properties and to suppress the development of autoimmune disease[46]. Thus, the up-regulation of IL17 expression in response to maternal infection is consistent with our observation that autoimmune-related pathways (autoimmune thyroid disease, systemic lupus erythematosus, type I diabetes mellitus) and immune activation related (asthma) disease pathways in the pup brains were down-regulated in response to maternal infection. Second, it is possible that *H. bakeri* derived microRNA[47] might reach the neonatal circulation and influence Wnt signaling in the postnatal brain. We recorded up-regulated expression of *Aspm* gene which interacts with small interfering RNA[48] and is a positive regulator of Wnt signaling in the brain[22].

Previously, a few beneficial effects of nematode parasites have been demonstrated in humans including use of nematode eggs to treat autoimmune diseases[49], lower allergic responses in nematode-infected infants and children[50] and favourable effects of nematodes on maternal reproduction as evidenced by improved conception, implantation and overall fecundity[51]. Regarding cognition, although there is some evidence from school-age children that direct infection with intestinal nematodes impairs cognition[52,53], a meta-analysis of the human studies concludes that the body of evidence does not support this claim[54]. In rodent study, an intestinal nematode infection with an extra-intestinal phase has been shown to impair reference memory in adult mice[55]. However, prior to the present study, nothing was known about the impact of the maternal nematode infection directly on postnatal brain gene expression or more specifically on key neuro-developmental pathways, although

two epidemiological studies are of relevance. Both have identified a negative, not positive, association between maternal infection with two intestinal nematodes during pregnancy and cognitive scores in infants[56,57]. Of note, however, both of the human nematodes have an extra-intestinal phase[58], in contrast to our model where the nematode remains in the intestine[16]. It is likely that researchers did not know whether the mothers had been infected during pregnancy, and that maternal infection during pregnancy and lactation may have heightened long-term potentiation with long-lasting beneficial effects for their children. Of course, this possibility can only be entertained if the rodent-based findings extend to humans.

In conclusion, our study demonstrated that a gastrointestinal nematode infection restricted to the maternal intestine positively influenced expression of postnatal brain genes and pathways associated with long-term potentiation. To the best of our knowledge, this is the first study showing that maternal nematode infection influences postnatal brain gene expression. This sheds light on an unappreciated effect of maternal nematode infection on postnatal brain development, which may have important long-term consequences for learning and cognition. However, further research is needed to determine whether differential gene expression leads to altered neurophysiology and altered cognitive behaviors that persist as the pups grow. If also applicable to human populations, this finding may have relevance to decisions regarding deworming treatments during pregnancy and early lactation.

Materials and Methods

Experimental design. We used a randomized design where half of the pregnant mice were infected with an intestinal nematode, and half were given a sham infection. Litters were sacrificed either on postpartum day 2 (P2) and postpartum day 7(P7). The protocol (# 2000-4601) was approved by the McGill University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

Parasites and mice. *Heligmosomoides bakeri* (= *H. polygyrus*; *Nematospiroides dubius*) is a murine nematode model of intestinal nematodes of livestock and humans[16]. When ingested, infective third stage larvae (L_3) penetrate the submucosa of the small intestine and undergo two moults before returning to the intestinal lumen where adult worms release eggs in the faeces[16]. Repeated exposure to larvae stimulates an ongoing Th2 immune response[18].

A total of 20 primiparous 8 to 9-week-old timed pregnant (gestation day, E4) outbred CD1 mice were housed individually in Nalgene cages (Fisher Scientific, Canada) with stainless steel racks to prevent coprophagia, at 22-25°C, 40-60% relative humidity and a 12 h light and dark cycle. Mice were fed a 24% protein diet (Harlan Teklad, TD. 90017) *ad libitum*. On embryonic day 5 (E5), mice were separated randomly into two groups (control and infected). The infected group was intubated with 100± 3 (L_3) suspended in distilled water on E7, E12 and E17, as well as on P3, for those mice necropsied on P7 (Fig 11). Establishment of *H. bakeri* infection in infected dams was confirmed at necropsy by presence of lesions on the duodenal wall and adult worms in the intestinal lumen. The control group was intubated with the same volume of distilled water on all infection days.

Pup brain and liver collection. We did not obtained litters from one control and two infected dams on P2. Therefore, four litters from control and three litters from infection group were selected and exsanguination on P2. Similarly, on P7, the remaining five litters from each group were sacrificed and the whole brain and liver tissue were collected. All tissue samples were snap frozen in liquid nitrogen and stored at -80$_0$C.

Genetic determination of sex of pups from liver tissue. Genomic DNA was extracted from pup liver tissue by DNeasy Blood & Tissue Kit® (Qiagen, Germany) following the manufacturer's guideline and amplified using the SX_Forward, 5′-GATGATTTGAGTGGAAATGTGAGGTA-3′ and SX_Reverse, 5′-CTTATGTTTATAGGCATGCACCATGTA-3′ primer pair according to published protocol[59]. Polymerase chain reactions were performed in a final volume of 25 µl using GoTaq® Green Master Mix (Promega, USA) with the following protocol: initial denaturation at 95°C for 5 min, 30 cycles with 95°C for 1 min, 85°C for 30 secs, 75°C for 30 secs, 53°C for 1 min, 72°C for 1 min. and the final elongation at 72°C for 5 min. The amplified products were run on 2% agarose gel with 2-Log DNA Ladder (0.1-10.0 kb, New England BioLabs). The primers amplified a Y-chromosome specific fragment in males that produced a 280 bp amplicon and two fragments of X-chromosome in females producing 480 and 660 bp amplicons.

RNA extraction and Illumina Hi-Seq sequencing of the male pup brain. For this study, we only studied the brains of male pups as the hippocampus develops larger in males than females in early

postpartum[60]. At both P2 and P7 time points, one brain per litter was randomly selected, and the total RNA of each brain was extracted using a commercial kit (NucleoSpin® RNA Plus, Macherey-Nagel, Germany) following the manufacturer's protocol. No pooled samples were used. The concentration of RNA was determined spectrophotometrically using NanoDrop. Seven RNA samples from P2 (four from the control and three from the infection group) and 10 RNA samples from P7 (five from the control and five from the infection group) were sent to a local genome centre for cDNA library preparation and Illumina Hi-Seq sequencing using the Illumina HiSeq4000 sequencer. After initial processing of raw data (FASTQ) including filtering of low tags, trimming, adaptor removal and alignment to the mouse reference genome (GRCm38), the sequencing centre provided us with the paired-end BAM files. The data files have been submitted into the Gene Expression Omnibus (GEO) repository (GSE118064).

Counting reads and analysis of differential gene expression. The paired end BAM files were sorted by read name using SAM tools[61]. HTSeq-count (v0.6) was used to count the reads for expressed exons within each gene[62]. All the count tables from different BAM files were compiled categorically (infection *vs* control) in a tab delimited text (.txt) file for each time point (P2 and P7) and the text files were uploaded to *NetworkAnalyst* (http://www.networkanalyst.ca), a web-based tool for comprehensive gene expression profiling[63]. The *edgeR* method was selected with adjusted *P* value < 0.05 and fold change > 1 to identify genes differentially expressed in the pup brain in response to maternal nematode infection. Principal component analysis confirmed that the effect of maternal nematode infection was homogeneous among the experimental replicates (Supplementary Fig.3).

Time-series analysis of gene expression. For the time-series gene expression analysis, we used gene expression data of three time points, namely E18, P2 and P7. The E18 data were obtained from our previous experiment [14] using the same experimental system (GSE96103) where pregnant mice had been given a sham infection or infected with 100 ± 3 L_3 on E5, E10 and E15 (vs. E7, E12, E17 and P3 in the present study), and where brains of unsexed fetuses (vs. only male pups in the present study) were harvested on E18. The previous study had included both protein-sufficient and protein-deficient dams, but fetal brain gene expression data only from the protein-sufficient arm of the study were used here.

We used *MetaboAnalyst* 4.0 pipeline with time series and one experimental factor as study design[64]. A heatmap was created for the direct visualization of the relative levels of gene expression over E18, P2 and P7 time points and between the control and infection groups and further modified by elimination of non-significant genes by ANOVA2 with Bonferroni multiple testing. Multivariate Empirical Bayes time-series analysis (MEBA)[65] was used in the pipeline to rank the genes whose temporal expression patterns differed. The 100 most highly ranked genes were selected and analyzed for gene ontology biological processes.

Gene ontology enrichment analysis of differentially expressed genes. The differentially expressed genes were analysed for enrichment with Gene Ontology biological process by PANTHER overrepresentation test. The significance of enriched GO terms was determined after Bonferroni correction for multiple testing. Furthermore, the enriched GO terms were summarized by removing redundancy and clustering based on their semantic similarity using ReviGO[66].

Pathway analysis and interactive network visualization. Protein-protein interaction networks of the differentially expressed genes were generated using the IMEx interactome database and the biological significance of up- and down-regulated nodes was determined by functional exploration against the KEGG pathway database in *NetworkAnalyst.*

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Supplementary data

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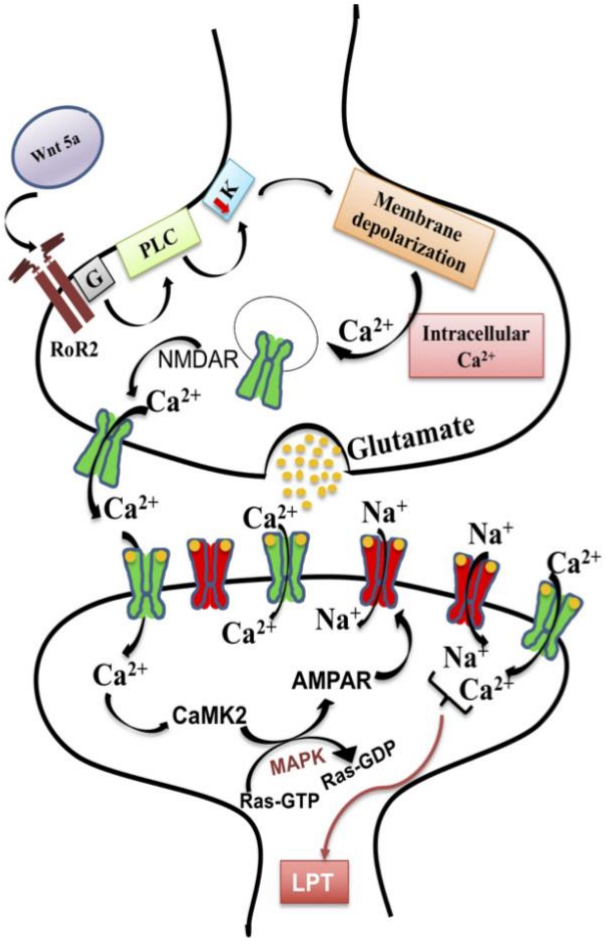

Figure 1.

Schematic of pathways associated with long-term potentiation in the pre-and post-synaptic membrane. Binding of Wnt5a to surface receptors on the pre-synaptic cell membrane (initiation of the Wnt signaling pathway) leads to mobilization of Ca2+ from intracellular stores (initiation of Ca2+ signaling pathway) and expression of N-methyl-D-aspartic acid receptors (NMDARs) on both pre-synaptic and postsynaptic membranes (initiation of the glutamatergic synapse pathway)[3]. Expression of NMDARs on the post-synaptic membrane facilitates the entry of Ca2+ which in turn activates calcium-calmodulin dependent protein kinase II (CaMKII) and mitogen-activated protein kinases (MAPKs) (initiation of the MAPK signaling pathway)[34]. Together NMDARs and MAPK lead to phosphorylation and translocation of α-amino-3-hydrozy-5-methylisoxazole-4-propionic acid receptors (AMPARs) to the post synaptic membrane[36] where they increase synaptic conductance leading to long- term potentiation.

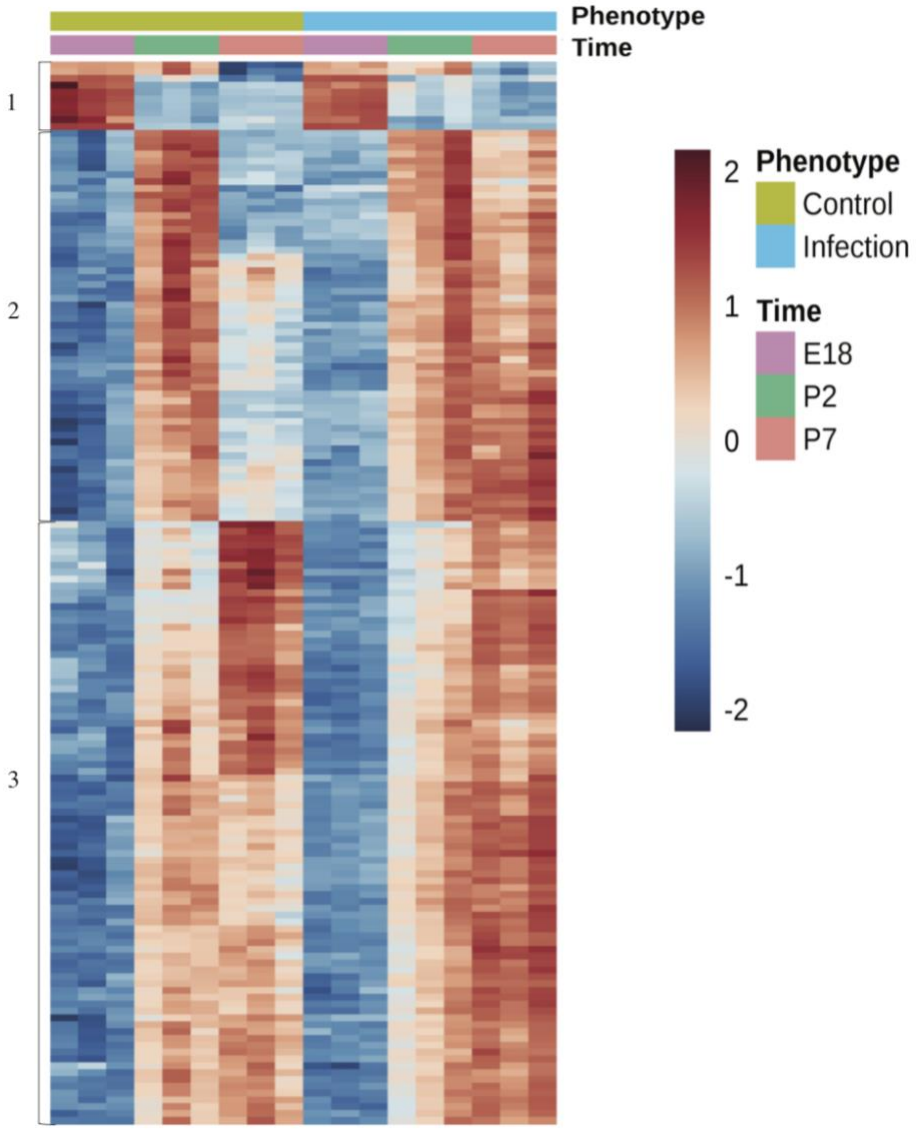

Figure 2.

Heatmap generated after elimination of non-significant genes by ANOVA2 with Bonferroni multiple testing revealing 3 temporal patterns of differential gene expression between control and infection groups at embryonic day 18 (E18), postnatal day 2 (P2) and postnatal day 7 (P7). Pattern 1 gene expression was higher on E18 than P2 and P7 in both control and infected groups. Expression of pattern 2 genes increased from E18 to P2 in both control and infected groups and highly expressed at P7 in the infected group but not the control group. Expression of genes in patterns 3 progressively increased from E18 to P2 to P7 irrespective of the infection status of the dams.

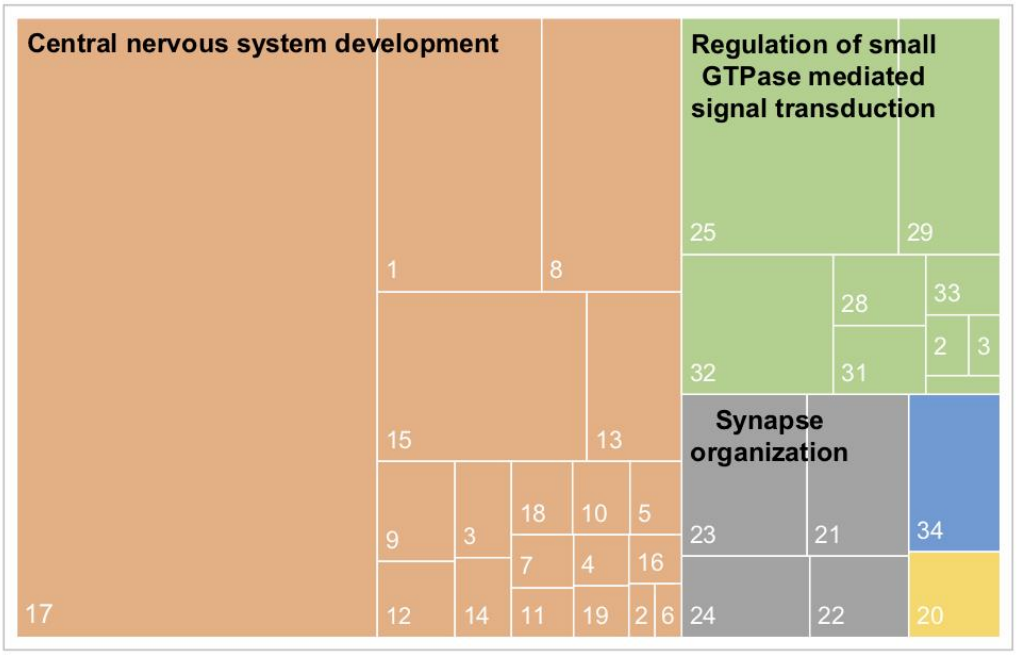

Figure 3.

TreeMap visualization of biological process GO terms (small rectangles) up-regulated in REVIGO. Superclusters of loosely related terms are visualized using different colors. The size of each rectangle reflects the p-value of the GO term. Where 1. Central nervous system development; 2. Regulation of neuron migration; 3. Neuromuscular process; 4. Neuromuscular process controlling balance; 5. Nerve development; 6. Embryonic camera-type eye morphogenesis; 7. Glomerulus development; 8. Post-embryonic development; 9. Gland morphogenesis; 10. Artery development; 11. Neuroepithelial cell differentiation; 12. Embryonic pattern specification; 13. Developmental growth involved in morphogenesis; 14. Neural tube closure; 15. Head development; 16. Cranial nerve development; 17. Cell morphogenesis; 18. Peripheral nervous system development; 19. Autonomic nervous system development; 20. Neural precursor cell proliferation; 21. Synapse organization; 22. Regulation of synapse structure or activity; 23. Plasma membrane organization; 24. Regulation of cell size; 25. Regulation of small GTPase mediated signal transduction; 26. Positive regulation of JUN kinase activity; 27. Negative chemotaxis; 28.Posttranscriptional gene silencing by RNA; 29. Steroid hormone mediated signaling pathway; 30. Regulation of glutamate receptor signaling pathway; 31. Regulation of synaptic plasticity; 32. Negative regulation of translation; 33. Semaphorin-plexin signaling pathway; 34. Locomotory behavior

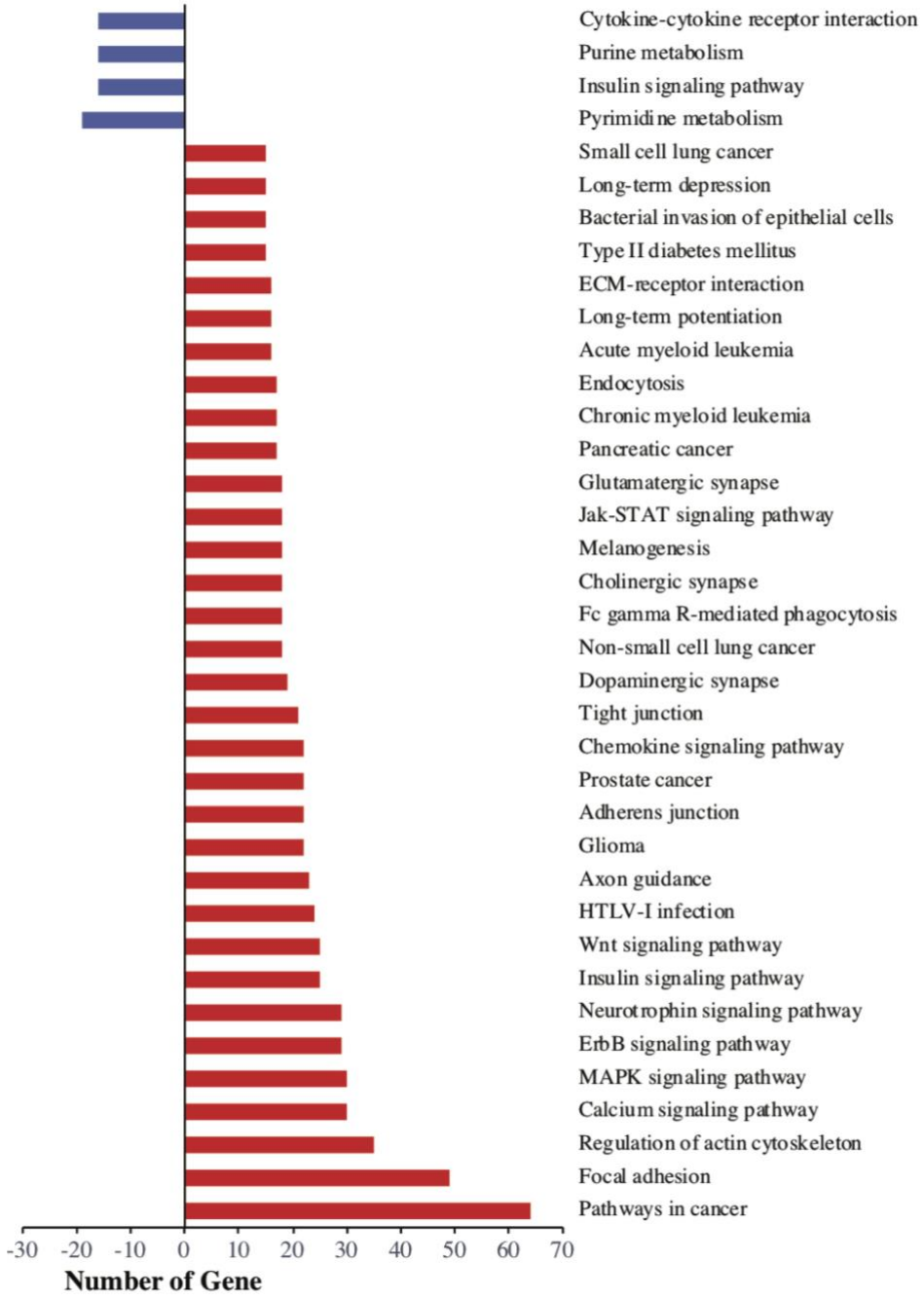

Figure 4.

Bar diagram of KEGG pathways that were significantly down-regulated (blue) or up-regulated (red) by maternal nematode infection in the postnatal day 7 (P7) pup brain, and that had more than 15 down- or upregulated genes respectively.

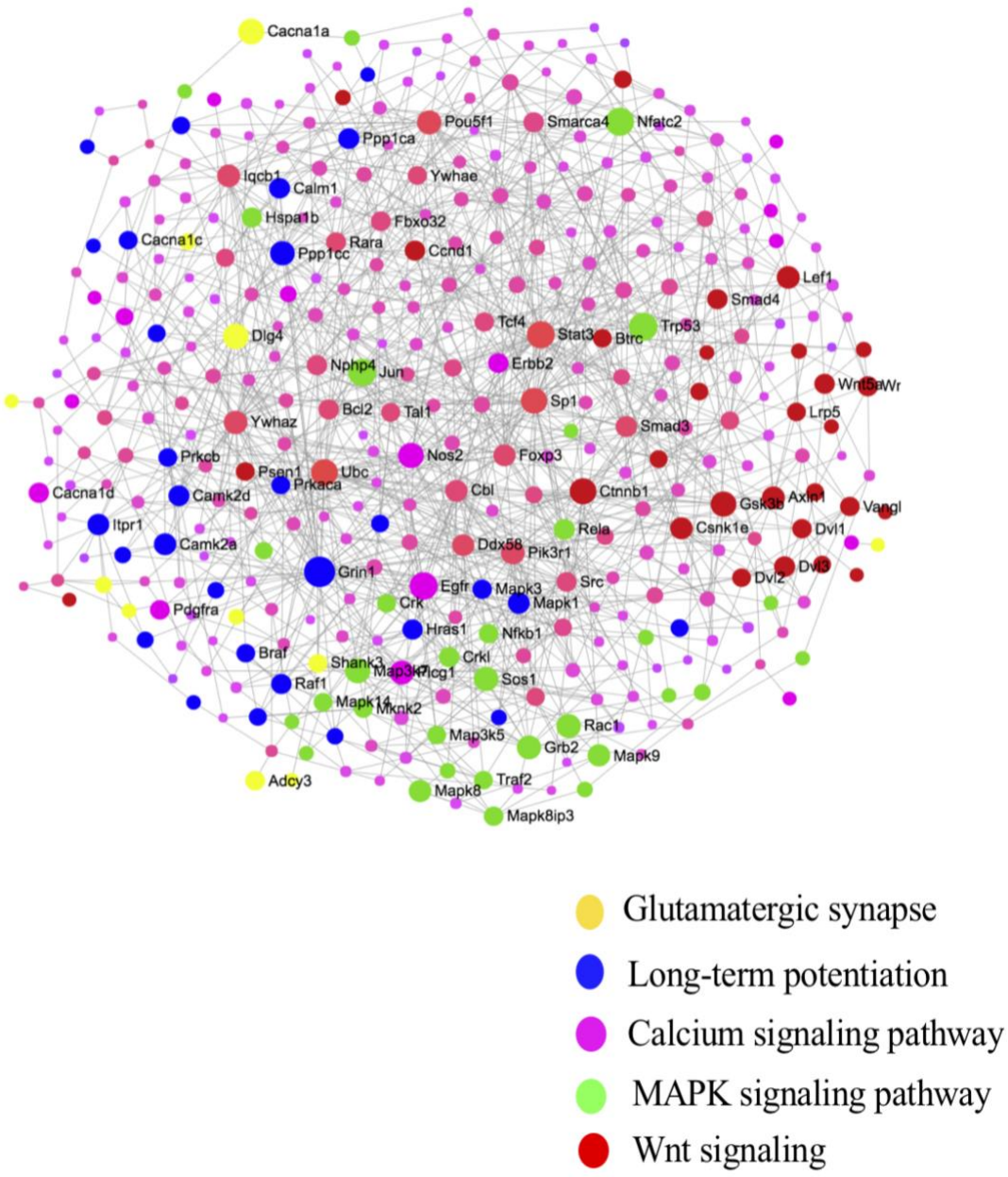

Figure 5.

IMEX Network of the postnatal day 7 (P7) brain showing the interactions among Wnt signaling, MAPK signaling, calcium signaling, long-term potentiation and glutamatergic synapse pathways in response to maternal nematode infection.

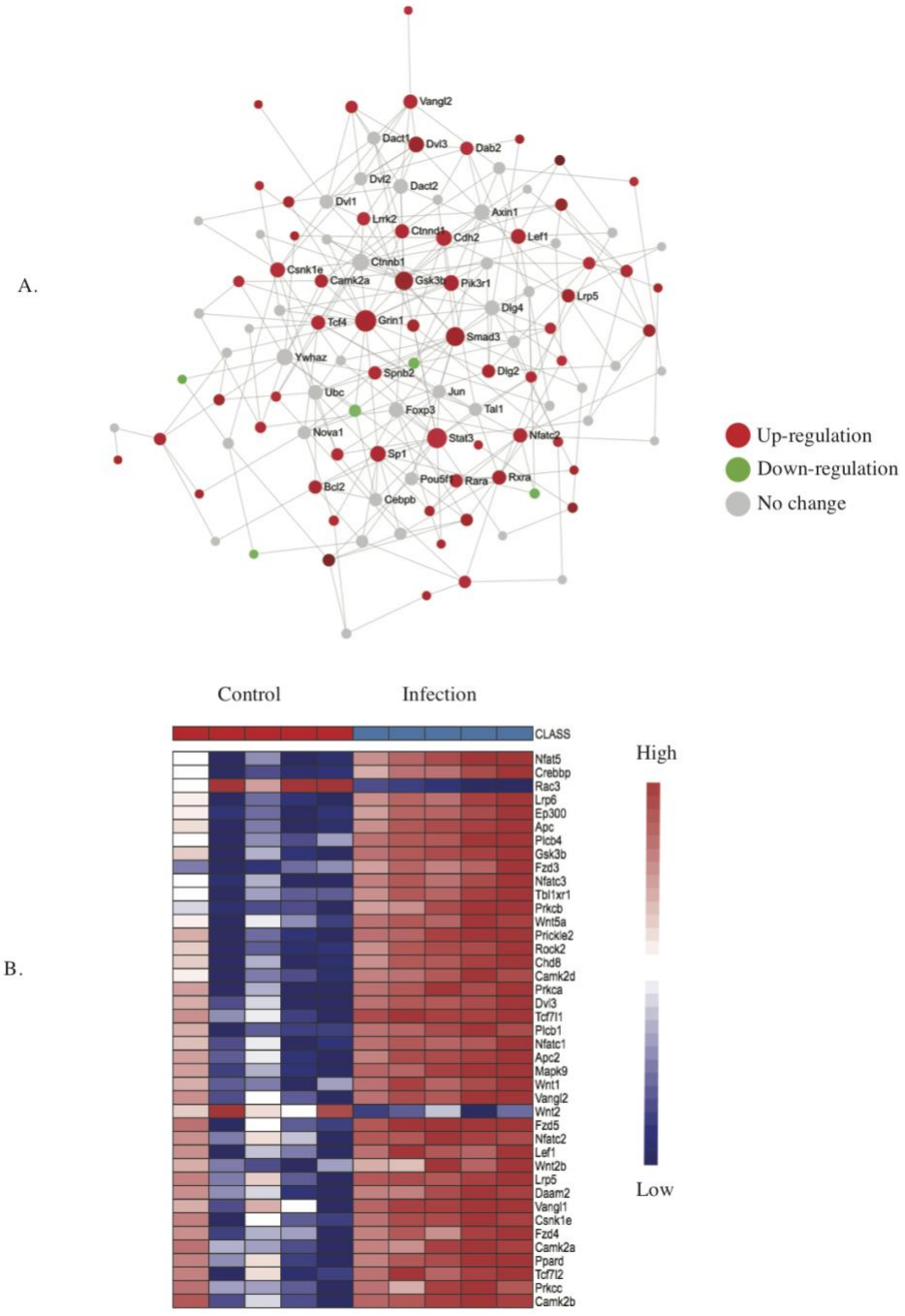

Figure 6.

KEGG pathway analysis (A) and heatmap (B) of the postnatal day 7 (P7) brain showing up- and down-regulated genes involved in the Wnt signaling pathway in response to maternal nematode infection.

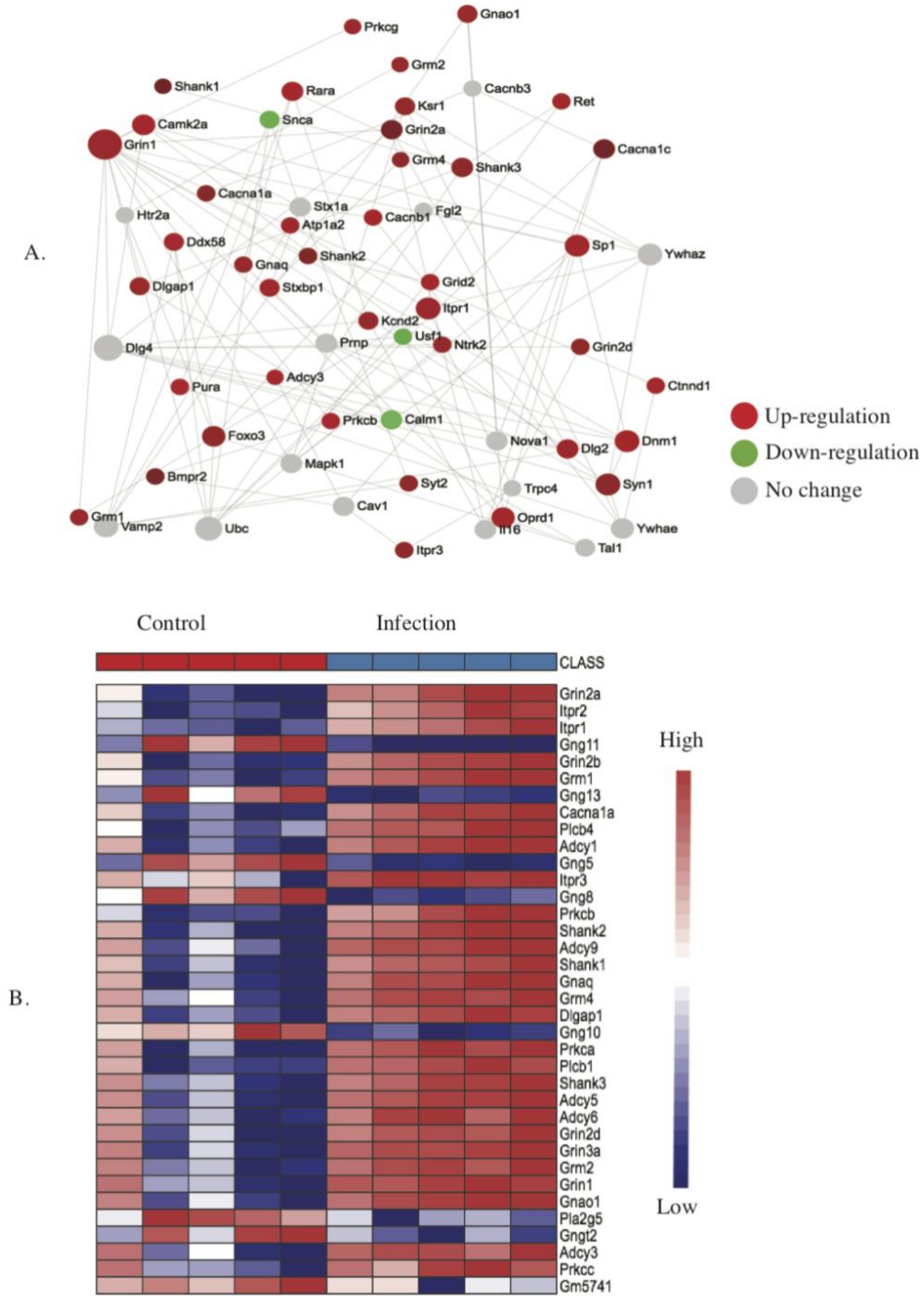

Figure 7.

KEGG pathway analysis (A) and heatmap (B) of the postnatal day 7 (P7) brain showing up and downregulated genes involved in glutamatergic synapse signaling pathway in response to maternal nematode infection.

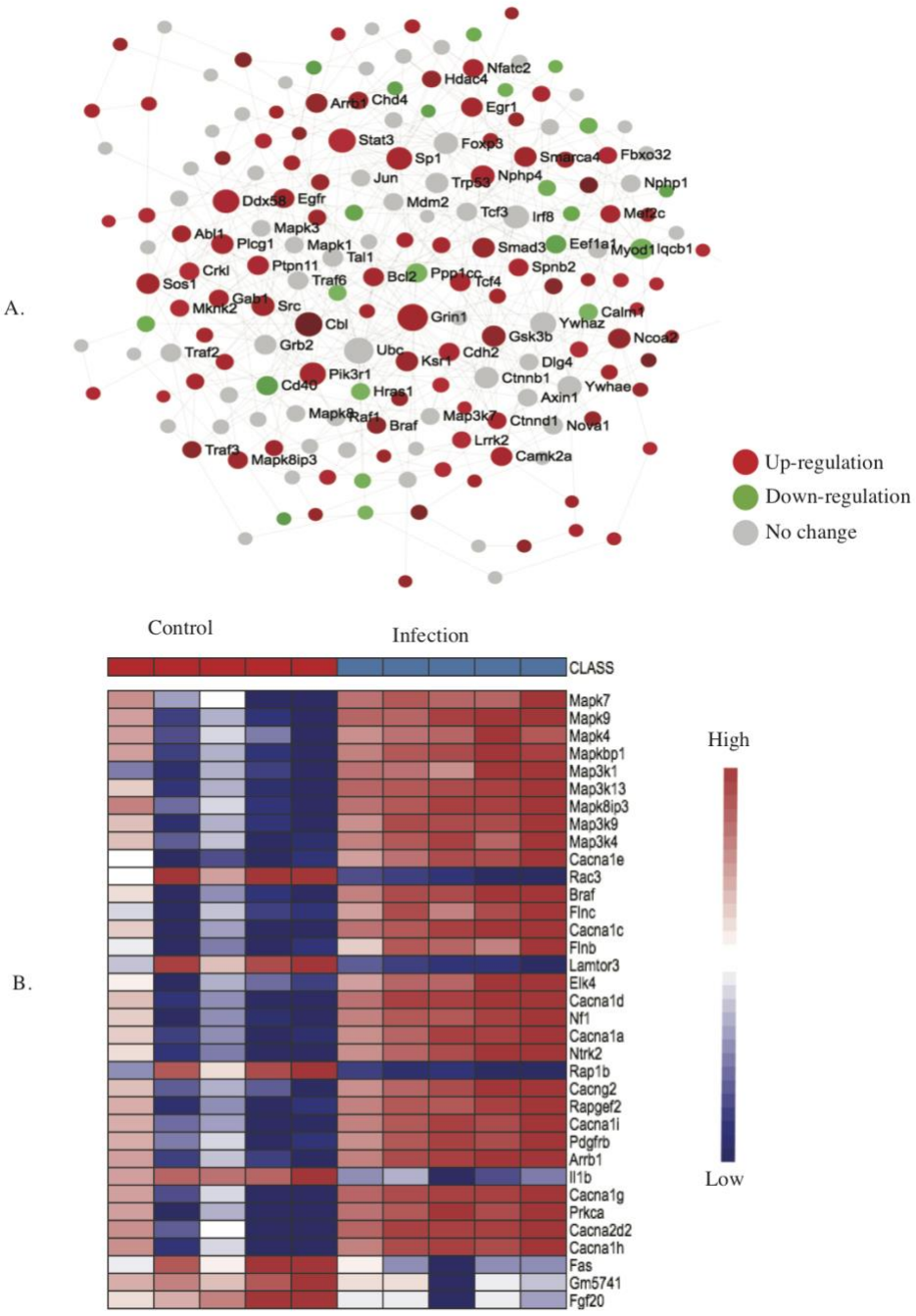

Figure 8.

KEGG pathway analysis (A) and heatmap (B) of the postnatal day 7 (P7) brain showing up and downregulated genes involved in MAPK signaling pathway in response to maternal nematode infection.

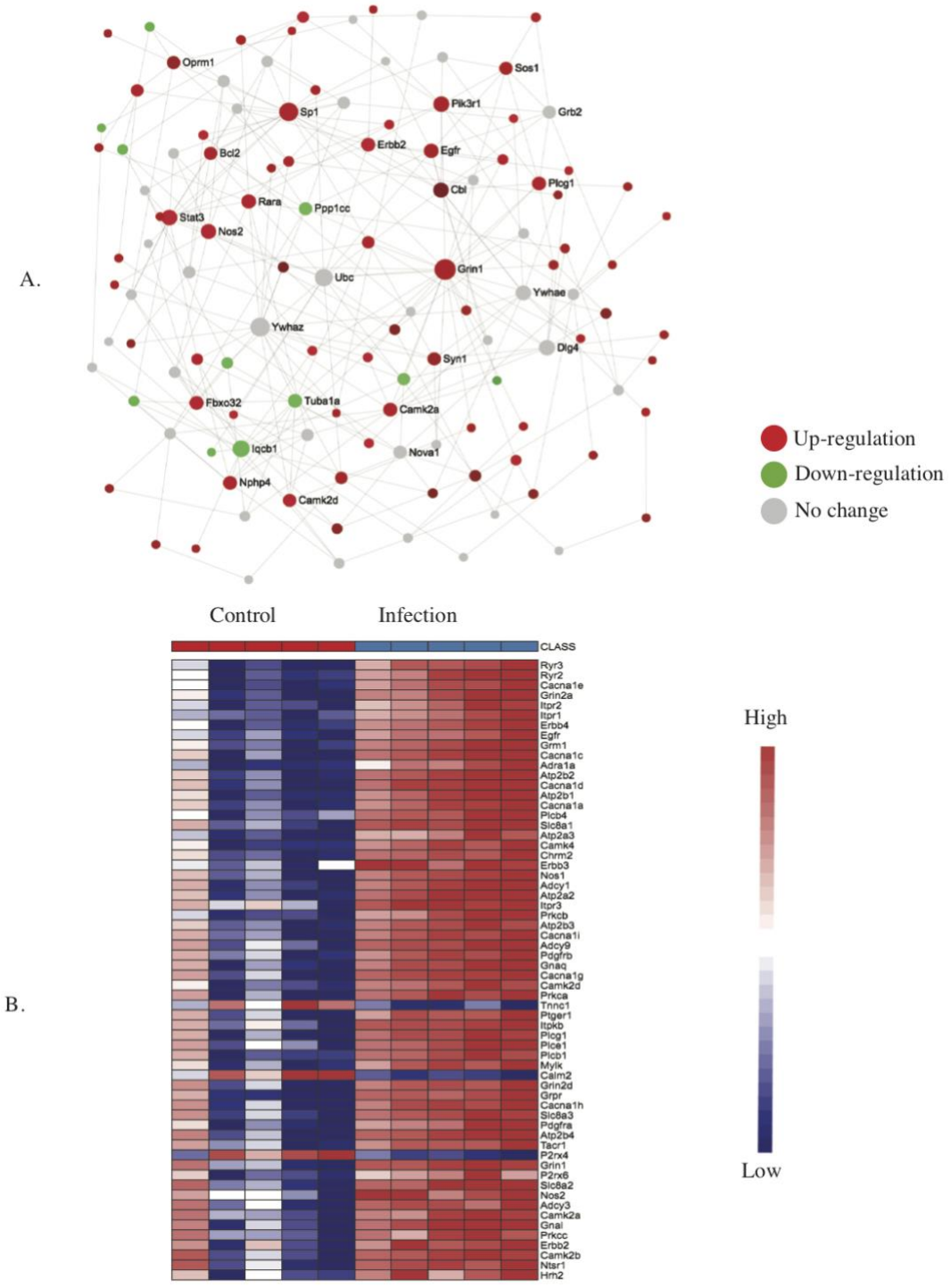

Figure 9.

KEGG pathway analysis (A) and heatmaps (B) of the postnatal day 7 (P7) brain showing up and down-regulated genes involved in calcium signaling pathway in response to maternal nematode infection.

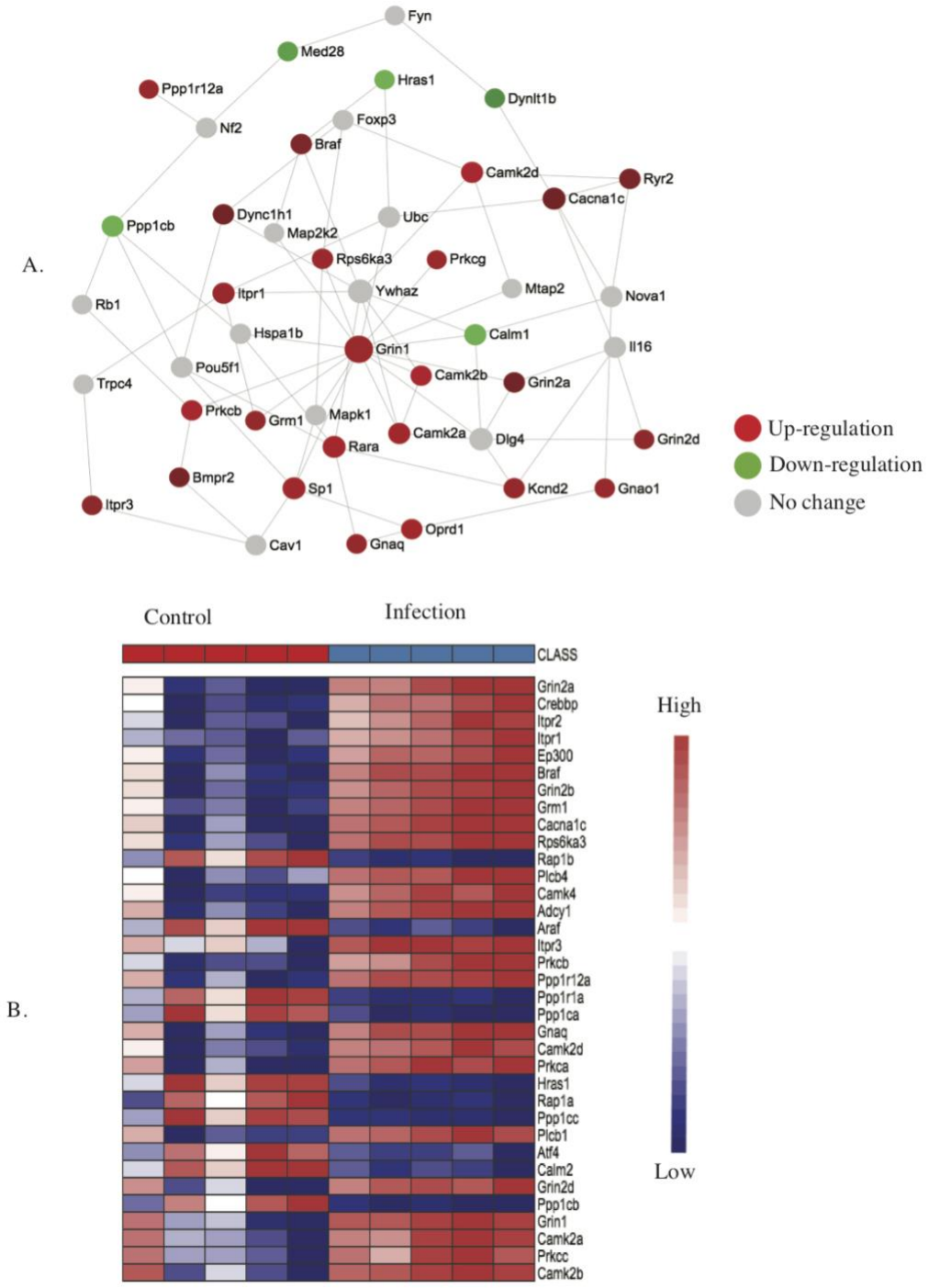

Figure 10.

KEGG pathway analysis (A) and heatmap (B) of the postnatal day 7 (P7) brain showing up and down-regulated genes involved in long-term potentiation signaling pathway in response to maternal nematode infection

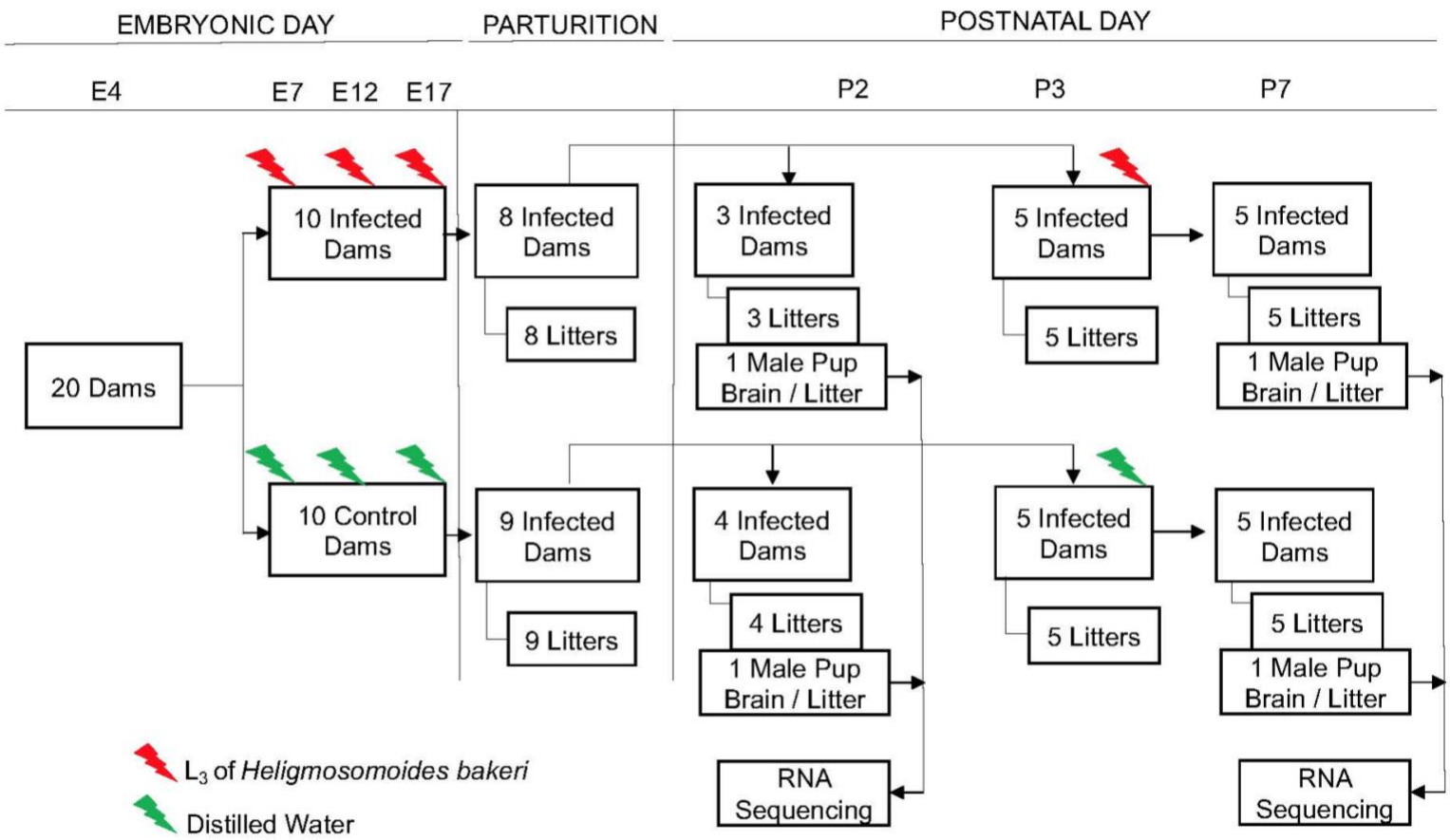

Figure 11.

Schematic representing experimental design. One male pup brain per litter was collected for RNA sequencing at postnatal (P) days 2 and 7. Although 20 timed-pregnant dams were received and allocated to treatments on embryonic day € 4, only 17 delivered litters at parturition.

Connecting Statement II

In Chapter-IV, I tested the hypothesis that maternal nematode infection alters brain gene expression in uninfected post-natal pups. Surprisingly, I recorded that maternal nematode infection altered over 6000 genes in the day 7 pup brain, and that several beneficial pathways were up-regulated. The results from Chapters III and IV raised an intriguing question. How does a nematode infection restricted to maternal GI tract exert an impact on neonatal brain gene expression?

Studies have shown that neonatal microbial colonization occurs at the time of birth and that the neonatal microbiome plays an important role in the post-natal brain maturation (Heijtz et al, 2011; Braniste et al, 2014). Given that the maternal vaginal microbiome is the primary source for the neonatal microbiome and that maternal stress is known to alter maternal vaginal microbiome (Jašarević et al, 2015), I hypothesised that maternal *H. bakeri* infection altered the maternal vaginal microbiome which in turn changed the microbial colonization in neonates. Therefore, in Chapter-V, I examined the effect of maternal nematode infection on the maternal microbiome, neonatal colonization and the functional composition of pup stomach microbiome.

Reference

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Chapter V

Maternal and neonatal microbiome diversity and metabolic pathways are modified by a gastrointestinal nematode in pregnant and lactating mice

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Authors contributions

Haque conceived and designed the study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. Koski (Co-supervisor) provided input on the study design and data interpretation and provided critical suggestions that have been incorporated into the manuscript. Scott (Supervisor) provided input on the study design and data interpretation, provided critical suggestions that have been incorporated into the submitted manuscript, and obtained funding for the research.

Abstract

The maternal microbiome is understood to be the principal source of the neonatal microbiome but the consequence of intestinal nematodes on pregnant and lactating mothers and implications for the neonatal microbiome are unknown. Using pregnant CD1 mice infected with *Heligmosomoides bakeri,* we investigated the microbiomes in maternal tissues (intestine, vagina, and milk) and in the neonatal stomach using MiSeq sequencing of bacterial 16S rRNA. Our first hypothesis was that maternal nematode infection altered the maternal intestinal, vaginal, and milk microbiomes and associated functional metabolic pathways. Maternal nematode infection increased both beta-diversity and the abundance of nine anaerobic fermenting bacteria as well as *Lactobacillus* and down-regulated carbohydrate, amino acid and vitamin biosynthesis pathways in the maternal caecum two days after parturition. Maternal nematode infection did not alter the vaginal or milk microbiomes. Our second hypothesis was that maternal infection would shape colonization of the neonatal microbiome. The pup stomach microbiome was similar to that of the maternal vaginal microbiome. However, pups of infected dams had higher beta-diversity at day two, and a dramatic expansion in the abundance of *Lactobacillus* between day two and seven compared with pups of uninfected dams. Our third hypothesis was that maternal nematode infection altered the functional composition of neonatal microbiomes. We observed up-regulation of several putatively beneficial pathways associated with synthesis of essential and branched-chain amino acids, vitamins, and short-chain fatty acids in the neonatal microbiome of infected dams. This is the first study to show that a nematode living in the maternal intestine altered the the composition and functional composition of the neonatal microbiome.

Keywords: 16S rRNA sequencing; *Heligmosomoides bakeri*; Maternal nematode infection; Microbiome; Mouse; Nematode parasite; Neonatal colonization; Pregnancy

Introduction

Gastrointestinal (GI) nematodes coexist with trillions of microbes in the GI tract and their impact on the diversity and abundance of the microbiome (Peachey et al., 2017) has been shown in non-pregnant animals for several nematode parasites across a diverse range of hosts (Scotti et al., 2020). *Trichuris* increased gut microbial diversity and abundance of members of Paraprevotellaceae family in humans (Lee et al., 2014), *Trichuris suis* altered the colon microbiome in pigs (Li et al., 2012; Wu et al., 2012), and *Toxocara cati* increased the prevalence of the phyla Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes in the feline gut (Duarte et al., 2016). The murine nematode used in the present study, *Heligmosomoides bakeri*, has been shown to shape the microbial community in both the lumen and mucosal epithelium of regions of the small and large intestine (Rapin et al., 2020), and in the lumen of the ileum (Walk et al., 2010) and caecum (Rausch et al., 2013) with results that differ depending on mouse and parasite strains and microbial conditions (Rapin et al., 2020). Release of endogenous antimicrobials by *H. bakeri* limited growth of pathogenic strains of *Escherichia coli*, *Staphylococcus aureus, Salmonella enterica* and *Enterococcus faecium* at least *in vitro* (Rausch et al., 2018) and the Th2-mediated immuno-regulation induced by *H. bakeri* (Brosschot and Reynolds, 2018) led to increased concentrations of bacteria-derived short-chain fatty acids (SCFAs) (Zaiss et al., 2015). In addition to shaping the microbiome, *H. bakeri* has been shown to respond to the bacterial community. *H. bakeri* establishment was enhanced in the presence of a high abundance of *Lactobacillus taiwanensis* that inhibits pro-inflammatory cytokine production through a regulatory T-cell (Treg) response (Reynolds et al., 2014), and the presence of bacteria was required for *H. bakeri* to express antimicrobial genes and induce lower local Th2 and higher Treg responses (Rausch et al., 2018).

The relationship between *H. bakeri* and the microbiome may be especially important during pregnancy. Like *H. bakeri*, pregnancy induces Th2-mediated immune regulation by suppressing the pro-inflammatory Th1 response to accommodate the antigenically foreign fetus (Szekeres-Bartho and Polgar, 2010), and Th2 cytokine concentrations have been shown to be further elevated during *H. bakeri* infection of pregnant mice (Odiere et al., 2013). Pregnancy has been shown to promote those species of *Lactobacillus* in the vaginal microbiome that

metabolize glucose into lactic acid and hydrogen peroxide (H_2O_2) and to lower the vaginal pH safeguarding the female reproductive tract from the invasion of opportunistic pathogens (Mijac et al., 2006; Tomas et al., 2003). Obnoxious maternal stimuli such as stress (Jašarević et al., 2015), bacterial infection (Greenbaum et al., 2019), and viral infection (Torcia, 2019) have all been shown to alter the normal vaginal microbiome in pregnancy.

In addition to having relevance for the dam, there may be consequences for the neonate as the dam microbiome is the initial source of the neonatal microbiome. Although the possibility of colonization *in utero* has been raised (Tanaka and Nakayama, 2017), evidence strongly suggests that the vaginal microbiome is the primary source of the neonatal gut microbiome at the time of delivery (Jašarević et al., 2015; Walker, 2017) and that colonization continues during lactation via transfer of the milk microbiome (Browne et al., 2019) supplemented by environmental sources including maternal feces (Liu et al., 2019a). Thus, if *H. bakeri* influences the microbiome of the vaginal tract or milk in addition to the intestine of the pregnant dam, this could alter composition of the neonatal microbiome.

The composition of the neonatal microbiome can also shape the microbiome-derived metabolite pool that is critical for neonatal growth (Wang et al., 2013). Bacteria-derived metabolites such as amino acids, vitamins, and SCFAs also play a critical role in postnatal development including immune system maturation (Dzidic et al., 2018), brain maturation via gut-brain cross talk (Heijtz et al., 2011), blood-brain barrier development (Braniste et al., 2014), hypothalamus-pituitary axis maturation (Sudo et al., 2004), intestinal development (Hooper, 2004), glucose transport (Doi et al., 2003), and neonatal metabolism (Nash et al., 2017) including fatty acid metabolism (Nishimura et al., 2010). Any alteration of neonatal colonization may have consequences on metabolites involved in postnatal development (Jašarević et al., 2015).

Given the complexity of microbiome-*H. bakeri* interactions in the infected mouse and the importance of the maternal microbiome in bacterial colonization of the neonate, it is likely that any impact of *H. bakeri* on the intestinal, vaginal or milk microbiome of pregnant and lactating hosts may extend to the neonate. The present study examined three questions using the pregnant/lactating mouse-*H. bakeri* model. First, does maternal nematode infection alter the maternal intestinal, vaginal, and milk microbiomes and their associated microbial metabolic

pathways? Second, does maternal nematode infection alter microbial colonization of the neonatal stomach? Third, are predicted microbiome metabolic functional pathways of the neonatal microbiome altered by maternal nematode infection?

2. Materials and methods

2.1. Experimental model

We used primiparous 8 to 9-week-old timed pregnant (gestation day, GD4) outbred CD1 mice infected with *Heligmosomoides bakeri* (= *H. polygyrus*; *Nematospiroides dubius*), a murine model of intestinal nematodes of livestock and humans (Behnke and Harris, 2010). Once ingested, the infective third stage larvae (L_3) penetrate the submucosa of the small intestine and undergo two moults before returning to the lumen of the duodenum where adult worms release eggs in the faeces (Reynolds et al., 2012). The adult nematodes induce a strong Th2 immune response and Treg mediated immune regulation following a single infection (Reynolds et al., 2012). We used a trickle infection protocol that leads to localized inflammation as each repeated dose of larvae penetrates the submucosa (Reynolds et al., 2012) and also generates a Th2 and Treg response in the distal intestine (Setiawan et al., 2007). The animal use protocol (# 2000-4601) was approved by the McGill University Animal Care Committee and complied with Guidelines of the Canadian Council on Animal Care (CCAC, 2020).

2.2. Study design and protocol

The study design involved two infection groups (maternal infection and control) and two postpartum microbiome sampling times for both dams (maternal day 2 [M2] and M9) and pups (day 2 pups [P2] and P7). The dams were sacrificed on M9 not M7 because M9 was the earliest time when a sufficient volume of milk could be collected (Willingham et al., 2014). A total of 20 timed pregnant mice, obtained from Charles River Laboratory (Kingston, NY, USA) on GD 4, were housed individually in Nalgene cages (Fisher Scientific, Canada), at 22-25°C and 40-60% relative humidity on a 12 h light and dark cycle. Mice had unrestricted access to a 24% protein diet (Harlan Teklad, TD. 90017) and water. On GD 5, dams were randomly assigned to the infected or control group (10 mice / group).

The infected group was intubated with 100± 3 L_3 suspended in distilled water on GD 7, 12 and 17, as well as on M3 for those dams sacrificed on M9. Establishment of *H. bakeri* infection was confirmed at necropsy by presence of lesions on the duodenal wall and adult worms in the intestinal lumen of dams. The control group was intubated with the same volume of distilled water on all infection days. On M2, half the dams in both groups (5 dams per group) and all their pups (P2) were sacrificed, and pup stomach contents from 2 pups per dam (a total of 10 pups from each group) were processed for the microbiome. On P7, pups of the remaining dams (5 per group) were sacrificed, and stomach contents were collected from 2 pups per dam (10 pups per group).

2.3. Sample collection

To examine the consequences of direct nematode infection of the dam on her intestinal microbiome, we selected the four regions of the intestine most likely to be affected (duodenum, ileum, caecum and colon) based on previous studies (Rausch et al., 2013; Walk et al., 2010), and collected the luminal contents from each region at M2 and M9. Approximately 2- 3 cm of proximal duodenum, distal ileum, and distal colon, and 1-1.5 cm at the caecal tip were aseptically incised, and the luminal contents of each region were collected as a single replicate, snap-frozen in liquid nitrogen and stored at -80° C until further processing.

To explore colonization of the pup stomach microbiome, we collected the maternal vaginal lavage at M2 and pup stomach microbiome at P2. This time point was selected in order to minimize disturbance of the litter immediately after delivery but maximize the likelihood that the pup microbiome reflected initial colonization. Vaginal lavage was collected by washing the vaginal wall with sterile saline using a sterile P-200 pipetman. The P2 pup stomach was removed and the entire stomach contents were collected as a single replicate. Both vaginal and pup stomach samples were snap-frozen in liquid nitrogen and stored at -80° C until DNA extraction.

As milk is a source of microbiome to the suckling pup, we wanted to determine whether maternal infection altered the milk microbiome and influenced the pup gut microbiome. Pup stomach samples were collected on P7 as described above. This sampling time most closely

aligned with when maternal milk could be collected (M9). Briefly, on M9, the dam was separated from her litter 2 hr prior to milking and then injected intraperitoneally with oxytocin (2 IU/kg body weight). Dams were then anesthetised with a gaseous anesthetic (isoflurane), the mammary gland region was cleaned with sterile alcohol prep pads, and the teat of each mammary gland was gently massaged and squeezed, to collect milk into a sterile P-200 pipetman. The pooled sample from teats of each dam was collected as a single replicate and stored at -80°C until further analysis.

2.4. DNA isolation and bacterial 16s rRNA sequencing

Genomic DNA was extracted from each sample (dam vaginal lavage, duodenum, ileum, caecum, colon, milk samples and pup stomach contents) using QIAamp® PowerFecal® DNA Kit (Qiagen, Germany) and stored at -20 $_{o}$C until further processing by the functional genomics platform of the McGill University and Génome Québec Innovation Centre, Canada, for Illumina MiSeq sequencing. The V4 region of the bacterial 16S rRNA was amplified using the 515F forward (GTGCCAGCMGCCGCGGTAA) and 806R reverse (GGACTACHVGGGTWTCTAAT) primer pair in a MiSeq Pe 250bp sequencer.

2.5. Preprocessing of data and analysis

We received Casava demultiplexed paired-end fastq files from the sequencing centre and the files were preprocessed, quality filtered and analysed using Quantitative Insights Into Microbial Ecology (QIIME 2) version 2018.11 software pipeline (Bolyen et al., 2019). The files were imported in QIIME 2 as '*SampleData[SequencesWithQuality*' file type to create a QIIME 2 artifact. We used the *q2-dada2* plugin, which filters phiX reads and chimeric sequences as a quality control step. The imported artifact was processed using the *qiime dada2 denoise-paired* command. The raw data files were submitted to Mendeley repository and are available *via.* https://data.mendeley.com/datasets/6dygr4kdz8/2.

Analyses of the various samples were separated into three sets with separate quality filtration because of large differences in the sequencing quality of the milk and vaginal lavage microbiomes compared with the dam GI and pup stomach microbiomes. For Set 1, milk and vaginal lavage sequence reads were trimmed at position 13 on the left and truncated at position

250 in both forward and reverse reads. For Set 2, maternal GI and pup stomach sequence reads were trimmed at position 24 on the left and truncated at position 250 in both forward and reverse reads. For Set 3, maternal vaginal microbiome and pup stomach sequence reads were trimmed at position 13 on the left and truncated at position 250 in both forward and reverse reads.

Alpha- and beta-diversity were analysed using *q2-diversity* plugin in QIIME2 at a *p-sampling-depth* 714 for Set 1, 32428 for Set 2, and 724 for Set 3. Alpha-diversity was measured using Shannon's diversity index, observed operational taxonomic units (OTUs), Faith's phylogenetic diversity and Pielou's evenness. Pairwise comparisons between groups were performed using Kruskal-Wallis test. Beta-diversity was measured as a qualitative (unweighted UniFrac distance) and quantitative (weighted UniFrac distance) measure of community dissimilarity and statistical differences in beta-diversity were tested using Permutational Multivariate Analysis of Variance (PERMANOVA).

We examined tissue specific microbiome composition using the Naive Bayes classifier and the *q2-feature-classifier* which was trained on the Greengenes13.8 99% OTUs, using 250 base sequences of the 16S (the V4 region, amplified by the 515F/806R primer pair). The composition data were plotted as pie charts to visualize the generic composition of the microbiome across different tissues. As the family Enterobacteriaceae is normally present at very low abundance (Osbelt et al., 2020), family level data are reported. When *Staphylococcus*, a member of the Enterobacteriaceae family was detected, it was shown along with the group of other Enterobacteriaceae. The category "others" included bacteria on unknown genus as well as genera whose abundance was <1%. Differential abundance was computed using the absolute difference in abundance of each genus between groups of interest. Microbiome data are compositional and an assumption of a specific distribution for parametric statistics could poteintially incorporate type 1 errors (Weiss et al., 2017). Therefore, we used the non-parametric ANCOM (Analysis of Composition of Microbiomes) test that makes no assumption of the distribution of the data (Mandal et al., 2015) using the QIIME2 *q2-composition* plugin for differential abundance of bacterial taxa at their genera level. When visual comparison of relative abundance between P2 and P7 suggested noteworthy differences, the absolute abundance of the

genera of interest were compared using a two-tailed *t* test. For all the aforementioned analyses statistical significance was determined for *p*-value cut off <0.05.

Finally, we used PICRUSt2 (Douglas et al., 2020) as a QIIME2 plugin for prediction of MetaCyc pathway abundance for the dam intestine and pup stomach microbiomes. The predicted pathway abundance was calculated for control and infection microbiomes in each tissue site and compared using an unpaired t-test for statistical significance ($p<0.01$).

3. Results

3.1. Diversity of maternal microbiomes

Infection had no impact on the alpha-diversity in any of the maternal tissues sampled (intestinal, vaginal and milk). A significant difference ($p=0.034$) in beta-diversity (unweighted uniFrac distance) was observed in the M2 caecal microbiome where infected dams had higher beta-diversity than control dams.

3.2. Composition and functional profile of maternal microbiomes

3.2.1 Composition of the GI microbiome

Visual inspection of the microbiome composition pie charts revealed variations in abundance of microbial genera among sites (duodenum, ileum, caecum and colon) of the maternal GI tract both in the infected and control dams at M2 and M9 (Fig. 1).

Duodenum: Lactobacillus was the predominant genus in the duodenum of both infected and control dams at both M2 and M9. At M2, *Bifidobacterium* was only detected in control dams whereas Enterobacteriaceae was only recorded in the infected dams at a relative abundance of 29%. At M9, the composition of the microbiome was similar between control and infected with the exception of the presence of *Turicibacter* in the control dams but not in infected dams (Fig. 1).

Ileum: As with the duodenum, *Lactobacillus* was the principle genus in the ileum of both control and infected dams at M2 and M9, and *Bifidobacterium* was detected in control but not infected dams at M2. At M9, the abundance of *Lactobacillus* was ~70% in infected dams and

~45% in control dams whereas the abundance of *Turicibacter* was 5% in infected dams and 26% in control dams (Fig. 1).

Caecum: A large proportion of caecal microbiome was recorded as "other" (either uncharacterised at the genus level and or abundance < 1%). Differential abundance analysis by ANCOM revealed that the caecal microbiome differed significantly between control and infected dams at M2. Infected dams had significantly higher abundance of the anerobic fermenting bacteria *Lactococcus, Adlercreutzia, Mogibacteriaceae, Dehalobacterium, Coprococcus* and *Ruminococcus* and lower abundance of *Mucispirillum, Parabacteroides* and *Oscillospira* compared with control dams (Table 1). Furthermore, the caecal abundance of *Lactobacillus* in infected dams was more than twice as high both at M2 and M9 in infected compared with control dams (Fig. 1) although the difference was not significant by ANCOM analysis. At M2, we also detected *Akkermansia* in both infected and control dams whereas *Oscillospira* was only detected in the control dams; at M9, *Ruminococcus* was detected only in the infected dams (Fig. 1).

Colon: We detected *Lactobacillus, Bacteroides* and *Akkermansia* in both infected and control dams at M2 and M9, but *Oscillospira* was only detected in control dams at M9 (Fig. 1).

3.2.2. Composition of the vaginal and milk microbiomes

Vagina: At M2 a high proportion of the vaginal microbiome was classified as "other" (47% in control dams and 66% in infected dams). The percent composition of *Lactobacillus* was 28% in infected and 3% in control dams but was not significantly different by ANCOM analysis. *Propioibacterium*, *Staphylococcus* and *Kocuria* species were only detected in control dams (Fig. 2).

Milk: We detected *Phenylobacterium* as well as a very low abundance of *Lactobacillus* in milk from both infected and control dams at M9, but *Proteus* was only detected in the milk of control dams (Fig.2). Of note, *Staphylococcus* made up 63% of the milk microbiome of control dams but only 27% of infected dams.

3.2.3. Predicted metabolic functional pathways

Predicted microbiome metabolic pathway analysis of the maternal microbiomes yielded significant (p<0.01) results only at M2 and only in the intestine. Infection down-regulated carbohydrate biosynthesis in the duodenum and down-regulated eight metabolic pathways involved in carbohydrate biosynthesis, amino acid biosynthesis and vitamin biosynthesis in the caecum (Table 2).

3.3. Influence of maternal nematode infection on neonatal microbial colonization and functional profile of pup stomach microbiome

3.3.1. Microbial diversity and composition

To determine if maternal nematode infection altered initial neonatal gut colonization, we compared the abundance of the P2 pup stomach microbiome with that of the maternal vaginal tract within both control and infected groups. In both cases, the P2 pup stomach microbiome reflected the M2 vaginal microbiome consistent with a vaginal source of colonization.

We then compared alpha- and beta-diversity scores, compositional pie charts, and differential abundance of the P2 and P7 pup stomach microbiomes between pups of infected and control dams. At P2, pups of infected dams had a significantly higher (p=0.008) total bacterial abundance and higher beta-diversity (weighted uniFrac). The higher beta-diversity was associated both with the presence of four genera (*Lactobacillus, Bacteroides, Prevotella* and *Oscillospira*) in pups of infected dams compared with only two genera (*Lactobacillus* and *Enterococcus*) in pups of control dams and with a very high abundance (83%) of other bacteria in pups of infected dams (Fig. 2). However, ANCOM analysis failed to detect differences in the abundance of individual genera.

At P7, the stomach microbiome of pups of the infected dams was primarily composed of *Lactobacillus* (99%). Of note, the expansion of *Lactobacillus* between P2 and P7 was significant in the infected group (p=2.1E-11) but not in the control group (p=0.52). *Proteus* (19%) and *Staphylococcus* (12%) were present only in the control pup stomachs (Fig. 2). However, ANCOM analysis showed no difference in the abundance of genera between infection and

control groups but the alpha-diversity (Pielou's evenness index) was higher (p= 0.041) in pups from infected dams.

3.3.2. Functional profile

The functional pathway analysis of the neonatal stomach microbiome indicated that maternal nematode infection altered the metabolic profile at P2 but not P7. At P2, 72 metabolic pathways were significantly (p<0.01) up-regulated (Table 3). These were broadly categorized as amino acid biosynthesis, energy and carbohydrate metabolism, vitamin-B biosynthesis and co-factors. Of note, several *de novo* biosynthesis pathways for essential and branched chain amino acids (isoleucine, methionine, valine, histidine, lysine and phenylalanine) were up-regulated (Table 3; Figure 3). Pathways associated with *de novo* synthesis of the SCFA acetate and the vitamin B-complex (thiamine, biotin, folic acid, cobalamin) were also up-regulated in response to maternal infection (Table 3; Fig. 4).

4. Discussion

Previous studies using non-pregnant mice have demonstrated alterations of the GI microbiome during *H. bakeri* infection, however ours is the first study to show that infection during pregnancy and lactation had an impact on the microbiome not only of the dam but also of their 2-day and 7-day old pups. We highlight five key findings. First, the maternal caecal microbiome was more affected than other intestinal regions, with higher beta-diversity, higher the abundance of anaerobic fermenting bacteria, and down-regulation of biosynthesis pathways. Second, bacteria in the Enterobacteriacea family were abundant in the duodenum of infected dams and not detected in control dams whereas *Lactobacillus* abundance did not differ, perhaps because inflammation associated with the L_4 larvae may have promoted growth of Enterobacteriacea but not *Lactobacillus*. Third, in contrast to the intestine, the vaginal and milk microbiomes were unaffected by maternal infection. Fourth, initial colonization of the pup stomach microbiome was consistent with vaginal colonization. However, the expansion of *Lactobacillus* in the neonate between P2 and P7 could not be linked to the milk microbiome given the very low abundance of *Lactobacillus* in maternal milk. Fifth, our most intriguing finding was the impact of maternal infection on the P2 neonatal microbiome, as evidenced by

increased microbial diversity as well as up-regulation of several putative *de novo* microbial biosynthesis pathways including essential and branched chain amino acids, vitamins and short-chain fatty acids that may promote neonatal growth and development.

Our first question was whether *H. bakeri* during pregnancy and early lactation altered the dam intestinal microbiome and putative microbial metabolic pathways at M2 and M9. Even though the nematode lives in the duodenum, the most dramatic impact was observed in the caecum. This is consistent with *H. bakeri*-induced accumulation of mucus (Sharpe et al., 2018) in the enlarged caecum (Kristen, 2002) and up-regulation of Th2 and Treg responses in the distal intestine (Setiawan et al., 2007). First, the accumulation of mucous would explain the observed expansion of *Ruminococcus* that ferments mucin (Belzer et al., 2017), *Coprococcus* that produces butyrate (Vital et al., 2017), *Adlercreutzia* and *Lactococcus* that ferment lipid and glucose (Yun et al., 2017; Passerini et al., 2013), and *Akkermansia* that degrades mucin (Derrien et al., 2008), all of which may have contributed to the observed higher beta-diversity in the caecum of infected dams. The higher abundance of anaerobic fermenting bacteria may also account for the down-regulation of the aerobic respiration (cytochrome C) pathway (Arai, 2011). Second, down-regulation of inflammation by Th2 and Treg responses would be consistent with the higher abundance of *Lactococcus* and *Dehalobacterium* which are associated with a Th2 environment (Liu et al., 2019b; Bian et al., 2017), and with the lower abundance of *Mucispirillum* that survives better in a a pro-inflammatory environment (Loy et al., 2017). Of note, *Akkermansia* has been associated with reducing low-grade inflammation, raising the possibility that nematode-induced shifts in the microbiome may in turn lower inflammation in the infected dam (Cani and de Vos, 2017).

Surprisingly, the only impact of *H. bakeri* on *Lactobacillus* in the maternal intestine was a trend toward higher abundance in the caecum at M2 and M9. The Th2- and Treg-mediated immune regulation (Allen and Maizels, 2011) induced by *H. bakeri* has been shown to extend to the distal intestine and dramatically reduce inflammation associated with colitis (Setiawan et al., 2007). Such an anti-inflammatory environment is known to favor the growth of *Lactobacillus* (Reynolds et al., 2014; Walk et al., 2010). Additionally, *Lactobacillus* directly inhibits the

release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Oh et al., 2018) that may enhance an anti-inflammatory environment (Reynolds et al., 2014) in the caecum.

In contrast to the caecum, *Lactobacillus* abundance was unaffected in the duodenum of infected dams. This differs from previous reports of higher abundance of Lactobacillaceae family in the duodenum (Reynolds et al., 2014) and ileum (Walk et al., 2010; Rausch et al., 2013) of infected mice. Furthermore, the relative abundance of Enterobacteriacea approached ~29% in the infected duodenum but was undetectable in control dams, a finding that is notable given that this family of facultative anaerobes normally represents <0.1% of the gut microbiome (Rivera-Chavez et al., 2017), and given that a single infection with *H. bakeri* increased Enterobacteriacea abundance in the ileum to only 1-2% of the microbiome (Rausch et al., 2013). We suggest that our trickle infection protocol may explain the absence of an expansion of *Lactobacillus* and the high abundance of Enterobacteriaceae in the duodenum. Repeated infection with nematode larvae that occurs during the trickle infection protocol induces much stronger and more persistent localized inflammation around the developing L_4 larvae compared with a single exposure to larvae in immunologically-naïve mice (Reynolds et al., 2012). The resulting pro-inflammatory environment in the duodenum may have inhibited the expansion of *Lactobacillus* which, as noted above, preferentially proliferates in an anti-inflammatory environment (Reynolds et al., 2014; Walk et al., 2010). Enterobacteriacea have been shown to grow under conditions of elevated oxygen (Rivera-Chavez et al., 2017) such as during pro-inflammatory responses (Heimesaat et al., 2006; Heimesaat et al., 2007; Nakanishi et al., 2015) that generate reactive oxygen metabolites (Rausch et al., 2013). Thus, the inflammation associated with the trickle infection may have promoted expansion of Enterobacteriacea. Taken together, these results add to the list of host and parasite conditions that influence the composition of the microbiome and thus contribute to lack of generalizability from one study to another (Rapin et al., 2020).

Our second question concerned the impact of maternal infection on colonization of the neonatal microbiome. As maternal stress has been shown to influence the vaginal microbiome (Jašarević et al., 2015), we had anticipated that *H. bakeri* might alter the vaginal microbiome. However, this was not observed when the vaginal microbiome was compared between control

and infected dams. Furthermore, the composition of the P2 neonatal stomach microbiome paralleled the vaginal microbiome of their respective dams. This is consistent with the understanding that neonatal colonization occurs during vaginal delivery (Jašarević et al., 2015) but does not discount the possibility of earlier colonization during the intrauterine phase (Younge et al., 2019). Despite this, however, maternal nematode infection increased the beta-diversity in the P2 pup stomach microbiome through an unknown mechanism. Thus, our data indicate that although the overall relative abundance of the pup microbiome is similar to that of the dam, the beta-diversity is affected, leading to greater microbial diversity among pups of infected dams compared to uninfected dams.

Interestingly higher beta-diversity in the pup stomach may underscore the up-regulatation of a large number of putative microbial pathways for *de novo* biosynthesis of amino acids, vitamins, and carbohydrates in response to maternal infection. Some studies have suggested that microbiome-derived amino acids enter host circulation (Metges et al., 1999; Mardinoglu et al., 2015; Ciarlo et al., 2016) and may contribute to the total pool of host amino acids (Lin et al., 2017). Microbiome-derived essential amino acids (isoleucine, valine, methionine, histidine and phenylalanine) that cannot be synthesized by mammals and are necessary for early postpartum growth (Pencharz and Ball, 2004) would be of particular importance. Furthermore, isoleucine and valine prevent accumulation of triglycerides in tissue (Nishimura et al., 2010), lower blood glucose via insulin-independent pathways (Doi et al., 2003), and are needed for intestinal barrier function and absorption (Teodoro et al., 2012) and immune function development (Negro et al., 2008), all critical processes during neonatal development (Jacobi and Odle, 2012). The upregulation of these pathways in response to a maternal intestinal nematode infection raises the intriguing possibility that the neonate may benefit from this pool of metabolites.

In addition, upregulated *de novo* biosynthesis pathways in the P2 pup stomach included isoleucine, catecholamine precursors, and acetate biosynthesis, all that potentially act as neurotransmitters or precursors of neurotransmitters (Rassin, 1994; Choi et al., 2011) that are important in gut-brain cross talk. In our previous study on the impact of maternal *H. bakeri* infection on gene expression in the pup brain, we reported up-regulation of several pathways that may have beneficial impact on pup cognition and long-term potentiation, and hypothesized that

this might have been mediated through an impact of the neonatal microbiome (Haque et al., 2019). Consistent with this hypothesis, the up-regulated biosynthesis of isoleucine, the precursor of taurine, may have a beneficial impact on postnatal brain development as higher taurine levels have been shown to have beneficial effects on neurodevelopment (Wharton et al., 2004). Furthermore, putative up-regulation of the biosynthesis of the catecholamine precursors, phenylalanine and tyrosine, may result in higher concentrations of catecholamine that is involved in bi-directional communication along the gut-brain axis (Asano et al., 2012). Maternal infection also upregulated the acetate biosynthesis pathway in the P2 pup stomach. This SCFA produced during microbial fermentation is known to participate in gut-brain cross-talk (Clemmensen et al., 2017) and to interact with the parasympathetic nervous system and influence brain gene expression (Perry et al., 2016). Acetate may also be converted to another short-chain fatty acid, butyrate, by the intestinal microbiome (Detman et al., 2019) and both acetate and butyrate have been shown to minimise stress-signaling and sensitivity of the hypothalamic pituitary adrenal (HPA) axis and favorably influence the postnatal brain development (Van de Wouw et al., 2018). It will be important to determine if the up-regulated biosynthesis pathways contribute to the metabolite pool of the pup and if this alters neonatal growth and development.

Finally, we observed a dramatic expansion of the abundance of pup stomach *Lactobacillus* between P2 and P7 in pups of infected dams. One of the potential modulators of the microbiome between P2 and P7 is maternal milk (Tanaka and Nakayama, 2017; Matsuyama et al., 2019), but milk was unlikely a direct source because the *Lactobacillus* abundance in dam milk was very low. An alternative explanation is that bioactive components in the milk (Gopalakrishna and Hand, 2020) of infected dams, such as cytokines, promoted proliferation of *Lactobacillus* in the pup stomach. A variety of cytokines have been found in milk, including transforming growth factor (TGF)-β in human, sow, rat, and mouse milk (Saito et al., 1993; Letterio et al., 1994; Penttila et al., 1998 and Nguyen et al., 2007) and IL-4 in human milk (Saso et al., 2019). Given that the *H. bakeri* infection induces production of IL-4 and TGF-β in mice (Reynolds et al., 2012) and that cytokines may be transferred to neonates via milk (Letterio et al., 1994), we hypothesize that infection-induced cytokines and immunomodulators may have been transferred via milk to the pup stomach. This may have established an anti-inflammatory

environment in the pup stomach that would have promoted the proliferation of the *Lactobacillus* that had colonized the pup stomach at delivery.

We acknowledge the following limitations of this study. In examining neonatal colonization, experimental and technical aspects precluded us from collecting colostrum which is also a potential source of the microbiome for pups (Toscano et al., 2017). Had we used a more comprehensive sequencing system like shotgun sequencing, we would have been able to identify those microbes that could not be characterized by 16S rRNA sequencing including protozoa and fungi. Additionally, although our number of replicates was sufficient to detect statistical differences between the control and infected groups, larger sample sizes might have revealed additional impacts of maternal infection on the maternal or neonatal microbiomes. Finally, direct measurement of metabolites in the pup would have allowed us to determine whether the predictions of the metabolic pathway analysis were reflected by higher concentrations of microbiome-derived metabolites in the total nutrient pool of pup.

To our knowledge, this is the first study to demonstrate that a maternal nematode infection restricted to the maternal GI tract increased the beta-diversity and up-regulated several developmentally relevant microbial metabolic pathways of biosynthesis in the P2 pup stomach. Further research is needed to determine whether the up-regulation of these pathways contributes to the metabolite pool in the pup and whether this altered pool in turn influences early growth and development perhaps through gut-brain crosstalk, host metabolism, and barrier functions.

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Competing Interests

The authors declare no competing interests

Table 1. Differential abundance of the dam caecal microbiome at P2 in response to maternal nematode infection, based on ANCOM analysis.

Bacteria	**W- value**	**Direction of change in response to infection**
Mucispirillum	8	Down
Parabacteroides	3	Down
Oscillospira	1	Down
Lactococcus	3	Up
Adlercreutzia	2	Up
Mogibacteriaceae	2	Up
Dehalobacterium	1	Up
Coprococcus	1	Up
Ruminococcus	1	Up

Table 2. Maternal nematode infection down-regulated microbiome functional metabolic pathways in the dam duodenum and caecum at M2.

Carbohydrate Biosynthesis Pathways - Down-Regulated		
Duodenum	Superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks biosynthesis	PWY-7332
Carbohydrate Degradation Pathways – Down-Regulated		
Caecum	Sucrose degradation II (sucrose synthase)	PWY-3801
Cofactor, Carrier, and Vitamin Biosynthesis – Down-Regulated		
Caecum	1,4-dihydroxy-6-naphthoate biosynthesis I	PWY-7374
	1,4-dihydroxy-6-naphthoate biosynthesis II	PWY-7371
	Superpathway of menaquinol-8 biosynthesis II	PWY-6263
	Superpathway of heme b biosynthesis from uroporphyrinogen-III	CODH-PWY
C1 Compound Utilization and Assimilation – Down-Regulated		
Caecum	Reductive acetyl coenzyme A pathway I (homoacetogenic bacteria)	PWY0-1415
Amino Acid Degradation – Down-Regulated		
Caecum	L-glutamate degradation V (via hydroxyglutarate)	P162-PWY
Electron Transfer Chains – Down-Regulated		
Caecum	Aerobic respiration I (cytochrome c)	PWY-3781

Table 3. Maternal nematode infection up-regulated microbiome functional metabolic pathways associated with amino acid biosynthesis, carbohydrate/energy metabolism, and vitamin biosynthesis in the pup stomach at P2.

Amino Acid Biosynthesis Pathways – Up-regulated	
Superpathway of L-isoleucine biosynthesis I	PWY-3001
L-isoleucine biosynthesis II	PWY-5101
Isoleucine biosynthesis III	PWY-5103
L-isoleucine biosynthesis IV	PWY-5104
L-isoleucine biosynthesis I (from threonine)	ILEUSYN-PWY
L-methionine biosynthesis I	HOMOSER-METSYN-PWY
Superpathway of L-methionine biosynthesis	PWY-5347
Superpathway of S-adenosyl-L-methionine biosynthesis	MET-SAM-PWY
Superpathway of L-lysine, L-threonine and L-methionine biosynthesis I	P4-PWY
L-valine biosynthesis	VALSYN-PWY
L-histidine biosynthesis	HISTSYN-PWY
L-lysine biosynthesis I	DAPLYSINESYN-PWY
Superpathway of L-phenylalanine biosynthesis	PWY-6628
L-ornithine biosynthesis I	GLUTORN-PWY
Superpathway of branched chain amino acid biosynthesis	BRANCHED-CHAIN-AA-SYN-PWY
Superpathway of L-serine and glycine biosynthesis I	SER-GLYSYN-PWY
Superpathway of L-aspartate and L-asparagine biosynthesis	ASPASN-PWY
L-arginine biosynthesis III (via N-acetyl-L-citrulline)	PWY-5154
L-arginine biosynthesis II (acetyl cycle)	ARGSYNBSUB-PWY
Superpathway of L-tyrosine biosynthesis	PWY-6630
Superpathway of sulfate assimilation and cysteine biosynthesis	SULFATE-CYS-PWY
L-arginine biosynthesis IV (archaebacteria)	PWY-7400

L-arginine biosynthesis I (via L-ornithine)	ARGSYN-PWY
Aspartate superpathway	PWY0-781
Superpathway of arginine and polyamine biosynthesis	ARG+POLYAMINE-SYN
Carbohydrate / Energy Metabolism – Up-regulated	
Pyruvate fermentation to isobutanol (engineered)	PWY-7111
TCA cycle V (2-oxoglutarate:ferredoxin oxidoreductase)	PWY-6969
Glycogen degradation I	GLYCOCAT-PWY
TCA cycle	TCA
Partial TCA cycle (obligate autotrophs)	PWY-5913
Pyruvate fermentation to acetone	PWY-6588
TCA cycle VIII (Helicobacter)	REDCITCYC
TCA cycle VII (acetate-producers)	PWY-7254
TCA cycle IV (2-oxoglutarate decarboxylase)	P105-PWY
NAD de novo biosynthesis I (from aspartate)	PYRIDNUCSYN-PWY
GDP-D-glycero-α-D-manno-heptose biosynthesis	PWY-6478
Starch degradation V	PWY-6737
NAD salvage pathway I (PNC VI cycle)	PYRIDNUCSAL-PWY
GDP-mannose biosynthesis	PWY-5659
Superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis	PWY-7328
Superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis	PWY-7323
CMP-3-deoxy-D-manno-octulosonate biosynthesis	PWY-1269
Colanic acid building blocks biosynthesis	COLANSYN-PWY
Methylerythritol phosphate pathway I	NONMEVIPP-PWY
Methylerythritol phosphate pathway II	PWY-7560
Superpathway of thiamine diphosphate biosynthesis I	THISYN-PWY
Mixed acid fermentation	FERMENTATION-PWY
Assimilatory sulfate reduction I	SO4ASSIM-PWY

Pathways Relevant to Vitamin Biosynthesis – Up-Regulated	
Biotin biosynthesis I	BIOTIN-BIOSYNTHESIS-PWY
Thiamine salvage II	PWY-6897
Superpathway of thiamine diphosphate biosynthesis II	PWY-6895
Flavin biosynthesis I (bacteria and plants)	RIBOSYN2-PWY
Tetrapyrrole biosynthesis I (from glutamate)	PWY-5188
Superpathway of adenosylcobalamin salvage from cobinamide I	COBALSYN-PWY
Superpathway of tetrahydrofolate biosynthesis and salvage	FOLSYN-PWY
Superpathway of tetrahydrofolate biosynthesis	PWY-6612
Thiazole biosynthesis I (facultative anaerobic bacteria)	PWY-6892
N10-formyl-tetrahydrofolate biosynthesis	1CMET2-PWY

Figure Legends

Fig. 1. Relative abundance of bacteria in different sections of the maternal GI tract of control and nematode-infected dams on postnatal days 2 and 9.

Fig. 2. Composition of microbiome in dam vagina on postnatal day 2 and milk on postnatal day 9, and in the pup stomach on postnatal day 2 and day 7.

Fig. 3. Putative metacyc *de novo* biosynthetic pathways associated with essential and branched chain amino acid biosynthesis that were up-regulated in neonatal stomach of infected compared with control dams on postnatal day 2.

Fig. 4. Putative metacyc *de novo* biosynthetic pathways associated with short-chain fatty acid [TCA cycle VII (acetate producers)] and with vitamin B complex biosynthesis that were up-regulated in neonatal stomach of infected compared with control dams on postnatal day 2.

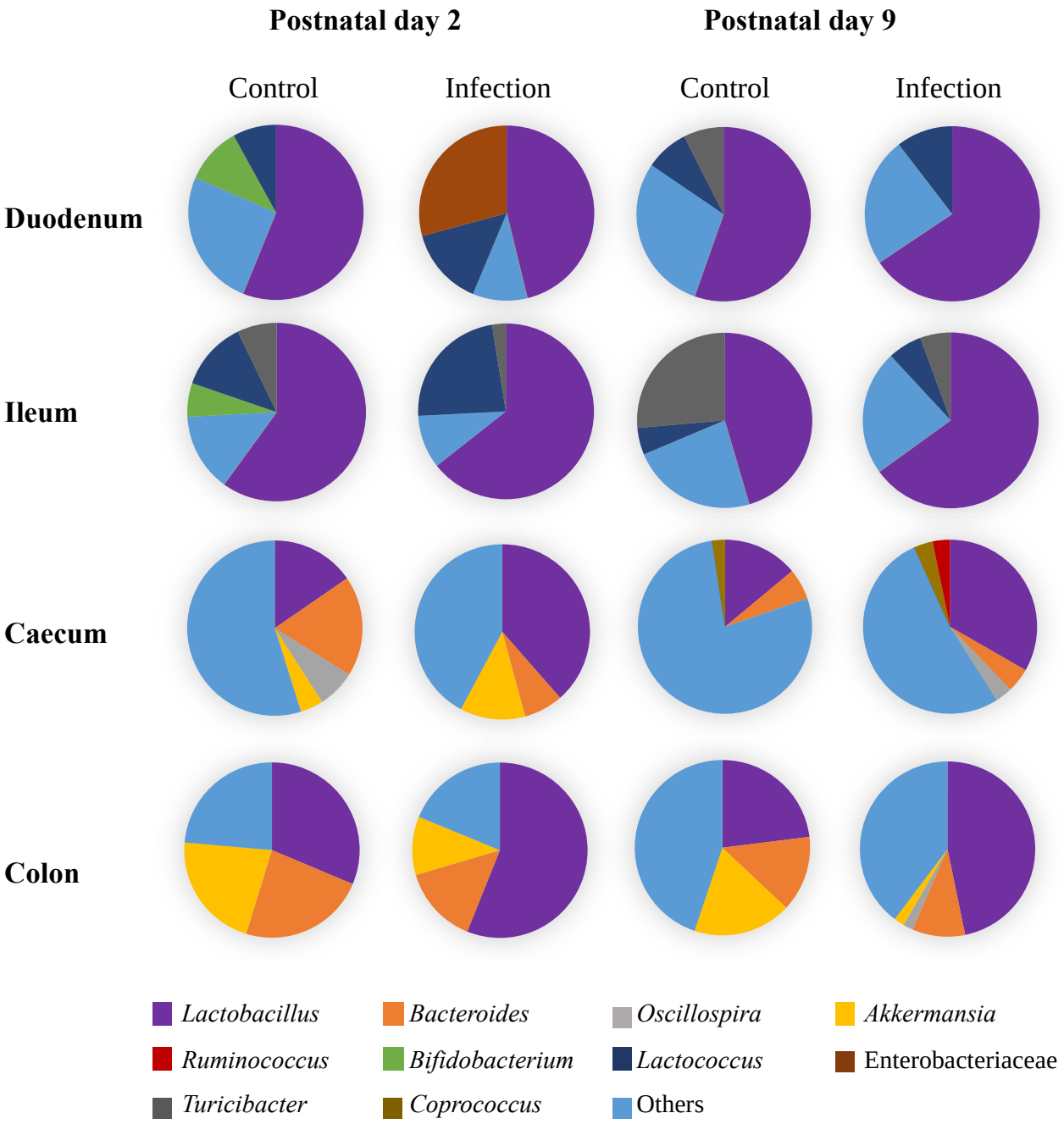

Postnatal day 2
Postnatal day 9
Control
Infection
Control
Infection
Duodenum
Ileum
Caecum
Colon
Lactobacillus
Bacteroides
Oscillospira
Akkermansia
Ruminococcus
Bifidobacterium
Lactococcus
Enterobacteriaceae
Turicibacter
Coprococcus
Others

Fig.1

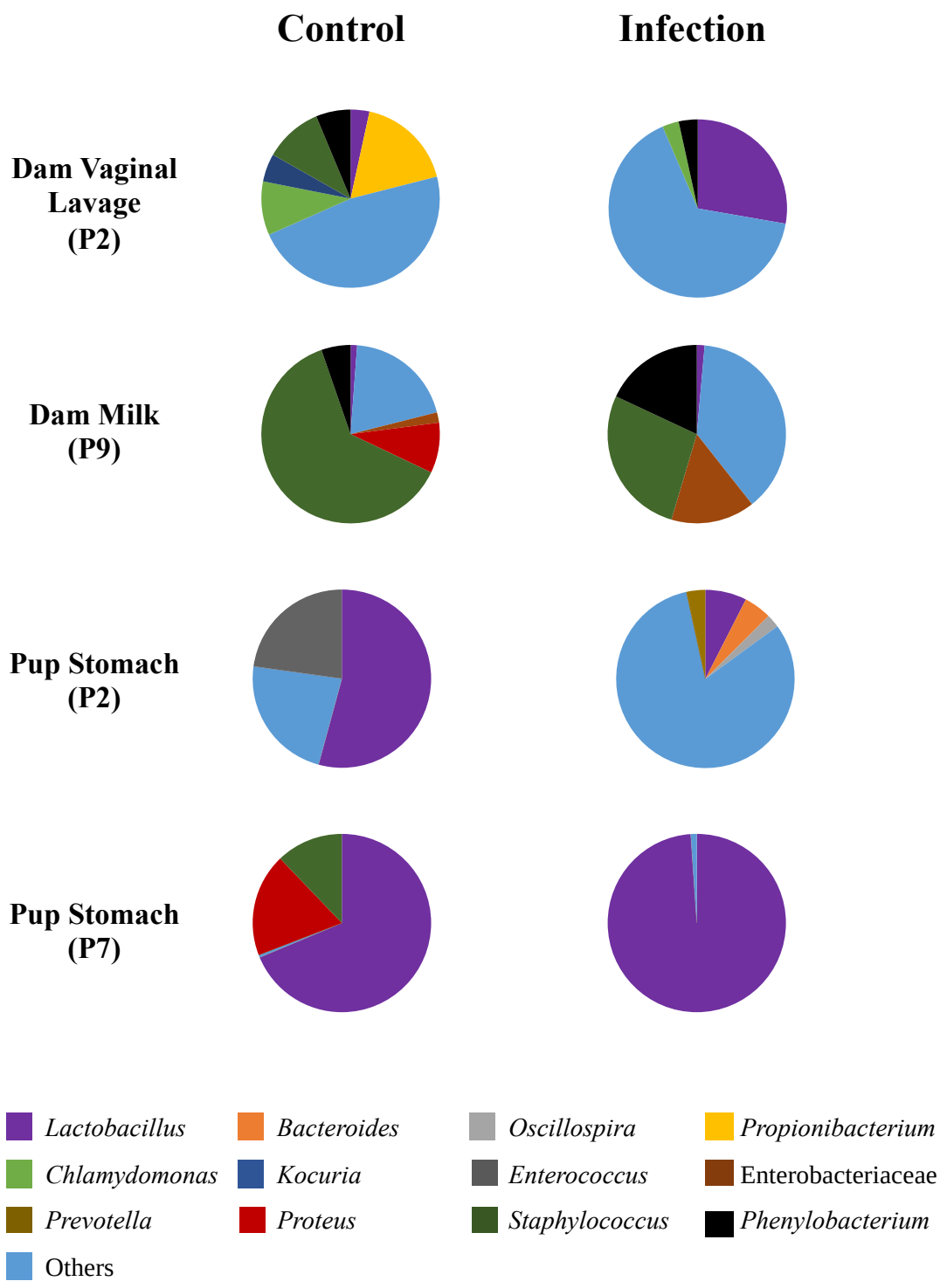

Control
Infection
Dam Vaginal Lavage (P2)
Dam Milk (P9)
Pup Stomach (P2)
Pup Stomach (P7)
Lactobacillus
Bacteroides
Oscillospira
Propionibacterium
Chlamydomonas
Kocuria
Enterococcus
Enterobacteriaceae
Prevotella
Proteus
Staphylococcus
Phenylobacterium
Others

Fig. 2

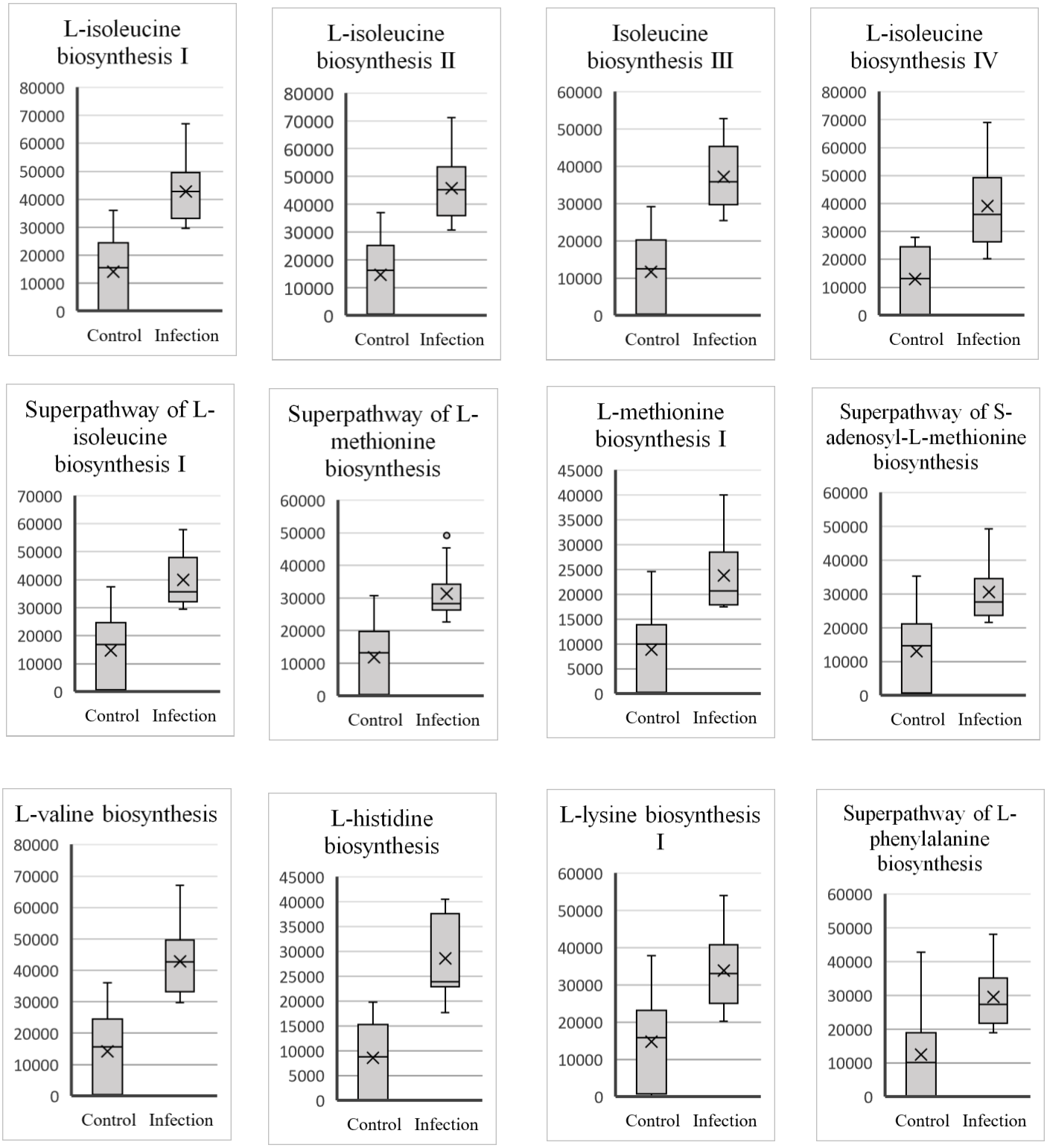
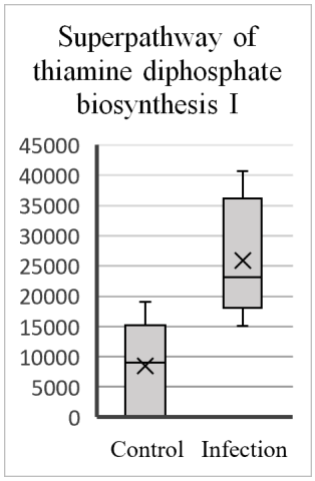
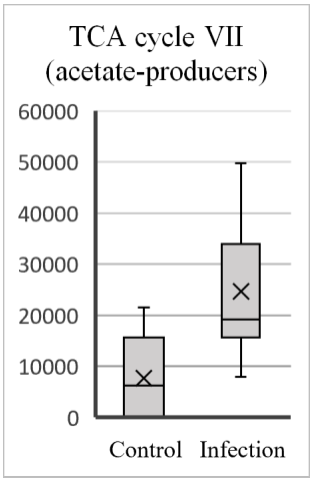
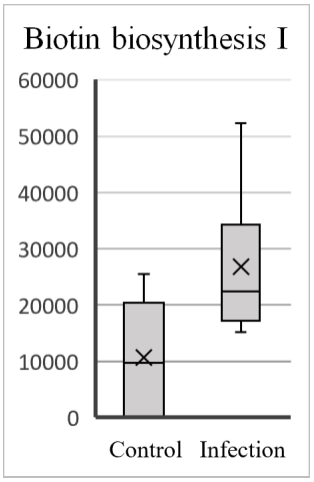
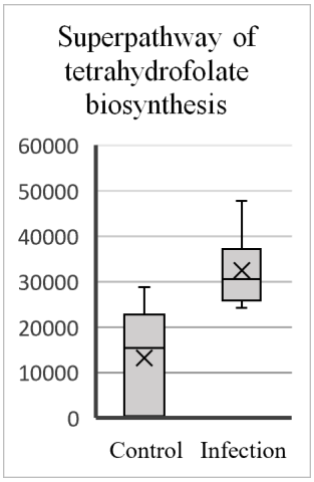

L-isoleucine biosynthesis I
80000
70000
60000
50000
40000
30000
20000
10000
0
Control Infection
L-isoleucine biosynthesis II
80000
70000
60000
50000
40000
30000
20000
10000
0
Control Infection
Isoleucine biosynthesis III
60000
50000
40000
30000
20000
10000
0
Control Infection
L-isoleucine biosynthesis IV
80000
70000
60000
50000
40000
30000
20000
10000
0
Control Infection
Superpathway of L-isoleucine biosynthesis I
70000
60000
50000
40000
30000
20000
10000
0
Control Infection
Superpathway of L-methionine biosynthesis
60000
50000
40000
30000
20000
10000
0
Control Infection
L-methionine biosynthesis I
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Control Infection
Superpathway of S-adenosyl-L-methionine biosynthesis
60000
50000
40000
30000
20000
10000
0
Control Infection
L-valine biosynthesis
80000
70000
60000
50000
40000
30000
20000
10000
0
Control Infection
L-histidine biosynthesis
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Control Infection
L-lysine biosynthesis I
60000
50000
40000
30000
20000
10000
0
Control Infection
Superpathway of L-phenylalanine biosynthesis
60000
50000
40000
30000
20000
10000
0
Control Infection

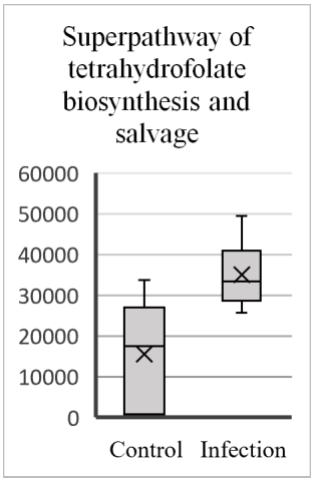
Fig. 3


Superpathway of thiamine diphosphate biosynthesis I
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Control
Infection


TCA cycle VII (acetate-producers)
60000
50000
40000
30000
20000
10000
0
Control
Infection


Biotin biosynthesis I
60000
50000
40000
30000
20000
10000
0
Control
Infection


Superpathway of tetrahydrofolate biosynthesis
60000
50000
40000
30000
20000
10000
0
Control
Infection


Superpathway of tetrahydrofolate biosynthesis and salvage
60000
50000
40000
30000
20000
10000
0
Control
Infection

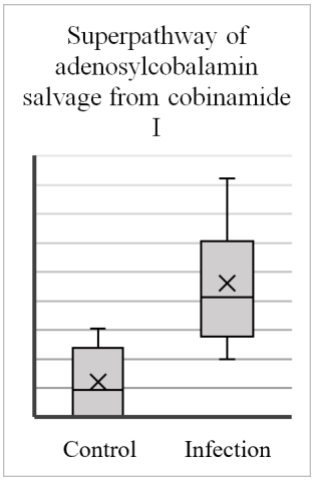

Superpathway of adenosylcobalamin salvage from cobinamide I
Control
Infection

Fig. 4

Chapter -VI

General Discussion

The possible consequences of maternal nematode infection and protein restriction on the neonatal brain were largely unknown when I began my PhD. Maternal protein restriction during the pre-implantation phase was known to reduce neuronal stem cells and progenitor cells in the neonatal mouse brain (Gould et al, 2018). A previous study showed that gene expression in the maternal placenta responded differently to maternal *H. bakeri* infection and to protein deficiency in a mouse model (Starr et al, 2016). However, it was not known whether these alterations were sufficient to influence the development of vital organs such as the brain in the developing neonate. In humans, some studies concluded that the presence of maternal nematode infection was associated with reduced early brain executive function and language (Nampijja et al, 2012) and impaired motor and cognitive development (Mireku et al, 2015) in infants. However, anthelminthic treatment of pregnant women was not associated with better psychomotor and cognitive function of their infants (Nampijja et al, 2012). Furthermore, a comprehensive meta-analysis failed to establish any connection between nematode infection and cognitive deficiency in a human population (Taylor-Robinson et al, 2015). As maternal nematode infection and protein malnutrition often co-occur among pregnant women, especially in developing countries (Crompton and Nesheim, 2002), this dissertation was designed to explore the possibility that maternal nematode infection and protein deficiency may influence the neonatal brain.

The key findings are that post-implantation maternal protein restriction did not alter fetal brain gene expression (GD18) whereas maternal nematode infection altered fetal (GD 18) and neonatal (P7) brain gene expression. Specifically, key gene expression pathways controlling synaptogenesis and long-term potentiation (LTP) were up-regulated in the neonatal brain (P7) as were genes associated with anti-inflammatory responses. On the other hand, the expression of vital pro-inflammatory genes was down-regulated, raising the possibility that an anti-inflammatory environment in the neonatal brain may positively contribute to synaptogenesis. Furthermore, maternal nematode infection increased the relative abundance of *Lactobacillus* in the vaginal tract of infected dams at P2, and PICRUSt prediction of the P2 pup stomach microbiome showed that putative pathways involved in amino acid biosynthesis, carbohydrate,

and energy metabolism, and vitamin biosynthesis were up-regulated in response to maternal nematode infection. Among these up-regulated putative pathways, several have the potential to participate in gut-brain cross-talk. Thus, these results show for the first time that a maternal nematode infection restricted to the maternal GI tract influenced pup brain gene expression and functional composition of microbiome.

6.1 Four possible pathways by which maternal nematode infection transmits signals to the perinatal brain

The alteration of gene expression in the brain of pups was intriguing as the pups were uninfected and the nematode infection remained restricted to the maternal gastrointestinal tract. The following potential pathways could explain how cues of a maternal nematode infection might have interacted with the developing neonatal brain (Figure 1).

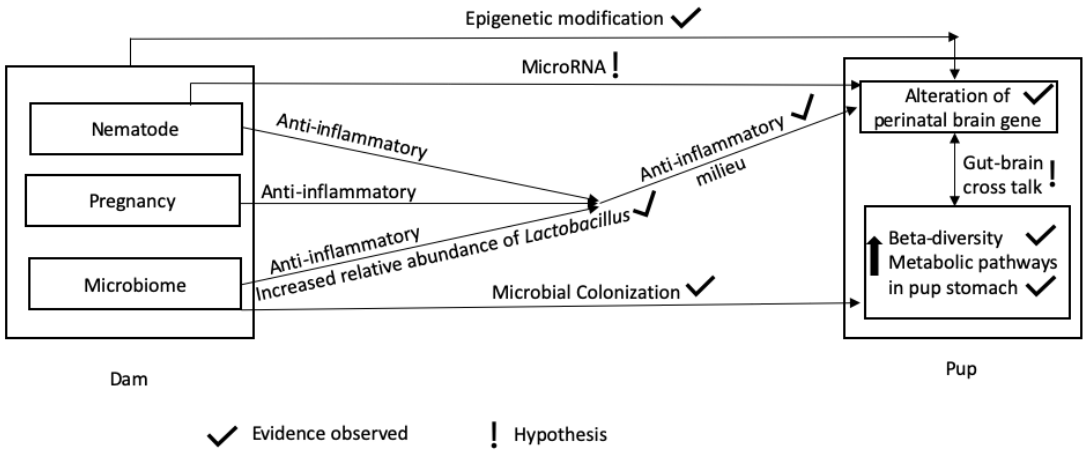


Fig.1. Suggested pathways by which maternal nematode infection might interact with perinatal brain gene expression based on our results.

Maternal nematode infection is known to alter concentrations of cytokines and growth factors in the maternal and fetal circulation. Using the same experimental model, Odiere et al. (2010) demonstrated higher concentrations of IL-4 and IL-1β in fetal circulation in response to maternal nematode infection. These cytokines may be one of the triggers for alteration of fetal brain gene expression as it has previously been demonstrated that maternal immune activation

can alter fetal brain gene expression. For example maternal IL-6 mediated pro-inflammatory response was shown to disrupt normal neurodifferentiation and axonal growth in the fetal brain (Garbett et al, 2012). However, drawing a definitive causal relationship between maternal immune activation and alteration of fetal brain gene expression is not possible without directly measuring cytokine concentrations in the pup brain. This would be a valuable follow-up experiment, and those cytokines known to increase in helminth infection (IL-4, TGF-β, IL-10, IL-13) (Maizels et al, 2012) would be of particular interest, especially given that the expression of IL-4 and TGF-β genes was up-regulated in the brain of pups from infected dams.

The alteration of neonatal brain gene expression might be mediated by epigenetic methylation induced by maternal *H. bakeri* infection. The prenatal environment has been shown to influence neurodevelopment through epigenetic modification, mainly by DNA methylation (Kundakovic and Jaric, 2017). Furthermore, a higher level of DNA methylation has been linked to higher synaptic plasticity and LPT in the brain (Levenson et al, 2006; Munoz et al, 2016). In my study, expression of genes involved in DNA methylation (*Dnmt1, Dnmt3a, Mecp2, Mbd5* and *Mbd6*) was up-regulated in the pup brain, in response to maternal nematode infection. Given the up-regulation both of LTP pathways and of genes involved in DNA methylation in the P7 pup brain, it is possible that maternal nematode infection altered perinatal brain gene expression through changes in DNA methylation. Therefore, measuring genome wide quantitative DNA methylation at the single-CpG-site using Infinium Methylation Array (Bibikova et al, 2011) may indicate whether the maternal nematode infection mediated epigenetic methylation of the pup brain genes, as suggested by my gene expression data.

H. bakeri has been shown to release microRNA within exosomes (Buck et al, 2014) and exosomes containing microRNA have been shown to participate in embryonic development and to influence cell-to-cell communication (Gross et al, 2017). Thus parasite-derived exosomes might reach the neonatal circulation and influence pup brain gene expression. Although our data was unable to produce any evidence of parasite-derived microRNA in the pup brain as the data were annotated against mice genome, an obvious next step would be to screen for parasite microRNA in the raw FASTQ sequences against the parasite genome.

Finally, a fourth possibility is that an altered neonatal microbiome might have influenced postnatal brain maturation. Neonatal colonization at the time of birth occurs principally from the maternal vaginal and skin microbiome. Studies have shown that the neonatal microbiome contributes to the development of the neonatal brain (Heijtz et al, 2011), the blood brain barrier (Braniste et al, 2014) and the HPI axis (Sudo et al, 2004). However, the exact mechanism(s) is not known. There are reports that microbiome-derived short-chain fatty acids may interact with the brain and influence its functions (van de Wouw et al, 2018; Park et al, 2019). Past studies in non-pregnant mice infected with *H. bakeri* infection showed that the GI infection altered the host gut microbiome (Walk et al, 2010; Rausch et al, 2013). Together this body of literature raises the possibility that an altered maternal microbiome may have led to changes in the neonatal microbial composition and function which may have influenced postnatal brain gene expression. This is of particular interest given the role of the gut microbiome in gut-brain cross-talk (Clemmensen et al, 2017). This possibility was explored in Chapter V, and the results of the PICRUSt analysis showed up-regulation of several biosynthesis pathways including amino acids and short-chain fatty acids which are known to influence in gut-brain cross talk. Given these promising findings, it would be important to examine through metabolic profiling whether these key molecules involved in gut-brain cross talk are present in higher concentrations.

6.2 Anti-inflammatory nature of the experimental model

Maternal *H. bakeri* infection may have altered the inflammatory environment for fetal and perinatal development and this may have contributed to altered gene expression as a recent study suggested that probiotic-suppressed maternal pro-inflammatory response minimised neuro-developmental disorders and improved brain function (Wang et al, 2019). Maternal inflammation is often echoed in the neonate including in the neonatal brain as a detrimental cue (Rudolph et al, 2018). For example, elevated expression of *BDNF* in the fetal hippocampus is a reference marker of maternal inflammation (Gilmore et al, 2003). However, there is reason to believe that our experimental model may have induced an anti-inflammatory rather than a pro-inflammatory environment in the neonatal brain. Expression of *BDNF* was not elevated in the pup brain. Furthermore, expression of key anti-inflammatory cytokine genes (*IL-4*, *TGF-β*) was up-regulated and expression of a pro-inflammatory cytokine gene (*IL-1β)* was down-regulated.

The anti-inflammatory nature of our experimental model may result from three factors. First, pregnancy itself is known to induce an anti-inflammatory response that protects the antigenically foreign embryo (Williams, 2012). This establishes a baseline anti-inflammatory state in the pregnant dam. Second, nematode infection is known to stimulate an anti-inflammatory response in the mouse (Sorobetea et al, 2018). Trickle infection with *H. bakeri* increases concentrations of anti-inflammatory cytokines over and above those in uninfected pregnant dams (Odiere et al, 2013) even though pro-inflammatory cytokines are also detectable in maternal serum (Odiere et al, 2010). Third, many species of *Lactobacillus* generate an anti-inflammatory environment (Oh et al, 2018) and vaginal *Lactobacillus* as well as intestinal nematode infection have been associated with an anti-inflammatory environment in pregnant women (Gonzalez-Fernandez et al, 2017). Consistent with this, the higher relative abundance of *Lactobacillus* in the pup stomach in response to maternal nematode infection may have contributed to an anti-inflammatory environment in the neonate.

6.3 Maternal nematode infection influenced neonatal stomach microbiome

Several previous studies have reported that GI parasites alter the gastrointestinal microbiome of their hosts (Walk et al, 2010; Rausch et al, 2013; Reynolds et al, 2015) but have not considered whether there is an intergenerational effect. This dissertation, for the first time, demonstrates that a nematode restricted to the maternal gut increased the early postpartum beta-diversity and later postpartum relative abundance of *Lactobacillus* in the pup stomach. In addition, putative metabolic pathways involved in amino acid biosynthesis, carbohydrate and energy metabolism, and vitamin biosynthesis were up-regulated in the P2 stomach and these may have important positive consequences for early postnatal growth (Dimmitt et al, 2010). The up-regulation of these putative metabolic pathways also supports the hypothesis that the neonatal microbiome of nematode-infected dams may mediate gut-brain cross talk and contribute to the observed differential brain gene expression noted above, as several of the up-regulated microbiome-derived amino acids act as neurotransmitters or neurotransmitter precursors (Rassin, 1994). Therefore, their increased production in the pup gut may have interacted with the enteric nervous system or participated in gut-brain cross talk and influenced the brain gene expression. Also, the up-regulation of the pathway for short-chain fatty acid biosynthesis includes synthesis

of acetate, which is known to participate in gut-brain cross talk (Bercik et al, 2011; Clemmensen et al, 2017). Future studies such as liquid-chromatography mass-spectrometry based metabolic profiling are required to establish whether maternal *H. bakeri* induced alteration in the functional composition of microbiome alters the metabolomic profile in pups and if so, the consequences of these changes for pup physiology and neurodevelopment.

6.4 Maternal nematode infection up-regulated pathways related to long-term potentiation in the P7 pup brain

One of the novel findings was that maternal nematode infection up-regulated key cell signaling pathways associated with long-term potentiation (LTP) in the P7 pup brain. LPT contributes to the synaptic plasticity and long-term memory in the developing brain (Sossin, 2018). Based on the transcriptome data and the fact that the five key interacting pathways for LTP were all up-regulated, it is hypothesized that pups of nematode-infected dams may have better cognitive function and long-term memory. However, it cannot be assumed that transcriptome data necessarily translate to biological function. In order to determine whether up-regulation of LTP pathways influences cognitive function and long-term memory of maternally exposed pups, a series of additional studies would be needed.

Behavioural studies: It would be important to conduct standardized behavioural tests in the pups. A logical first step would be to use an Object Location Test to explore the short-term memory that first occurs when young pups begin to remember the relative location of two objects. Those with improved LTP and enhanced memory would be expected to preferentially explore the object that has been moved (Lueptow, 2017). Longer-term spatial memory could be tested using the Morris Water Maze Test or the Barnes Maze Test where the interval between training and testing is longer (a week rather than a few hours) and where the spatial memory task is more complex (Gawel et al, 2019). Should phenotypic differences be detected in the behaviour of young mice, it would also be useful to see if the alterations persist when pups mature.

Brain ultrastructure: Although maternal nematode infection was not associated with differences in the relative or absolute mass of the fetal brain (Starr et al, 2015) and although the

mass of pup brains in Chapter IV was not affected by maternal infeciton, our unpublished data from a pilot study showed that the absolute mass of the P7 brain was lower in pups of infected dams. If this observation were confirmed, it would be consistent with my observation that expression of the *miRNAlet-7* gene was up-regulated in the P7 brain (Haque et al, 2018) as *miRNAlet-7* promotes cellular differentiation but inhibits cell proliferation by suppressing the expression of *cylin D1* (Zhao et al, 2010). Beyond the possible effect on brain size, it would also be useful to examine the effect of maternal nematode infection on the ultrastructure of the perinatal brain through histological and histochemical studies. Of particular interest would be the number and distribution of synaptic receptors, such as glutamate receptors which play a critical role in the brain. Depending on the distribution and number in nerve cells, glutamate receptors may function abnormally in the central nervous system (Newcomer et al, 2000). One type of ionotropic glutamate receptor, NMDAR, was up-regulated in the pup brain in response to maternal infection, but I was unable to differentiate between the up-regulation of synaptic and extra-synaptic NMDAR in my data. This information may be relevant as these two types of NMDARs have contrasting roles in regulation of the transcription factor CREB, expression of the neurotrophic factor, and neural survival during neurodevelopment (Hardingham et al, 2002; Vanhoutte and Bading, 2003), and an imbalance between synaptic and extra-synaptic NMDAR has been shown to lead to neurodegenerative diseases in adults (Hardingham and Bading, 2010). Scanning electron microscopy following immunogold labelling of the NMDARs would be helpful in detecting any alteration of distribution of the two types of NMDAR in nerve cells (Baude et al, 1995), and in determining if pup brain ultrastructure is altered by maternal nematode infection.

6.5 Maternal protein deficiency had a minor effect on the fetal brain

It was interesting to note that maternal protein deficiency exerted only a minor impact on fetal brain gene expression in that altered expression was detected for only one gene. This minimal impact of maternal protein deficiency on fetal brain gene expression may be explained by the experimental protocol. The pre-implantation period is most vulnerable to maternal protein deficiency (Wu et al, 2004), hence I began my experiments on embryonic day 5, one day after implantation is completed in mice (Yoshinaga, 2013) in order to ensure that implantation did occur. Also, a marginal level of protein (6%) was used in order to minimize the direct

effects of protein deficiency on fetal growth and resorption of embryos (Odiere et al, 2010; Starr et al, 2015). Thus, the combination of a marginal dietary protein restriction together with starting the experiment after implantation may have minimized the impact of the dietary protein treatment on fetal brain gene expression. This finding also supported the contemporary findings that neonatal brain development is spared in mild to moderate maternal nutrient deficiency (Prado and Dewey, 2014).

The minimal impact of protein deficiency could also reflect the ability of the placenta to protect key organs from maternal stress. The maternal placenta is known to undergo modifications that ensure that the needs of developing fetal brain are met during short-term maternal nutritional deficiency (Broad and Keverne, 2011). Placental expression of several embryonic growth-promoting genes including *Gata3* (GATA binding protein 3) was up-regulated in dams fed a 6% protein diet, compared with control dams fed a 24% protein diet (Starr et al, 2016), and *Gata3* has been shown to promote fetal brain growth (Pandolfi et al, 1995). Thus, the placental growth factors including *Gata3* that were up-regulated in response to maternal protein deficiency may have protected the fetal brain. It is important to note that I did not examine the effect of maternal protein deficiency on post-natal put brain development and therefore follow-up studies would be necessary to explore the effect of maternal protein deficiency on the post-natal pup brain.

6.6 Challenges of the computational approach to data interpretation

Bioinformatic pathway enrichment analysis revealed a few pathways that were both significantly up-regulated and also significantly down-regulated. Pathway enrichment analysis asks whether a large number of genes in the pathway are up-regulated, in which case the pathway is considered to be up-regulated. It also considers whether a large number of genes in the pathway are down-regulated, in which case the pathway is significantly down-regulated. One example was the insulin signaling pathway, where the pathway was both down-regulated (expression of 12 genes was down-regulated) and up-regulated (expression of 18 genes was up-regulated). Upon further examination, one of the down-regulated gene (*Fbp1*) acts as a negative regulator of the pathway (Zhang et al, 2010). Hence, down-regulation of inhibitor would have a positive impact on the pathway up-regulation. Nevertheless, this highlights a limitation to

bioinformatic analysis of gene expression pathways. There is a need for additional bioinformatics tools that incorporate function as well as direction of differential expression in assigning direction to the shift in the pathway. In the case of the insulin signalling pathway, it maintains energy homeostasis that is needed for survival of neurons in the brain (Plum et al, 2005). Given the statistical finding that this pathway was both up-regulated and down-regulated, it would be important to determine whether it was functionally up-regulated and hence promoted neuron survival, or was functionally down-regulated, possibly leading to abnormal neurodevelopment. This would involve direct measurement of protein such as phosphatidylinositol 3 -kinase, critical for insulin signaling in the brain (Plum et al, 2005).

With advances in research, our understanding of the multiple functions of individual genes is expanding, and this influences the interpretation of gene expression data. For example, *S100A8* and *S100A9* were first described as pro-inflammatory proteins because they were secreted by neutrophils and macrophages (Gebhardt et al, 2006). Thus, the finding that their expression was up-regulated in the fetal brain was used to support the possibility that maternal infection could potentially cause neuroinflammation in the fetal brain, as noted in Chapter 3 (Haque et al, 2018). However, *S100A8* and *S100A9* also act as calcium-binding proteins (Hoyaux et al, 2000) and given that calcium signaling was up-regulated in the pup brain, these two proteins could have participated in the initiation of the calcium signaling pathway. Moreover, a more recent study has demonstrated that *S100A8* and *S100A9* also activate brain macrophages (microglia) to initiate apoptosis (Wu et al, 2018) which plays a pivotal role both in the cellular homeostasis during neural development and in neuropathology (Yamaguchi and Miura, 2015). Therefore, future studies would be required not only to confirm higher concentrations of *S100A8* and *S100A9* protein but also to determine whether the higher expression *S100A8* and *S100A9* genes in the fetal brain was associated with the calcium signaling, neuro-developmental apoptosis, neuro-inflammation related anomalies, or other functions that may become evident in the future. This example highlights the importance of conducting functional studies in order to determine the consequences of differential gene expression, especially as additional functions are uncovered.

6.7 Broader implication of maternal nematode infection on perinatal outcomes

It seems counterintuitive to think that a nematode infection may have benefits to its host, but this possibility is not without precedent. GI nematodes have co-evolved and have shaped many aspects of host physiology, metabolism, and immunology. Usually, they do not impose life-threatening risk to their hosts, unless present in large numbers. The hygiene hypothesis acknowledges the potentially beneficial aspects of the presence of nematode infection in the host (Briggs et al, 2016) that are attributed to the fact that they induce a Th2 immune response and immune regulation that creates an anti-inflammatory environment (Briggs et al, 2016). Based on this, efforts are currently underway to utilize nematodes or their secretory and excretory products for the treatment of inflammatory bowel diseases (Xu et al, 2019), colitis (Wangchuk et al, 2019), and obesity and diabetes (Yang et al, 2013). Another example of a benefit of nematode infection emerges from a recent longitudinal study among Bolivian forager-horticulturalist women living in a region with 70% helminth prevalence. The study showed that parasitic nematodes favoured conception, implantation and overall fecundity among women (Blackwell et al, 2015).

Prior to my research, there was little evidence of intergenerational effects in nematode infected dams. Using the *H. bakeri* mouse model, it had been shown that maternal infection impaired fetal and neonatal growth (Odiere, et al, 2010; Starr et al, 2015) and that it may influence immune responses of the F1 generation when exposed to the same parasite (Kristen 2002). In humans, two studies have directly considered the impact of maternal nematode infection during pregnancy on brain development of infants (Nampijja et al, 2012; Mireku et al, 2015). They provide some evidence for impaired gross motor responses at 1 year (Mireku et al, 2015), and impaired executive function and self-control at 15 mo (Nampijja et al, 2012). The only positive impact was better self-control if mothers had been infected with *Strongyloides* during pregnancy, although significance was lost when other maternal and child factors were included (Nampijja et al, 2012).

The data presented in this dissertation both adds to our understanding on intergenerational impacts of nematode infections and highlights potential benefits to the pup.

The maternal nematode infection altered both neonatal brain gene expression and the early neonatal microbiome, and both in a direction that may have benefits to the pup. If parallel phenotypic benefits are found, this would be another example that nematode infections can have positive effects on their host, and a first example where the benefit is expressed in the next generation. It is important to recognize, however, that even if phenotypic benefits are observed, the net effect of all consequences of maternal infection may be detrimental to the developing pup and furthermore, the effects observed were the consequences of a low dose of maternal nematode infection and had the dose been higher, we might have found a detrimental outcome on the neonatal brain. Furthermore, I used a trickle infection protocol with the goal of maintaining an immune response in the dam. Had a single infection been administered, or a challenge infection that results is expulsion of the worms, the implications for the pup would likely have differed.

The possibility that maternal nematode infection may influence postnatal development, perhaps with beneficial effects, has intriguing evolutionary implications and warrants further exploration both in laboratory models as outlined above, and in human populations. A first step in human populations would be to consider that nematode infection during pregnancy might be influencing learning and cognition in infants. A meta-analysis comparing randomized controlled studies (RCTs) and quasi-RCTs associating anthelmintic treatment for soil-transmitted helminths with placebo or control on learning and cognition among children aged 16 years or less was inconclusive (Taylor-Robinson et al, 2015). This could be due to the fact that these studies explored the impact of direct infection of the children and did not control for maternal helminth infection. Perhaps a clearer picture would emerge if studies controlled for maternal nematode infection during pregnancy and lactation.

Despite the intriguing possibility of a positive association of a chronic gastrointestinal nematode infection in pregnancy and neonatal brain development, it is important to acknowledge that the associations reported here were based on up- and down-regulation of genes and pathways without further verification of protein expression or phenotypic consequences. Future behavioural studies on pups exposed to the maternal nematode infection would reveal if their cognitive and motor performance abilities have been affected. Similar behavioural tests should also be performed once the pups mature to examine whether any alterations are transient or long-

lasting. Given that gene expression patterns may differ between male and female pups (Trabzuni et al, 2013), it would be useful to determine if maternal nematode infection has a similar impact on brain gene expression in female pups as a previous study suggested that maternal stress differentially influenced brain development and behaviour between male and female newborns (Weinstock, 2007). The putative contribution of the microbial functional metabolic pathways in the pup stomach to the total nutrient availability of pups of infected dams warrants further verification using metabolomics approaches to determine whether the microbiome-derived metabolites were available to pups.

In conclusion, my studies, for the first time, shed light on a possible unappreciated consequence of maternal nematode infection on perinatal brain gene expression. Furthermore, my findings showed that maternal nematode infection exerted an intergenerational influence by altering the neonatal stomach microbiome. Additionally, several of the functional metabolic pathways with potential to participate in gut-brain cross-talk were up-regulated in the pup stomach microbiome in response to maternal nematode infection, providing some support for the hypothesis that altered brain gene expression results from maternal effects on colonization of the neonatal microbiome. Therefore, the results have raised some important hypotheses about the impact of maternal nematode infection on perinatal brain development and neonatal microbial colonization, hypotheses that warrant further exploration and if proven to be true, could lead to a paradigm shift that highlights not only the negative but also positive impacts of nematode infections on their host and on the next generation.

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