THE IMPACT OF ANTIBIOTICS ON THE GUT-BRAIN AXIS

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ABSTRACT

The gut and brain are involved in a bi-directional communication system, referred to as the gut-brain axis. While it has been established that antimicrobials induce dysbiosis in the gut, which further disrupts immune and metabolic homeostasis, research on brain and behaviour development is becoming a topic of interest. We propose that alterations via antibiotics at the level of the gut microbiota impacts the gut-brain axis. The primary interest of this thesis is to understand the effects that antibiotics have on brain and behaviour development in conjunction with changes in the immune system and metabolism using the antibiotic mouse model. Mice treated with antibiotics revealed behavioural differences in the open field apparatus and three-chamber social behaviour apparatus, but not in the elevated plus maze and auditory fear conditionings enclosures. Evaluation of intestinal permeability revealed that female Balb/C mice administered a combination of bacitracin, neomycin and primaricin and another group administered a combination of ampicillin, neomycin and primaricin showed reduced intestinal permeability. Furthermore, the immune system condition was evaluated using flow cytometric analysis of spleens, which revealed no effect of treatment on immune cell profiles in CD1 mice treated with ampicillin. Evaluation of serum cytokine levels showed minimal differences in Balb/C and C57Bl/6 mice treated with antibiotics. Body weight and water and food consumption were evaluated in mice administered antibiotics. Weight loss differences were observed in two groups of female Balb/C mice, with the first group administered bacitracin, neomycin and primaricin and the second group administered ampicillin , neomycin and primaricin. Antibiotic treatment dependent differences in water

and food consumption were observed. Serum insulin and leptin level investigation revealed that female Balb/C mice administered ampicillin, neomycin and primaricin had reduced serum insulin levels compared to strain matched controls. These findings indicate that antibiotic treatment impact metabolic function. This pilot study using antibiotic treated mouse models provides insight on the microbiota's effects on the gut-brain axis, which can help to potentially identify methods of preventing gut microbiota mediated pathology in humans.

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ABBREVIATIONS

5HT1A	**Serotonin Receptor 1A**
AMPK	**Adenosine Monophosphate-Activated Protein Kinase**
Angptl4	**Angiopoietin-like protein 4**
ANS	**Autonomic Nervous System**
BDNF	**Brain-Derived Neurotrophic Factor**
CNS	**Central Nervous System**
CS	**Conditioned Stimulus**
DGGE	**Denaturing Gradient Gel Electrophoresis**
ENS	**Enteric Nervous System**
EPM	**Elevated Plus Maze**
F	**Female**
FIAF	**Fasting-Induced Adipose Factor**
FITC	**Fluorescein Isothiocyanate**
GF	**Germ Free**
GI	**Gastrointestinal**
GPCR	**G Protein-Coupled Receptor**
HPA	**Hypothalamic-Pituitary-Adrenal**
IBD	**Inflammatory Bowel Disease**
Ig	**Immunoglobulin**

IL	**Interleukin**
LPL	**Lipoprotein Lipase**
LPS	**Lipopolysaccharide**
M	**Male**
NMDA	**N-methyl-D-aspartate**
PCR	**Polymerase Chain Reaction**
PYY	**Peptide YY**
SCFA	**Short Chain Fatty Acid**
SPF	**Specific Pathogen Free**
TNF-α	**Tumor Necrosis Factor-α**
TLR	**Toll Like Receptor**
US	**Unconditioned Stimulus**

TABLE OF CONTENTS

LIST OF FIGURES

STATEMENT OF CONTRIBUTIONS

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1.0 Introduction

1.1 The Gut-Brain Axis

The gut-brain axis, which is interchangeably referred to as the brain-gut axis — depending on the perspective from which it is studied — is characterized by its bi-directional communication facilitated by the autonomic nervous system (ANS), enteric nervous system (ENS), neuroendocrine system, and immune system. As described by Cryan and O'Mahony (2011) this back and forth signaling between the gastrointestinal (GI) tract and the central nervous system (CNS) is crucial in the maintenance of bodily homeostasis. An important pillar for gut-brain communication and overall homeostasis is the microbiota residing in the GI tract of each human. The gut microbiota's relationship with the gut-brain axis impacts health on multiple levels including metabolism, and brain development and function (Chen, D'souza and Hong, 2012; Foster and McVey Neufeld, 2013). Disturbances in the gut microbiota can lead to neuronal and developmental disorders such as depression and anxiety, autism, and immune and metabolic based disorders such as inflammatory bowel disease (IBD), obesity, and diabetes (Backhed *et al.*, 2012).

1.2 The Gut Microbiota

Round and Mazmanian (2009) describe the gut microbiota as an amalgam of microorganisms, which when combined form a complex and diverse community residing in the GI tract. By means of microevolution the relationship between the gut microbiota and the host has become mutualistic in nature, meaning that both parties continue to co-

evolve and gain increased fitness (Backhed *et al.*, 2011; Round and Mazmanian, 2009). The gut microbiota is involved in the development of its host's intestinal architecture, which is regarded as the first benefit that the host gains from its gut microbiome (Backhed *et al.*, 2011; Round and Mazmanian, 2009). The second benefit the host gains from the bacteria colonizing its GI tract is increased nutrient absorption. The gut microbiota can digest complex nutritional compounds such as certain polysaccharides into simpler structures. This allows the host to harvest energy from nutrients it normally would not be able to break down on its own (Backhed *et al.*, 2011; Round and Mazmanian, 2009). Finally, the bacterial population of the gut plays a critical role in defending its host against pathogen invasion, by limiting available space, energy, and resources and by inducing activation of the immune system via pro- and anti-inflammatory responses. In return, the gut microbiota receives from its host a balanced and nutritionally rich environment conducive to healthy growth and development (Backhed *et al.*, 2011; Round and Mazmanian, 2009). As mentioned previously, in addition to the impact the gut microbiota has on the development of metabolic processes and the immune system, the gut microbiota also plays a critical role in behaviour and central neurotrophin expression. (Cryan and O'Mahony, 2011, Bercik *et al.*, 2011).

1.3 Composition and Diversity of the Microbiome

Colonization of the GI tract begins at birth in humans and rodents alike. The newborn is first colonized by the microbes inhabiting the mother's vaginal tract, composed of *Lactobacillus*, *Prevotella*, or *Sneathia spp*, following vaginal birth or by the

microbiota found on the mother's skin, composed of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium spp*., following Caesarian births. During infancy, the gut microbiota is much simpler in composition and diversity compared to adulthood when it becomes much more complex and diverse. Therefore, the process of birth not only gives rise to a new organism, but also to one's intestinal microflora (Honda and Littman, 2012; Dominguez-Bello *et al.*, 2010).

The GI tract is home to the highest proportion of bacteria, an estimate of around 70% of the total microbiome present in the body. Of this large number, the colon specifically holds 10^{12} -10^{13} of the 10^{14} bacterial cells found in the GI tract (Backhed *et al.*, 2011; Cani *et al.*, 2009; & Ley *et al.*, 2008). Of these bacteria some are allochthonous, meaning they only reside temporarily in the GI tract, and the rest are autochthonous, which occur naturally in the GI tract and tend to colonize specific animal species. It is the autochthonous bacteria which impact the host's development and physiology as they bind to the epithelial wall of the GI tract and form a permanent bio-layer (Honda and Littman, 2012; Berg, 1996; Xu and Gordon, 2003).

The gut microbiota profile becomes more complex in composition and diversity after the infant's weaning from the mother when strict anaerobes start to dominate (Honda and Littman, 2012). Initially, profiling of polymerase chain reaction (PCR) derived amplicons targeting the 16S rRNA gene (V3-region) by denaturing gradient gel electrophoresis (DGGE) proved to be a very efficient method to determine the gut microflora profile (Hufeldt *et al.*, 2010; Bech-Nielsen *et al.*, 2011). Currently, the primary choice of method in gut microbiota profiling has become metagenomics, as seen through

the establishment of the International Human Microbiome Consortium in 2008 (Blottiere *et al.*, 2013). The two dominant phyla, which comprise over 90% of the bacteria residing in the GI tract, are the Bacteriodetes and the Firmicutes. Of the Firmicute bacteria found in the gut the most prevalent are two groups, which are part of the clostridium genus (gram-positive bacteria), clostridial clusters IV and XIVa, including the *Lachnospiraceae* (Honda and Littman, 2012). Less abundant phyla include Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria. Overall, from these phyla the gut microbiota contains approximately 200 dominant bacterial species and close to 1000 less prevalent ones (Zoetendal *et al.*, 2006). Based on microbiome studies using DGGE, Vrieze *et al.* (2010) estimated that the intestinal lumen contains 150 times more bacterial genetic material than human genetic material. Therefore, the gut microbiota can be seen as an 'exteriorized organ' of the human body.

1.4 Methods Used to Study the Gut-Brain Axis

The microbiota in conjunction with the gut-brain axis have been intensely studied by various methods of investigation including: germ-free, antibiotic, probiotic, infection, and fecal transplantation studies (Cryan and Dinan, 2012). Here we will focus on the first two methods of study listed.

1.4.1 Germ-Free Mice Studies

Previous work completed in our lab has built upon the existing knowledge and postulation of the existence of the gut-brain axis by using the germ free (GF) mouse (Neufeld *et al.*, 2011a, Neufeld *et al.*, 2011b). Initially, the GF mouse model was used for immunological studies; however, it is also used to research the impact of the intestinal commensals on the gut-brain axis by examining what occurs in the absence of the gut microbiota. GF mice are bred and raised in facilities that are free of any microbial organisms, such as bacteria, archaea, viruses and unicellular eukaryotes that normally colonize a host's body (Macpherson & Harris, 2004). As a result of the strict lack of exposure to microbes, GF mice do not contain mucosal resident bacteria, and therefore have no commensal intestinal flora. Mice that are raised with exposure to microbial organisms and that possess normal gut microbiota, with the exception of a defined list of mouse pathogens, are referred to as specific pathogen free (SPF) mice (Macpherson & Harris, 2004). Most studies that focus on the gut microbiota, some of which also use the GF mouse as an animal model, will use the SPF mouse or the conventionalized GF mouse as a control in order to evaluate the changes induced by the gut microbiota on the area of interest. The conventionalized GF mouse is a GF mouse that has been introduced to and populated by the microbiome of a normal SPF mouse (Macpherson & Harris, 2004). This is achieved by exposing the GF mouse to the feces of an SPF mouse over a period of a few weeks. The conventionalization process of GF mice allows researchers to study the impact of the gut microbiota on the various systems it interacts with at different time points throughout life.

An area of interest in relation to the gut microbiota is its effects on the hypothalamic-pituitary-adrenal (HPA) axis. The first study to demonstrate the regulatory effect of the gut microbiota on the development of the HPA stress response was conducted by Sudo and colleagues in 2004. Since then, our published work has shown that the gut microbiota has modulatory effects on behaviour and that the absence of the gut microbiota leads to neurochemical changes in the CNS (Neufeld *et al.*, 2011a). Neufeld *et al.* (2011a) reported that GF mice showed reduced anxiety-like behaviour compared to SPF mice when tested in the elevated plus maze (EPM). Changes in expression of plasticity-related genes, which have been established to be involved in emotional behaviour in mice, were observed at the level of the CNS. Specifically, the study by Neufeld *et al.* (2011a) found a significant decrease in mRNA expression of the N-methyl-D-aspartate (NMDA) receptor subunit NR2B in the central amygdala. In the dentate granule layer of the hippocampus a significant increase in mRNA expression of brain-derived neurotrophic factor (BDNF) and a significant decrease in mRNA expression of serotonin receptor 1A (5HT1A) was observed (Neufeld *et al.*, 2011a). Upon conventionalization of GF mice with SPF feces in order to establish a normal gut microbiota repertoire, it was observed that conventionalized mice retained the reduced anxiety-like behavior (Neufeld *et al.*, 2011b) suggesting that microbiota influenced the development of CNS circuitry related to behaviour that was no longer reversible in adulthood.

A study by Heijtz *et al.* (2011) further reinforces the influence of the gut microbiota on brain development and behavioural phenotypes in adulthood. Heijtz and

colleagues (2011) found that GF mice display increased motor activity levels and reduced anxiety- like behaviour compared to SPF mice, as well as alterations in expression of anxiety and synaptic plasticity-related genes in the CNS. Additionally, Heijtz and team (2011) showed that conventionalization of GF mice in early life resulted in normal behaviour phenotypes in adulthood, similar to those of SPF mice. Contrary to GF mice but similar to SPF mice, conventionalized mice exhibited in the striatum reduced levels of synaptophysin and PSD-95, which influence synaptogenesis (Heijtz *et al.*, 2011). Another recent study by Clarke *et al.* (2012) focused on elucidating whether the gut microbiota has critical neurodevelopmental implications. Besides demonstrating the sex dependent nature by which the gut-brain axis regulates the hippocampal serotonergic system during early life, Clarke and colleagues (2012) showed that conventionalization of GF mice at weaning age resulted in the reversal of reduced anxiety-like behaviours to normal phenotypes seen in SPF mice.

From these studies it was understood that neural pathways become altered during early development due to the lack of normal gut microbiota and that microbial recolonization in adulthood does not have an effect on behaviour. This finding underlines the crucial significance of the gut-brain communication axis to the CNS development of stress systems and further establishes the possible existence of a critical window for behavioural phenotoype flexibility (Neufeld *et al.*, 2011b).

1.4.2 Antibiotic Studies

An alternative to the GF mouse as a tool for the study of the gut-brain axis is the administration of oral antibiotics to SPF mice. Antimicrobials are known to disrupt the gut microbiota by reducing bacterial numbers and diversity, inducing dysbiosis, which in turn can affect bodily homeostasis (Verdu *et al.*, 2006; Cani *et al.*, 2008; Stecher, Maier and Hardt, 2013; Cryan and O'Mahony, 2011). Recent studies have observed that disruption of the gut microbiota by means of antibiotic administration has impacts on behaviour. Bercik and colleagues (2011) have shown that administration of antibiotic (Neomycin, Bacitracin and Primaricin) treatment in mice results in alterations of the gut microbiota profile and furthermore leads to increased exploratory behaviour and hippocampal expression of BDNF.

Furthermore, antibiotic treated mice have also been used in metabolic and immune related studies. Cani and colleagues (2008) conducted a study in which C57Bl/6 male mice and *ob/ob* mice were administered a combination of ampicillin and neomycin in order to modulate the gut microbiota. Cani *et al.* (2008) have argued that modulation of the gut microbiota influences metabolic endotoxemia, inflammation and other associated metabolic disorders by means of intestinal permeability status, which is a key player in immune defense. A more recent study by Bech-Nielsen and colleagues (2011) showed that antibiotic treatment (ampicillin and erythromycin) altered the composition of the gut microbiota profile and altered glucose metabolism in female C57Bl/6 mice. Interestingly this was not correlated with weight development in mice (Bech-Nielsen *et al.*, 2011).

The antibiotics used in the studies mentioned above and also in this thesis are known to modulate the intestinal microflora targeting different bacterial populations. For instance, ampicillin, bacitracin and erythromycin primarily target Gram-positive and some Gram-negative bacteria, while neomycin primarily targets Gram-negative and some Gram-positive bacteria. The reasoning behind Bercik et al's and our use of primaricin, an antifungal, is based on the knowledge that although the intestinal microflora is largely comprised of bacteria, it also contains a significant amount of fungi (2011).

1.5 Gut Microbiota and the Immune System

It is of no surprise that the gut microbiota plays a significant role in the development and maintenance of the intestinal epithelial barrier, a key factor of the host's immune system. Therefore, the gut microbiota is crucial to the development of the mucosal and systemic immune system. The protective effect of the commensals in the GI tract begins with the competitive exclusion of pathogenic microorganisms, since the commensals limit available space and resources that could otherwise be used by pathogenic organisms (Honda and Littman, 2012).

As previously mentioned in Section 1.4.1, the GF mouse model has played a significant role in understanding the impact of the gut microbiota on the immune system. GF mice exhibit undeveloped mucosal and systemic immune systems. This was observed in the hypoplastic Peyer's patches that had few germinal centres and very low levels of immunoglobulin (Ig) A producing plasma cells and lamina propria CD4+ T cells. Furthermore, the architecture of the spleen and lymph nodes of GF mice lacked in

structure as exhibited through poorly developed B- and T- cell areas. GF mice suffer from hypogammaglobulinemia as seen through reduced levels of IgG and reduced numbers of B cells in serum. The lack of commensal bacteria in the GI tract also resulted in an altered gene-expression profile of the intestinal epithelial-cell layer and abnormally high endothelial venule morphology in GF mice (Macpherson & Harris, 2004, Cebra, 1999). These changes observed in the GF mouse model support the notion that the gut microbiota plays a significant role in the development of the mucosal immune system of the gut. It impacts the organization of the Peyer's patches and isolated lymphoid follicles, production of antimicrobial peptides by the epithelial cells of the intestinal lumen, and the localization of a variety of immunocytes at the mucosal sites (Honda and Littman, 2012).

An important modulatory effect the gut microbiota has on the immune system is modulation of the "leakiness" of the intestinal wall. Intestinal permeability is controlled by the expression and function of tight junctions, which maintain the structure and organization of the gut epithelial cells to form a selective barrier. This barrier separates the intestinal lumen and the microflora found within it from peritoneal tissues, maintaining bodily homeostasis. Gut microbiota and intestinal permeability status are most highly impacted by diet. When the tight junctions of the intestinal epithelial barrier are compromised, bacterial bodies and products, such as lipopolysaccharides (LPS) produced by gram-negative bacteria, cross the mucosal barrier eliciting inflammatory responses (Honda and Littman, 2012; Carvalho and Saad, 2013; Cani *et al.*, 2008). Immune recognition of LPS is mediated through the toll like receptor (TLR)-4- receptor complex found in intestinal epithelial cells, which in turn elicit an intracellular signaling

cascade and ultimately the release of pro-inflammatory cytokine such as interleukin (IL)-1α, IL-1β, IL-4, IL-6 and tumor necrosis factor (TNF)-α. In turn pro-inflammatory responses are regulated by anti-inflammatory cytokines, such as IL-10 (Rodes *et al.*, 2012, Sudo *et al.* 2004).

The gut microbiota impacts the function of the immune system starting at the primary site, the intestinal epithelial barrier, which has also been shown to have implications on homeostatic metabolic function. It has been suggested that a compromised intestinal epithelial barrier resulting in increased gut permeability may impact the absorption of antigens that attack and damage pancreatic beta-cells, resulting in metabolic alterations as seen through reduced insulin production.

1.6 Gut Microbiota and Metabolism

The condition of the gut microbiota has several distinct avenues by which it impacts bodily homeostasis. Of particular interest is the link between the gut microbiota profile and metabolic disorders such as obesity and diabetes mellitus. At the core of metabolism are two hormones, leptin and insulin. Leptin is a polypeptide hormone secreted by the adypocytes with pleiotropic effects on appetite and energy metabolism (Halldén & Aponte, 1997). Leptin is released into the circulatory system and travels to the hypothalamus, the primary centre for regulation of food intake and body weight, where it performs its role (Klok, Jakobsdottir and Drent, 2006). Insulin is a hormone synthesized by the beta cells of the pancreas and plays a critical role in energy metabolism. Insulin regulates excess energy storage by promoting fat synthesis from surplus glucose in the

blood. Insulin insensitivity, also referred to as insulin resistance, is a result of the inability of body cells to respond appropriately to insulin. This inability of body cells to respond to insulin causes blood glucose levels to be elevated, leading to hyperglycemia. Hyperglycemia in turn leads to an overproduction of insulin by the pancreatic beta-cells as they attempt to lower blood glucose levels. Insulin insensitivity is a contributing factor to type 2 diabetes mellitus, which is characterized by an inability of body cells to respond to insulin. Therefore, insulin tends to be overproduced. A complete inability of the body to produce insulin is a hallmark of type 1 diabetes mellitus, where pancreatic beta-cells are unable to produce insulin (Guyton and Hall, 2006).

Diet is one of the key factors that influences the status of the gut microbiota profile, which in turn impacts other bodily systems that are in communication in order to maintain homeostasis. Research has shown that dysbiosis seen in the gut microbiota, by means of diet or antibiotic intake, is linked to immune dysregulation which furthermore leads to metabolic dysfunction (Carvalho and Saad, 2013). It is important to note that a large overlap occurs between the immune system and metabolism in alterations that occur as a result of gut microbiota changes. These systemic changes impact insulin sensitivity and glucose tolerance leading to pathologies such as obesity or diabetes (Carvalho and Saad, 2013; Rodes *et al.*, 2012; Membrez *et al.*, 2008; Cho et al, 2012).

Backhed *et al.* (2004) lead the way in metabolism research by establishing that conventionally reared mice have 42% more total body fat and 47% more gonadal fat than GF mice fed high-energy content diets. The conventionalization of the GF mice showed an increase of 60% in body fat content and insulin resistance despite a lower-energy

content diet (Backhed *et al.*, 2004). Weight gain is attributed to increased intestinal glucose absorption and non-digestible food component breakdown for energy extraction, coupled with higher glycemia and insulinemia, components of lipogenesis regulation (Backhed *et al.*, 2004). However, when studied in antibiotic administered animal models, gut microbiota modulation was not associated with alterations in body weight (Membrez *et al.*, 2008; Bech-Nielsen *et al.*, 2011).

There are four mechanism that have been proposed by which the gut microbiota influences host energy metabolism and fat storage, most of which are in tandem with immune regulation (Musso *et al.*, 2011). The gut microbiota causes the suppression of intestinal secretion of fasting-induced adipose factor (FIAF); modulation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity; modulation of gut motility and nutrient absorption by microbial-derived short chain fatty acids (SCFAs); and finally modulation of host inflammatory response – chronic low- grade endotoxemia (Musso *et al.*, 2011; Vrieze *et al.*, 2010).

FIAF, also known as angiopoietin-like protein 4 (Angptl4), is a ~ 50 kDA glycoprotein which acts as an inhibitor of the adipose tissue lipoprotein lipase (LPL). Its role is to reduce adipocyte uptake of fatty acids and triglyceride accumulation (Kersten, 2005; Kim *et al.*, 2010). Backhed and colleagues (2004) established that the expression of FIAF is modulated by the gut microbiota. GF FIAF-knockout mice contained 67% higher epididymal fat pad LPL compared to GF wild-type mice, and no changes in epididymal fat pad LPL levels were observed upon conventionalization of GF FIAF knockout mice (Backhed *et al.*, 2004). Furthermore, in more recent research Kim *et al.* (2010)

demonstrated that FIAF is involved in the central regulation of metabolism. It was observed that hypothalamic FIAF regulates anorexigenic responses via suppression of hypothalamic AMPK activity (Kim *et al.*, 2010).

Similarly to, but separate, from the FIAF pathway, Backhed *et al.* discovered that the gut microbiota has an effect on AMPK regulation and fatty acids oxidation (2007). AMPK is a heterotrimeric enzyme that is conserved from yeast to humans and functions to monitor cellular energy status (Musso *et al.*, 2010). GF mice consuming a high-fat and sugar diet were shown to be protected from developing obesity (Backhed *et al.*, 2007). Backhed *et al.* (2007) attributed this protection to two independent mechanisms, the FIAF and AMPK pathways. The lean body phenotype of GF mice is associated with high skeletal muscle and liver levels of AMPK and its downstream targets that partake in fatty acid oxidation (Backhed *et al.*, 2007).

Furthermore, microbial-derived SCFAs such as propionate, acetate and butyrate act as signaling ligands for the G protein-coupled receptors (GPCRs), Gpr41 and Gpr43. Samuel *et al.* (2008) compared the effect of Gpr41 functional deletion on host adiposity and energy harvest from diet in GF, conventionalized, or GF mice colonized with two prevalent human gut fermentative commensals, *B. thetaiotaiomicron* and *M. smithii*. It was found that regardless of elevated levels of intestinal SCFAs, both conventionally raised Gpr41-knockout and GF Gpr41-knockout mice colonized with *B. thetaiotaiomicron* and *M. smithii* were significantly leaner, with reduced hepatic de novo lipogenesis and triglyceride content, compared to wild-type mice. Gpr41 deficiency was associated with decreased expression of peptide YY (PYY)—an anorexigenic peptide

secreted by the intestinal enteroendocrine cells—,faster intestinal transit rate, and reduced harvest of energy from the diet (Samuel *et al.*, 2008).

The link between the immune and metabolic systems are mediated by the gut microbiota that elicit immune responses at the intestinal level. Mice fed a high-fat diet exhibited an increase in the LPS-expressing bacteria in the gut, resulting in an elevation of plasma LPS levels in these mice (Cani *et al.*, 2007). This effect was termed as metabolic endotoxemia, the presence of metabolic endotoxins in the blood, resulting in inflammatory responses triggered by the immune system. Cani and colleagues (2007) concluded that obesity, insulin resistance and glucose intolerance can be caused by chronic inflammation, induced by low-grade endotoxemia.

1.7 Thesis Objectives

Hypothesis:

Antibiotic treatment in mice alter behaviour through barrier function, and via immune and metabolic changes.

Objectives:

The primary objective of this study was to elucidate the impact of antibiotics on behaviour. In addition we examined the impact of administration of antibiotic treatment on intestinal barrier function and circulating gut hormones and cytokines. As previously mentioned, recent studies have shown that compared to SPF mice, GF mice exhibit reduced anxiety-like behaviour in the paradigms of the open field test, EPM and

light/dark box test (Neufeld *et al.*, 2011a; Neufeld *et al.*, 2011b; Bercik *et al.*, 2011). To study the effects of antibiotics on the gut-brain axis we included several behavioural tests. These included tests of exploratory and anxiety-like behaviours (open field and elevated plus maze tests), social behaviour (three-chamber apparatus social behaviour test) and finally learning behaviour (fear conditioning test).

Previous research in our lab has confirmed the presence of a critical window for conventionalization of GF mice in terms of regulating stress circuitry. Moreover, recent research has shown that conventionalization of GF mice from birth normalized behaviour in the EPM, but not in the light/ dark test. However, the conventionalization of GF mice in adulthood does not lead to behaviour normalization of GF mice in the open field test (Heijtz *et al.*, 2011). Clarke *et al.* (2012) showed that conventionalization of GF mice at 3 weeks of age (weaning age) normalized anxiety-like behaviour in the light/dark box test. These observations together with previous findings from our lab led Foster and McVey Neufeld (2013) to postulate that adolescence may be the critical window during development when the gut microbiota influences CNS wiring pertaining to stress-associated behaviour. Therefore, study 1 was designed to examine whether adolescence is indeed the critical window when gut microbiota influences behaviour.

In study 2 we examined the impact of antibiotic treatment in adult mice on behaviour, barrier function, and immune and metabolic systems. Cani and colleagues (2008) showed that administration of antibiotics, ampicillin and neomycin, in mice caused alterations in the gut microbiota profile, reduced levels of metabolic endotoxemia, and reduced intestinal levels of lipopolysaccharide (LPS). It was observed that high-fat

feeding mice showed a robust increase in intestinal permeability, a key player in immune defense, and a decrease in gene expression of tight junction proteins, therefore leading to the idea that intestinal permeability may play a key role in gut associated disorders. This thesis explores the effects of antimicrobial treatment on intestinal permeability, spleen immune cell profile and serum cytokine and gut hormone levels in mice.

The use of the antibiotic treated mouse model has been highly prevalent among metabolic studies, regarding obesity and diabetes for example. Membrez and colleagues (2008) administered norflaxcin and ampicillin to mice and demonstrated that the gut microbiota is a main contributing factor to bodily insulin sensitivity, but has no correlation to obesity development. Bech-Nielsen and colleagues (2011) showed that antibiotic treatment (ampicillin and erythromycin) altered glucose metabolism in female C57Bl/6 mice. Moreover, like Membrez *et al.* (2008), Bech-Nielsen *et al.* showed that antibiotic treatment was not correlated to weight development in mice (2011). Knowing that gut microbiota status is greatly associated with metabolic homeostatsis and associated disorders, we decided to measure body weight changes, water and food consumption, and serum insulin and leptin levels in our experimental mice.

2. Methods

Study #1 - Rx1

2.1 Animals

Pregnant female CD1 mice were obtained from Charles River (Quebec, Canada), at 16 days pregnant. Pups were born in house at SJH animal facility; date of birth was designated postnatal day 0, P0. Four litters of pups were used in the experiment; litter 1 – n =14, 8 female (F) and 6 male (M); litter 2 – n= 10, 4 F and 6 M; litter 3 – n=10, 6 F and 4 M; litter 4 – n=14, 8 F and 6 M. At P21 mice were weaned and identified by ear punch, housed by sex 2 per cage. All animals were housed under 12 h light/12 h dark cycle, with lights on at 5AM.

2.2 Ethics

All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

2.3 Experimental Design

Antibiotic treatment of ampicillin sodium salt (Sigma-Aldrich, Missouri, USA) was administered in sterile drinking water at a concentration of 1g/l. Control group (CON) received sterile drinking water. The mice were divided into 3 groups: CON, antibiotic treatment starting at **4 weeks** old (RX4) and antibiotic treatment starting at **8 weeks** old (RX8). The treatment was administered in a volume of 200 ml, and was changed twice

weekly.

2.4 Weekly Data Collection

Weight and food and water consumption data collection occurred biweekly, during cage maintenance.

2.5 Behaviour Testing

Mice were tested in the open field at P25, prior to the first treatment start time. Starting at 10 w of age, mice were tested sequentially in the open field, three-chamber social behaviour apparatus, elevated plus maze, and auditory fear conditioning. Mice were handled twice weekly starting at weaning age (P21).

2.5.1 Open Field

Mice were tested in the open field test one hour into the active period at P25 and at 10 w of age. Animals were transported to the behaviour testing room for a 30 min period of habituation prior to testing. Plexiglas activity chambers (43x21x20 cm) with ventilated lids were connected to a computer that provided automated recording of locomotor activity using MotorMonitor software (Kinder Scientific, Poway, CA). Animals were placed in chambers and allowed to explore for 30 min while general locomotor activity for each animal was acquired. Enclosures were cleaned with water and dried between tests.

2.5.2 Social Behaviour

Mice were tested in the three-chamber social behaviour apparatus during the afternoon (light cycle) at 11 weeks of age. The Plexiglas three-chamber social behaviour apparatus (73x45x30.5 cm) was mounted with a recording camera with an aerial view of the apparatus in its entirety. The camera was connected to a computer that provided automated recording of the location of the experimental mouse within the apparatus using Ethovision software (Noldus, Leesburg, VA). Experimental and stranger animals were transported to the behaviour testing room for a 5 min period of habituation prior to testing. Each experimental animal was subjected to a 5 min habituation to the apparatus followed by a 10 min sociability test. During the 5 min long habituation period the experimental animal was placed in the center chamber of the apparatus without access to the outer chambers as they were blocked with clear Plexiglas dividers. Following habituation a stranger mouse was placed in one of the outer chambers under a cup, which allowed for interactions but kept the experimental and stranger mice separate. The doors to the outer chambers were opened and the sociability trial was conducted for a period of 10 min during which the experimental mouse was allowed to explore all three chambers of the apparatus. The three-chamber social behaviour apparatus was cleaned with water and dried between each animal.

2.5.3 Elevated Plus Maze

Mice were tested in the elevated plus maze apparatus during the light cycle at 12 weeks of age to assess anxiety-like behaviours. The elevated plus maze apparatus consists

of four black Plexiglas arms in a plus shape, with two opposing arms enclosed by 15.2 cm high black Plexiglas walls, while the other two opposing arms are open. Furthermore, the apparatus is elevated at 76.2 cm off the ground and equipped with thirty-two electronic photobeams for automated recording of locomotor activity (See Fig 1.; Sidor, Rilett & Foster, 2010). The elevated plus maze (Kinder Scientific, Poway, CA) was connected to a computer that collected behavioural data using MotorMonitor software. Mice were transported to the behaviour testing room for a 15 min period of habituation prior to testing. Mice individually commenced the test from the intersection of the apparatus. The animals were undisturbed throughout the 5 min duration of the test. The elevated plus maze apparatus was cleaned with water and dried between each animal.

2.5.4 Auditory Fear Conditioning

Mice were individually tested in the auditory fear conditioning enclosures during the light cycle at 13 w of age to assess emotional learning and memory. Plexiglas activity chambers (43x21x20 cm) with ventilated lids were connected to a computer that provided automated recording of immobility using MotorMonitor software (Kinder Scientific, Poway, CA). Furthermore, the enclosures were connected to generators that produced the conditioned aversive stimulus (CS), a 30 s tone and the unconditioned aversive stimulus (US), a 2 s electric shock that animals were presented with during day one of testing. The fear conditioning test measures immobility, known as freezing behaviour. According to Curzon *et al.* (2009) freezing behaviour is observed in rodents as a response to fear and is defined by the absence of movement with the exception of respiration. Animals were

transported to the behaviour testing room for a 5 min period of habituation prior to testing. The auditory fear conditioning test occurred over the span of two consecutive days, day one being the acquisition phase and, day two the cued and context phases. Day one: Mice were placed in the activity chambers and allowed to explore. Following the first 135 s of the test a tone was presented for the duration of 30 s. During the last 2 s of the tone presentation, a 0.5 mA floor grid shock was administered for the duration of 2 s. Mice were left undisturbed in the activity chambers until the completion of the test at 300 s, after which they were removed and placed back in their housing cages. Day two: The cued test was performed on the animals 24 h after day one. The cued test assessed the animal's ability to remember and associate the CS with the US, without the influence from the context, the environment. Mice were placed in cages different than those of day one. Mice were presented with the CS at 135 s (no shock) and behaviour monitored for 5 min duration of the test. An hour after, the contextual test was performed where animals were placed in the same cages designated on day one, without the presentation of the US. Animals were undisturbed for the 5 min duration of the test. The contextual test assesses whether the animal remembers and associates its environment with the US.

2.6 Tissue Collection

Tissue collection occurred within 1 week of the final behavioural test when spleens were removed from each animal and placed in test tubes containing 1% PBS until they were later processed for profiling of immune cells.

2.7 Flow Cytometric Analysis of Spleens

Spleens harvested at endpoint from experimental animals of litters 3 and 4 (n=23, CON: 4 F and 3 M; RX4: 6 F and 4 M; and RX8: 4 F and 2 M) were kindly processed by Kelly Rillet via flow cytometric analysis to define their immune cell profiles. For each spleen, the tissue was pulverized in 5 ml 1% PBS and centrifuged at 1500 rpm for 5 min. The pellet was then resuspended in 1 ml 1% FACS Buffer (FB; 1% Fetal Bovine Serum in 1% PBS). Two tubes per spleen containing 10^6 cells per tube were prepared for each spleen and stained with 100 μL antibody solution for 30 min in the dark. T cells were stained for using CD3 antibody tagged with PE-A fluorophore (1:300 dilution; eBioscience, San Diego, CA). B cells were stained for using CD19 antibody tagged with PerCP-Cy5.5 fluorophore (1:300 dilution; eBioscience, San Diego, CA). Macrophages were stained for using F4/80 antibody tagged with APC-A fluorophore (1:200 dilution; eBioscience, San Diego, CA). In addition to the samples, 4 tubes were prepared with 10^6 cells per tube for compensation. One tube was incubated in FACS buffer only as an unstained control. Each of the other tubes was labeled with one of CD3, CD19, or F4/80. Cells were resuspended in 400 ul of 2% PFA and incubated in the dark at room temperature for the duration of 30 min. Finally, cells were resuspended in 400 ul FB and stored in the dark at 4°C up to 2 days before flow cytometry was performed. Samples were transferred into FACS tubes immediately prior to FACS analysis and shielded from light throughout the flow cytometry process. Samples were run using the BD FACSCanto flow cytometer (San Jose, CA) connected to a computer on which data was analyzed using FlowJo (Ashland, OR).

2.8 Data Analysis

All data was analyzed using SPSS (Armonk, NY, USA). Body weight measurements and, water and food consumption statistical analysis was conducted using repeated measured three-way ANOVA tests with age, sex and antibiotic treatment as factors. Furthermore, statistical analyses of behavioural testing were conducted using three-way ANOVA tests with time, sex and antibiotic treatment as factors. Statistical analyses of flow cytometric tests were conducted using two-way ANOVAs with antibiotic treatment and strain as factors. Additional pair-wise two-tailed t-tests were conducted on all data sets. Statistical significance was set at $p<0.05$.

Study #2 - Rx2

2.9 Animals

Female Balb/C mice (n=24) and C57BL/6 (n=24) were obtained from Charles River (Quebec, Canada) at 8 weeks of age. The mice were maintained in standard housing, 2 per cage at SJH animal facility. For the first week in the mice were allowed to habituate to the new environment without any disruptions other than maintenance. Following the habituation period the mice were handled twice a week for the duration of one week before commencing the experiment. At 10 weeks of age the mice were identified by ear punch.

2.10 Experimental Design

Mice were divided into 4 treatment groups (n=6 per strain per treatment). The

antibiotic treatments were administered in sterile drinking water. The first treatment group of Balb/c mice was administered a combination of the following antibiotics: Bacitracin (5mg/ml), Neomycin (5mg/ml), Primaricin (1.2μg/ml) (Sigma-Aldrich, Missouri, USA). Following complications with the first group of Balb/c mice, the antibiotics concentrations in the first treatment for the C57BL/6 mice were modified to the following: Bacitracin (2mg/ml), Neomycin (2mg/ml), and Primaricin (1.2μg/ml) (Sigma-Aldrich, Missouri, USA). For the remaining 3 treatment groups the antibiotic concentrations were maintained the same for both the Balb/c and C57BL/6 cohorts. Treatment group 2 was composed of the following antibiotics: Ampicillin (1mg/ml), Neomycin (2mg/ml), and Primaricin (1.2μg/ml) (Sigma-Aldrich, Missouri, USA). Treatment group 3 was composed of the following antibiotics: Erythromycin (1mg/ml) and Primaricin (1.2μg/ml) (Sigma-Aldrich, Missouri, USA). Treatment group 4 (CON) consisted of sterile water. Across the four treatment groups the antibiotics were administered in a volume of 200 ml of sterile water, which was changed twice weekly for a period of 2 weeks.

2.11 Weekly Data Collection

Weight and, food and water consumption data collection occurred twice weekly, during cage maintenance. Two minute handling took place at the same time.

2.12 Behaviour Testing

Mice were tested in the elevated plus maze 2 w after commencing treatment

administration with the exception of treatment group 1 of the Balb/c mice, which performed the test 10 days from commencing the treatment. This was due to complications caused by the antibiotic concentration, which proved to be too high. It was later modified as mentioned above in Section 2.3 for the C57BL/6 cohort.

2.12.1 Elevated Plus Maze

Mice were tested in the elevated plus maze apparatus as described above in section 2.5.3.

2.13 Intestinal Permeability

Two days after the elevated plus maze test permeability of the small intestine was measured *in vivo* via gavage of a fluorescent probe, specifically fluorescein isothiocyanate (FITC) (F1906; Invitrogen, Eugene, OR) as quantification of its recovery in mouse serum. Mice were fasted without food, but allowed water, for 3 hours prior to gavage. Mice were administered 200 μl of 1.25 mg/ ml FITC solution by gavage technique. Weight measurements were recorded, which were later used for calculating intestinal permeability.

2.14 Tissue Collection

Tissue collection was completed 3 hours later from administration of FITC solution when blood was collected from each mouse.

2.15 Insulin and Leptin Analysis

Serum harvested at endpoint from experimental animals of control, treatment 2 and treatment 3 groups of both Balb/C and C57BL/6 mice were kindly processed by Robyn Mackenzie for insulin and leptin levels using the Mouse Adipokine Magnetic Bead Panel 96-well Plate Assay (MILLIPLEX MAP, Billerica, MA, USA; Cat. # MADKMAG-71K). The immunoassay was run in duplicate for each serum sample. Plate was washed using 200 μl Assay Buffer per well, sealed and mixed on shaker for 10 mins at room temperature (RT), followed by decanting. 10 μl of standards or controls were added into the appropriate wells, followed by 10 μl of Assay Buffer into the background and sample wells. 10 μl of Serum Matrix was added to background, standards, and control wells. 10 μl of sample were added to sample wells, finally followed by 25 μl Beads to each well. The plate was sealed, covered with foil, and incubated on the plate shaker overnight at 4°C. Well contents were removed and the plate was washed three times with 200 μl Wash Buffer per well. 50 μl of Detection Antibodies were added to each well, followed by a 30 min incubation period at RT. Following, 50 μl Streptavidin-Phycoerythrin were added per well, and left to incubate for another 30 min at RT. Well contents were removed and the plate was washed three times with 200 μl Wash Buffer per well. 100 μl Sheath Fluid was added to each well and the plate was read using the Bio-Plex 200 System (Luminex xMAP Technology - BIO-RAD, Hercules, CA) connected to a computer on which data was analyzed using Bio-Plex Manager 4.1.1 (BIO-RAD, Hercules, CA).

2.16 Cytokine Analysis

Serum harvested at endpoint from experimental animals of control, treatment 2 and treatment 3 groups of both Balb/C and C57BL/6 mice were kindly processed by Marg Coote for Interleukin (IL)- 1α, IL-1β, IL-6, IL-10 and TNF-α levels using the Mouse Cytokine/Chemokine Magnetic Bead Panel 96-well Plate Assay (MILLIPLEX MAP, Billerica, MA, USA; Cat. # MCYTOMAG-70K, MCYTOMAG-70K-PMX/ MCYTMAG-70K-PX32). The immunoassay was run in duplicate for each serum sample. Plate was washed using 200 µl Assay Buffer per well, sealed and mixed on shaker for 10 mins at room temperature (RT), followed by decanting. 25 µl of standards or controls were added into the appropriate wells, followed by 25 µl of Assay Buffer into the background and sample wells. 25 µl of appropriate matrix solution was added to background, standards, and control wells. 25 µl of sample were added to sample wells, finally followed by 25 µl Beads to each well. The plate was sealed, covered with foil, and incubated on the plate shaker overnight at 4°C. Well contents were removed and the plate was washed two times with 200 µl Wash Buffer per well. 25 µl of Detection Antibodies were added to each well, followed by a 1 hour incubation period at RT. Following, 25 µl Streptavidin-Phycoerythrin were added per well, and left to incubate for another 30 min at RT. Well contents were removed and the plate was washed two times with 200 µl Wash Buffer per well. 150 µl Sheath Fluid was added to each well and the plate was read using the Bio-Plex 200 System (Luminex xMAP Technology - BIO-RAD, Hercules, CA) connected to a computer on which data was analyzed using Bio-Plex Manager 4.1.1 (BIO-RAD, Hercules, CA).

2.17 Data Analysis

All data was analyzed using SPSS (Armonk, NY, USA). Body weight measurements and, water and food consumption statistical analysis was conducted using repeated measured three-way ANOVA tests with age, sex and antibiotic treatment as factors. Furthermore, statistical analyses of behavioural testing were conducted using three-way ANOVA tests with test, sex and antibiotic treatment as factors. Statistical analyses of intestinal permeability, gut hormone and cytokine levels were conducted using two-way ANOVAs with antibiotic treatment and strain as factors. Additional pair-wise two-tailed t-tests were conducted on all data sets. Statistical significance was set at $p<0.05$.

3. Results

Study #1 – Rx1

3.1 Body Weight + Water and Food Consumption

Body weight measurements of mice were recorded biweekly from weaning until tissue collection age. Figure 1 displays body weight measurements for female (A) and male (B) mice. A significant effect of age ($F_{5.3, 224}$=1084.2, p<0.0005) and an age by sex interaction ($F_{5.3, 224}$=54.4, p<0.0005) on weight gain was observed. A significant effect of sex was also observed ($F_{1, 42}$=240.8, p<0.0005) and a sex by treatment interaction was observed for weight gain ($F_{1,42}$=3.30, p=0.047). Sex differences in body weight (age and treatment matched) were observed between male and female CON mice from P28 onwards (p<0.05); between male and female RX4 mice from P28 onwards (p<0.05); and between male and female RX8 mice from P21 onwards. There were no significant effects of treatment on weight gain.

Water and food measurements were recorded biweekly in order to observe consumption trends for both female and male mice from weaning until tissue collection age. Figure 2 displays water consumption per day for female (A) and male (B) mice. Values for water consumption per day were generated by summing the biweekly measurements recorded for each cage of mice and dividing the sum by seven. A significant effect of age ($F_{3.3,162}$=2.69, p=0.049) and an age by sex interaction ($F_{3.3, 162}$=36.2, p=0.023) on water consumption was observed. However, no significant effects of sex or treatment were observed on water consumption so further pairwise analysis was not performed.

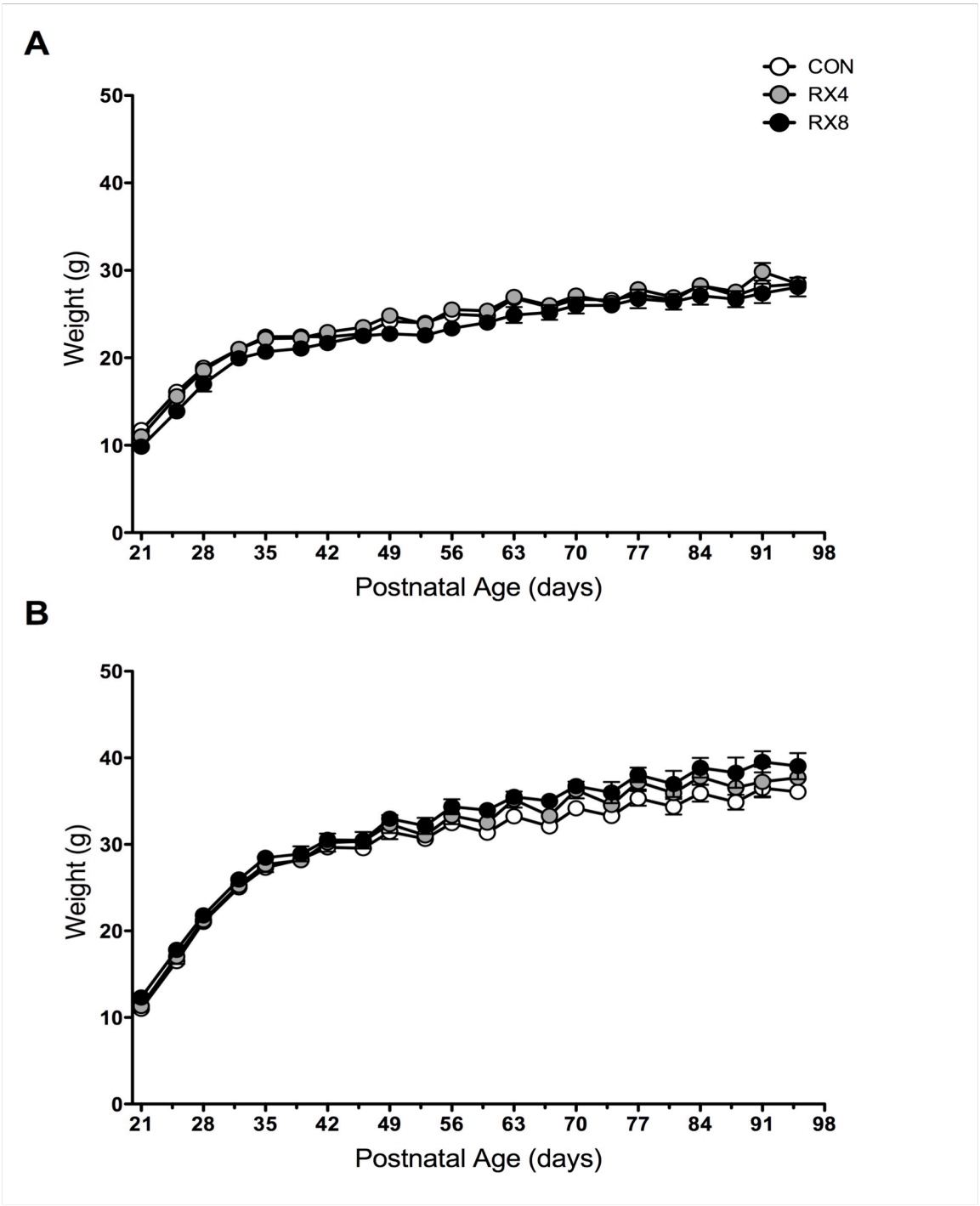

Figure 1. Body weights were measured for female (A) and male (B) mice biweekly from weaning age until tissue collection. A. Female mice showed no difference in body weight between treatment groups. B. Male mice showed no difference in body weight between treatment groups. Data is presented as mean +/- S.E.M.

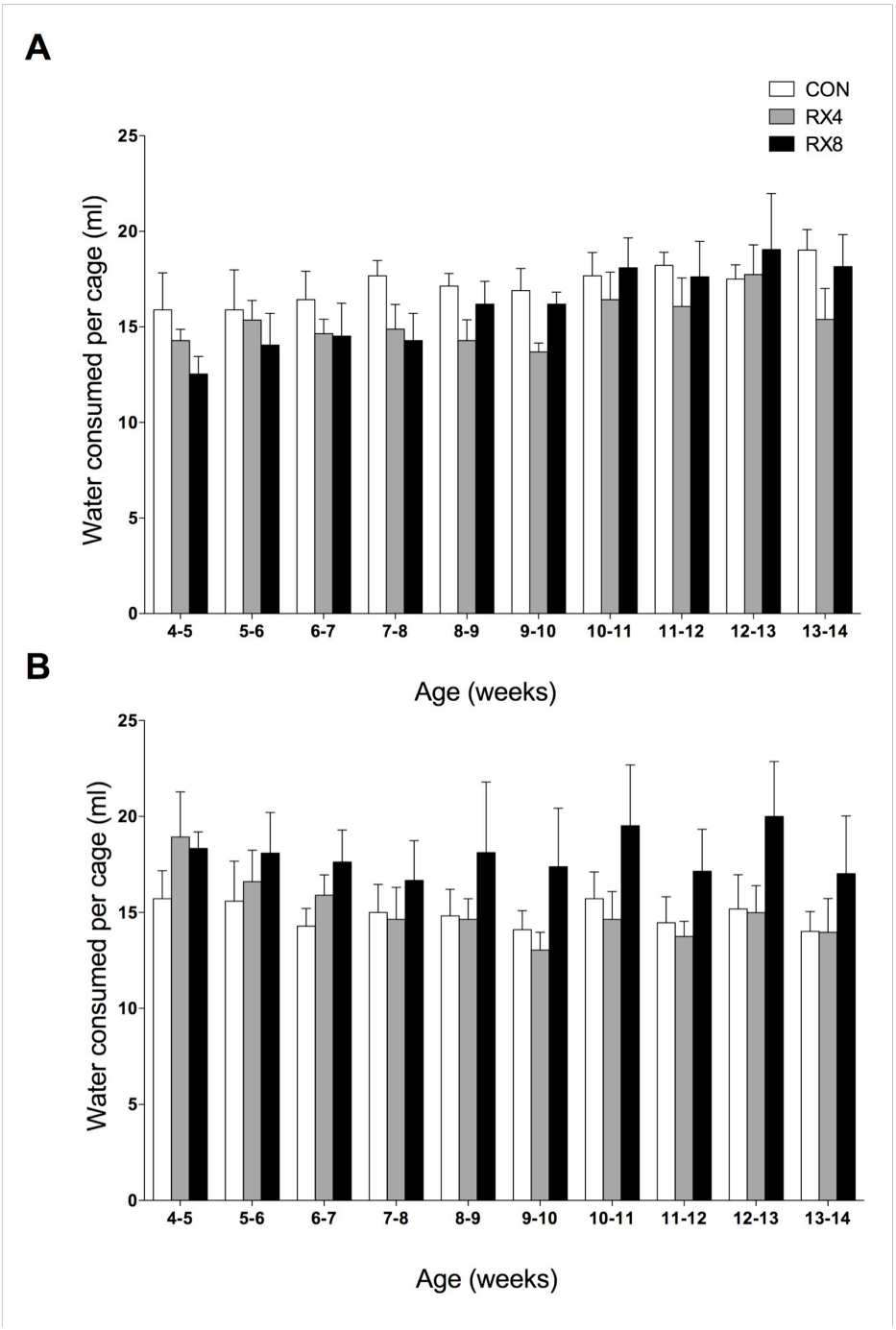

Figure 2. Water consumption was measured for the female (A) and male (B) mice biweekly from weaning age until tissue collection. Values for water consumption per day were generated by summing the biweekly measurements recorded for each cage of mice and dividing the sum by seven. No differences were observed between treatment groups. Data is presented as mean +/- S.E.M.

Figure 3 displays food consumption per day for female (A) and male (B) mice. Values for food consumption per day were generated by summing the biweekly measurements recorded for each cage of mice and dividing the sum by seven. A significant effect of age on food consumption was observed ($F_{3.69,162}$=2.73, p=0.04). A significant effect of sex was also observed ($F_{1, 18}$=5.04, p=0.038). Sex differences in food consumption were determined by independent t-test (age and treatment matched groups). Male CON mice had increased food consumption compared to female CON during week 4 and week 5 ($p<0.05$). Male RX4 mice had increased food consumption compared to female RX4 mice during week 6 ($p<0.05$). Male RX8 mice had increased food consumption compared to female RX8 mice during week 4 ($p<0.05$), however, this observation is comparable to the above-noted difference in CON mice as treatment started in this group at 8 weeks of age. As shown in Fig. 1 and noted above, these small changes in feeding did not impact weight gain during this period.

3.2 Open Field

Open field data collected at P25 were analyzed by repeated measures ANOVA with time as a within subject repeated factor, and sex and treatment as between subject factors. This analysis revealed a significant effect of time, but no significant effect of sex or treatment. It is possible that this study is not sufficiently powered to detect differences with 3 factors. Next, open field data collected at P25 was analyzed for male mice and female mice independently using repeated measures ANOVA with time as a within subject repeated factor and treatment as a between subject factor. Figure 4 displays

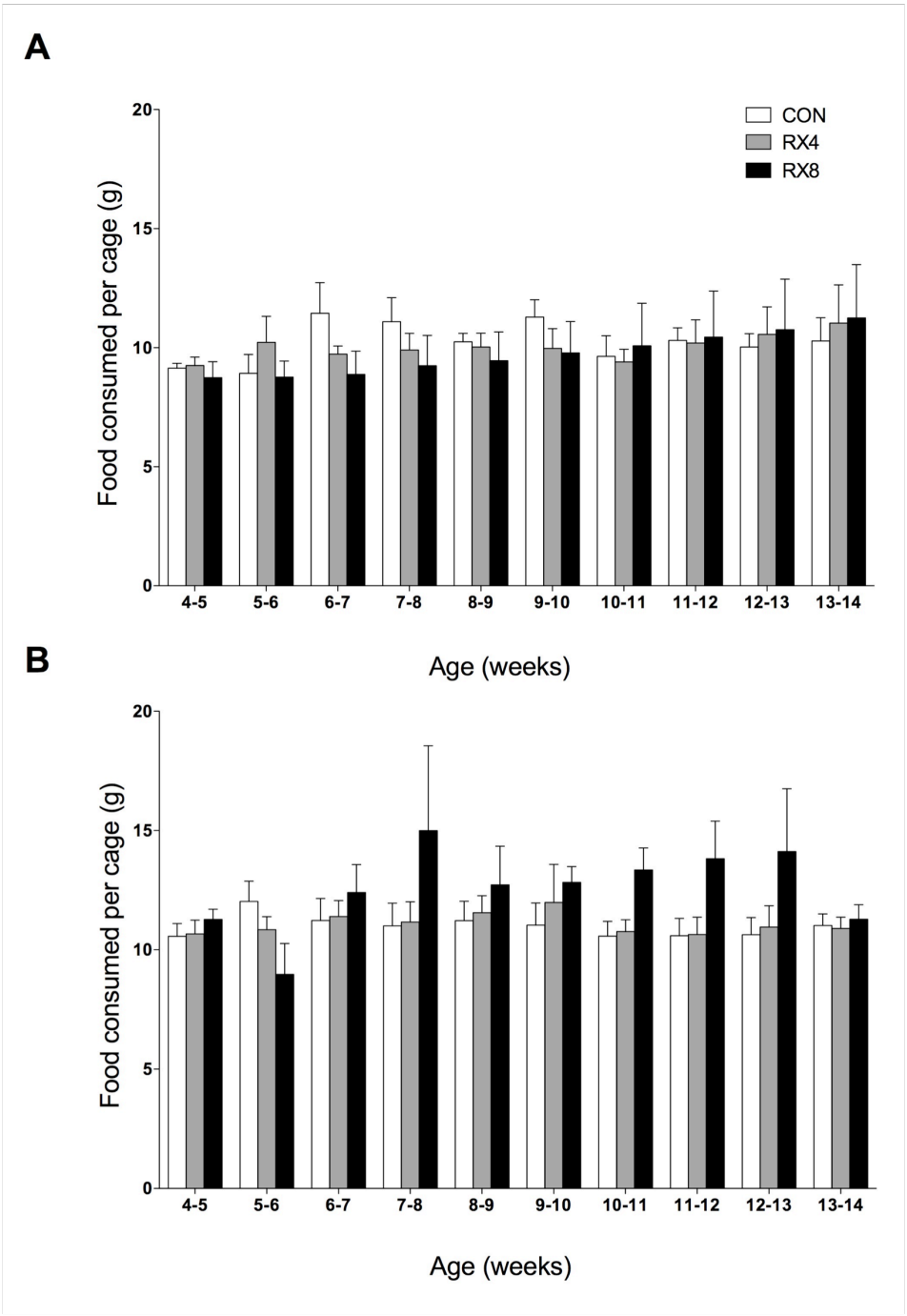

Figure 3. Food consumption was measured for the female (A) and male (B) mice biweekly from weaning age until tissue collection. Values for food consumption per day were generated by summing the biweekly measurements recorded for each cage of mice and dividing the sum by seven. No differences were observed between treatment groups. Data is presented as mean +/- S.E.M.

distance travelled (A), amount of time spent in the center of the enclosure (B) and rearing behaviour (C) exhibited by female mice in the open field test at P25. Data is presented at 5 min intervals over the 30 min test period. Habituation was evident in distance travelled over the 30 min testing period (Fig. 4A), and a significant effect of time on distance travelled was observed ($F_{4.1, 95}$=27.7, p<0.0005). No differences were observed between treatment groups in time spent in the center of the enclosure or rearing for female mice (Fig. 4B and 4C). Figure 5 displays distance travelled (A), amount of time spent in the center of the enclosure (B) and rearing behaviour (C) exhibited by male mice in the open field test at P25. Data is presented at 5 min intervals over the 30 min test period. A significant effect of time ($F_{5,95}$=40.8, p<0.0005) and a significant effect of treatment ($F_{2,19}$=4.98, p=0.018) was observed in male mice for distance travelled. Pairwise comparisons revealed increased locomotor activity during the first 10 min of the testing period in RX8 male mice compared to CON male mice (5 min, t=3.89, p=0.002; 10 min, t=2.48, p=0.29). This was an unexpected difference, as treatment had not begun at this time. No differences were observed for time spent in the center of the enclosure between groups. For rearing, a significant effect of time was observed ($F_{5,95}$=4.83, p=0.001) but no effect of treatment on rearing was observed in male P25 mice.

Open field data collected at P70 were analyzed by repeated measures ANOVA with time as a within subject repeated factor, and sex and treatment as between subject factors. This analysis revealed a main effect of time, but no significant effect of sex or treatment, similar to P25 data analysis noted above. Next, open field behaviour was

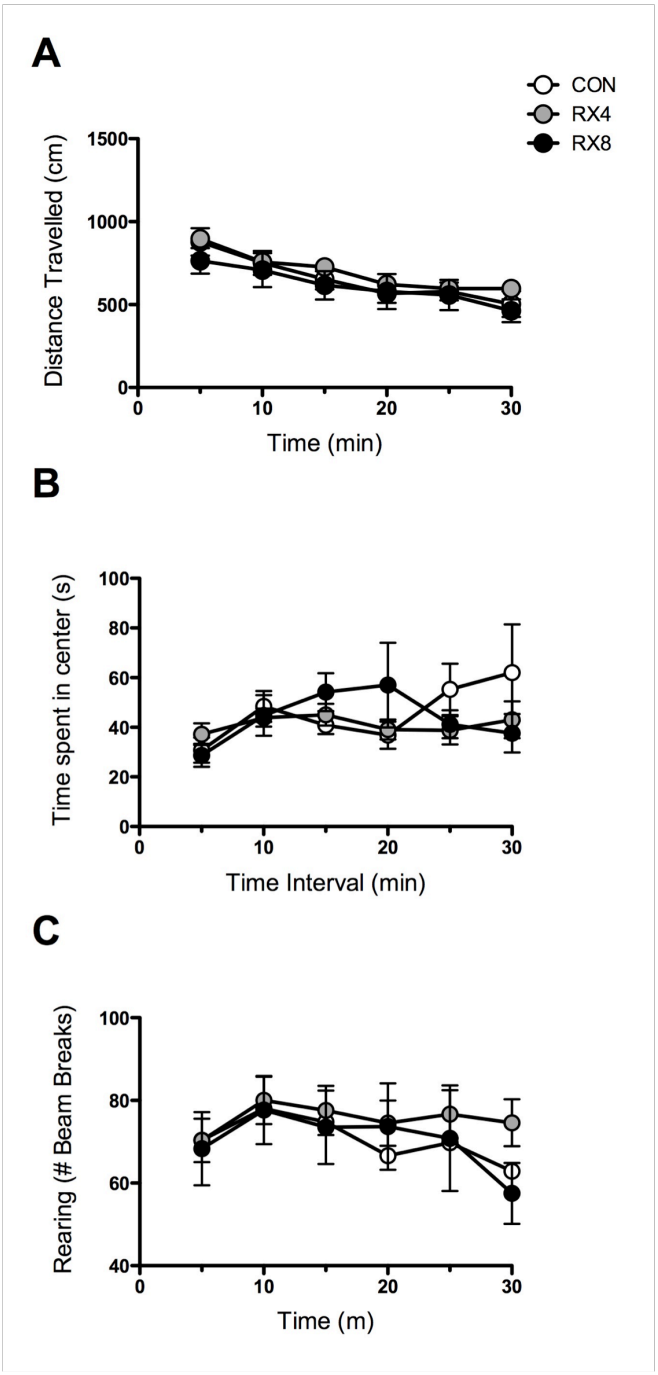

Figure 4. Locomotor activity was measured in female mice at P25 in the open field. Total distance traveled (A), duration of time spent in the center of the activity chamber (B) and rearing behaviour (C) are shown at 5 min intervals for 30 min testing period. No difference were observed between treatment groups in any of the locomotor measurements. Data is presented as mean +/- S.E.M.

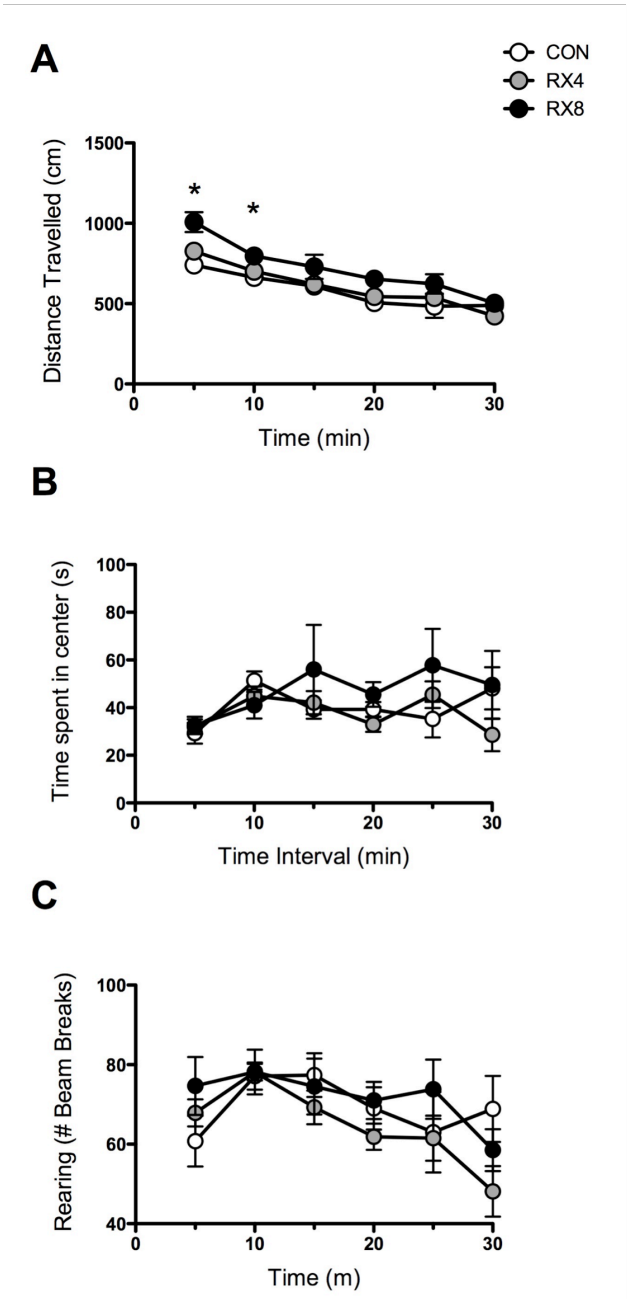

Figure 5. Locomotor activity was measured in male mice at P25 in the open field. Distance traveled (A), duration of time spent in the center of the activity chamber (B) and rearing behaviour (C) are shown at 5 min intervals for 30 min testing period. Increased activity during the first 10 min of the test was observed in RX8 male mice compared to CON male mice (*). No difference were observed between treatment groups for time spent in the center (B) or rearing (C). Data is presented as mean +/- S.E.M., *$p<0.05$.

analyzed for male mice and female mice independently using repeated measures ANOVA with time as a within subject repeated factor and treatment as a between subject factor. Figure 6 displays total distance travelled (A), amount of time spent in the center of the enclosure (B) and rearing behaviour (C) exhibited by female mice in the open field test at P70. Data is presented at 5 min intervals over the 30 min test period. For distance travelled, a significant effect of time was observed ($F_{3.2, 74}$=50.2, p<0.0005). As shown in Fig.6 A, mice habituated to the open field over the 30 min test period. No effect of treatment on distance travelled was observed in female mice (Fig. 6A). A significant effect of treatment was observed for center time ($F_{4.5, 187}$=116.7, p<0.0005). Specifically, female RX8 mice spent more time in the center of the activity chamber than female CON mice at the 15 min (t=2.89 p=0.014) and 30 min (t=3.07, p=0.01) time intervals. No significant differences were detected between CON and RX4 groups. Rearing behaviour of female mice did not differ between treatment groups at P70, however there was a significant effect of time on rearing ($F_{4.8, 110}$=12.4, p<0.0005) and a significant interaction between time and treatment ($F_{9.6, 110}$=2.93, p=0.003). No pairwise (time interval matched) differences in rearing were found between CON and RX4 or CON and RX8 female mice.

Figure 7 displays total distance travelled (A), amount of time spent in the center of the enclosure (B) and rearing behaviour (C) exhibited by male mice in the open field test at P70. Data is presented at 5 min intervals over the 30 min test period. Distance travelled by male mice in the activity chambers did not differ between treatment groups at P70, yet a time effect was observed for the total distance travelled by males ($F_{4.5, 85}$=69.6, p<0.0005). As shown in Fig. 7A, mice habituated to the testing apparatus over the 30 min

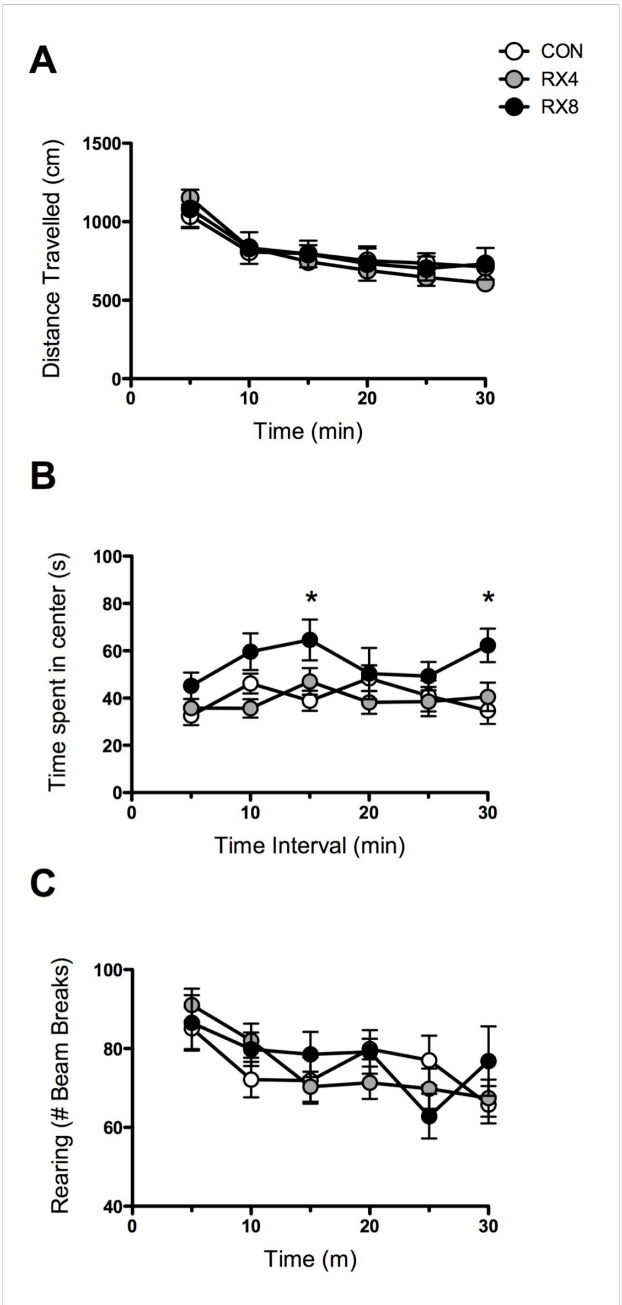

Figure 6. Locomotor activity was measured in female mice at P70 in the open field. Total distance traveled (A), duration of time spent in the center of the activity chamber (B) and rearing behaviour (C) are shown at 5 min intervals for 30 min testing period. B. During the 3rd and 6th time intervals Rx8 mice spent significant more time in the centre of the enclosure compared to CON mice (*). This indicates that Rx8 female mice exhibit reduced anxiety-like behaviour. No difference were observed between treatment groups in the other two locomotor measurements. Data is presented as mean +/- S.E.M., *$p<0.05$.

testing period. No differences in amount of time spent in the center of the open field activity chamber by male mice at P70 were observed between treatment groups. Rearing behaviour of male mice did not differ between treatment groups at P70, however a significant effect of time was observed ($F_{5, 95}$=17.6, p<0.0005), seen in Fig. 7C as habituation in all treatment groups over the 30 min testing period.

Overall, antibiotic treatment did not alter locomotor or exploratory behaviour in male or female CD1 mice. Treatment with ampicillin starting at 8 weeks of age reduced anxiety-like behaviour in female CD1 mice as shown by increased time spent in the center of the open field compared to control female mice. Treatment did not affect anxiety-like behaviour in the open field test in male mice. A baseline difference between randomized groups of male mice was observed in locomotor activity at P25, however this difference was not evident at P70.

3.3 Social Behaviour

Amount of time spent in the three different chambers of the social behaviour apparatus during the duration of the test is displayed in Figure 8 for female (A) and male (B) mice. A significant effect of location was observed ($F_{1.1, 45.5}$=78.7, p<0.0005), but no effect of sex or treatment was observed. Female and male mice spent significantly less time in the center chamber compared to the outer chambers. Female mice did not show a preference for the chamber that contained the stranger mouse, but instead spent similar amounts of time exploring the chamber with the empty cup as they did the chamber with the stranger mouse (Fig. 8A). Male CON mice did not show a preferences for the stranger

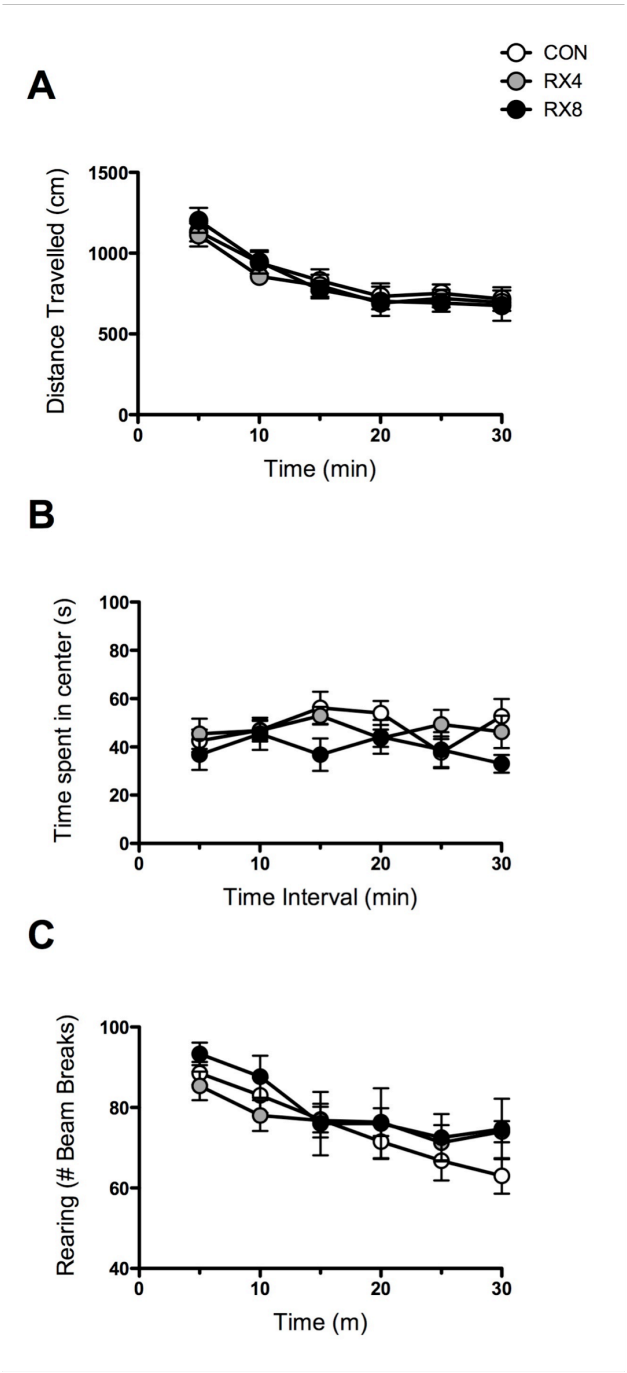

Figure 7. Locomotor activity was measured in male mice at P70 in the open field. Total distance traveled (A), duration of time spent in the center of the activity chamber (B) and rearing behaviour (C) are shown at 5 min intervals for 30 min testing period. No difference were observed between treatment groups in any of the open field measurements. Data is presented as mean +/- S.E.M.

mouse compared to empty cup, however, male RX4 ($t=5.2$, $p<0.0005$) and RX8 ($t=3.0$, $p=0.014$) mice showed a significant preference for the stranger mouse compared to the empty cup (Fig. 8B), suggesting increased sociability in these groups of mice.

3.4 Elevated Plus Maze

Animals were tested in the elevated plus maze (EPM), which measured the following:, amount of time spent in the open arms, intersection and closed arms of the apparatus, number of entries into the open and closed arms, and distance travelled in open and closed arms. Figure 9 shows the time spent in each of the EPM zones for female (A) and male (B) mice. Repeated measures ANOVA with zone as a within-subject factor and sex and treatment as between subject factors showed a significant effect of zone location ($F_{1.3,56.5}=570.6$, $p<0.0005$). All mice spent the most time in the closed arms and the least time in the open arms, with a mid-level time spent in the intersection of the maze (Fig. 9). No effect of sex or treatment on time spent in the different EPM zones was observed.

Both female (Fig. 10A) and male (Fig. 10B) mice showed a significant effect of zone on the number of entries into the open and closed arms of the EPM ($F_{1,42}=178.9$, $p<0.0005$). No significant effect of sex was observed on zone entries. No differences between treatment groups were observed for open arm entries, however, a significant decrease in the number of closed arm entries in male RX4 mice compared to control male mice (Fig. 10B, $t=2.4$, $p=0.029$) was observed.

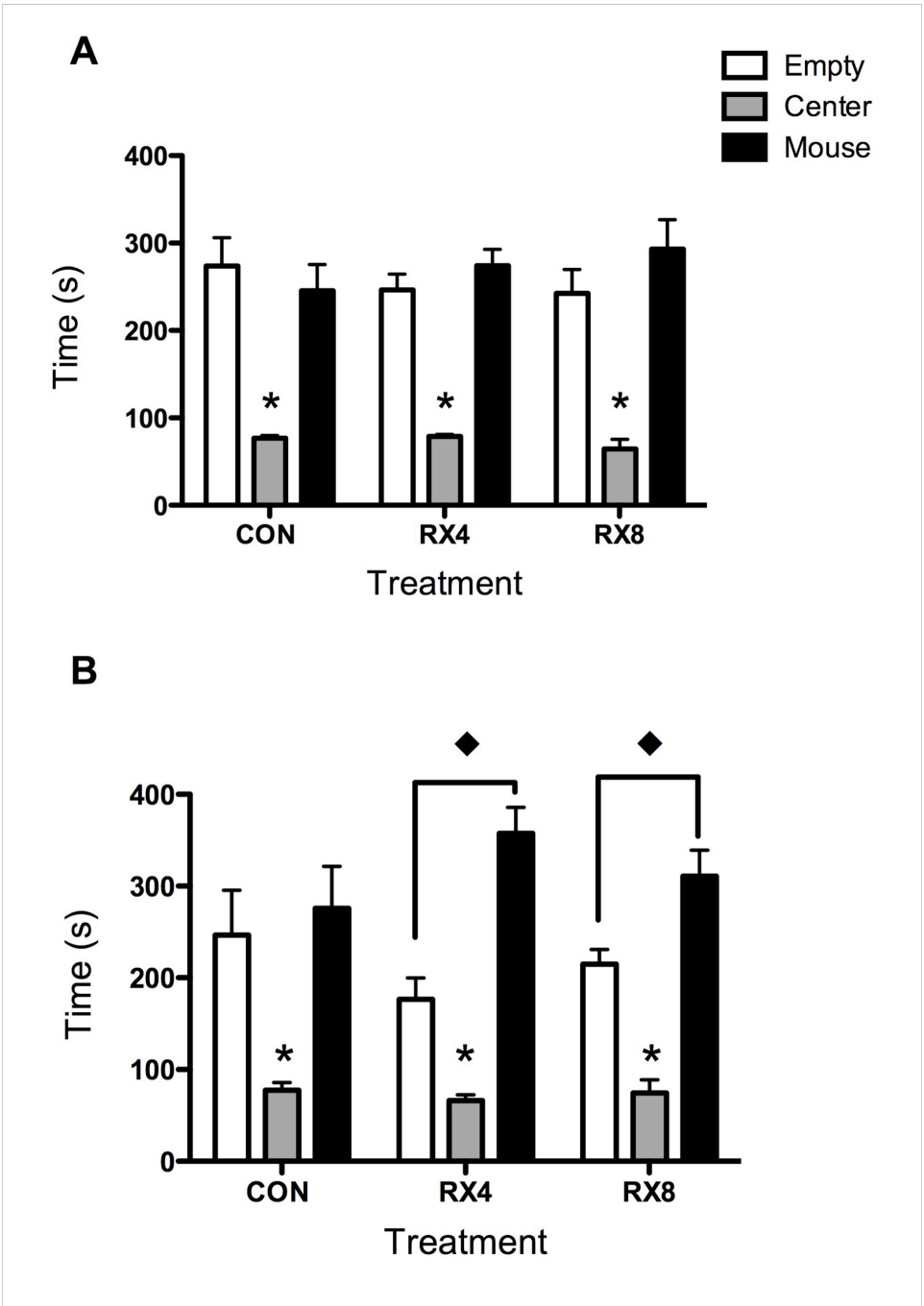

Figure 8. Social behaviour was measured in female (A) and male (B) mice in the three-chamber social behaviour apparatus. In all treatment groups, both female and male mice spent significantly more time in the empty and mouse chambers compared to the center chamber, *$p<0.05$. Male RX4 mice spend significantly more time in the mouse chamber compared to the amount of time spent in the empty cup chamber, ♦$p<0.05$ Data is presented as mean +/- S.E.M..

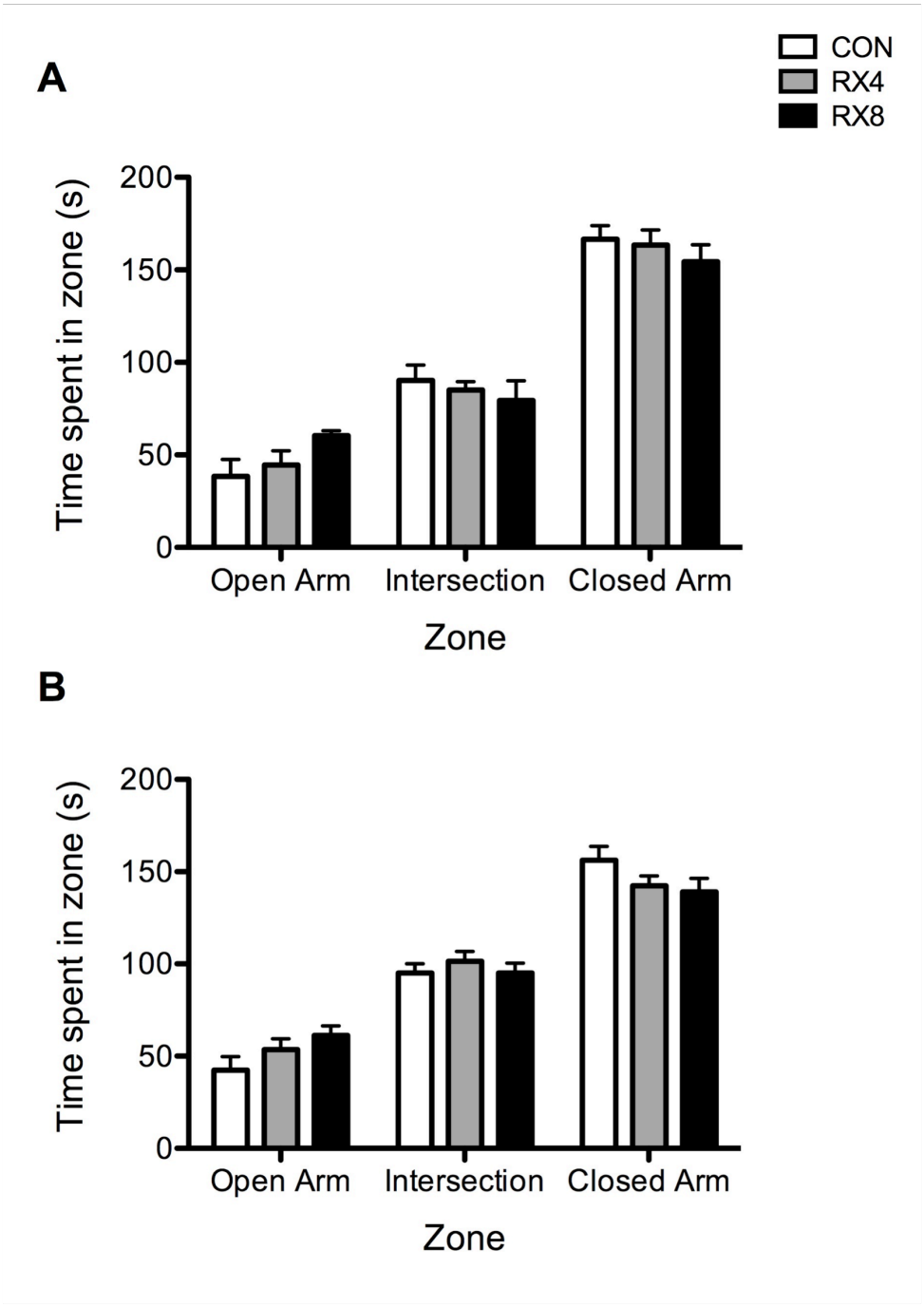

Figure 9. Amount of time spent in the different zones of the elevated plus maze (EPM) by female (A) and male (B) mice were measured. No effect of treatment was observed on the amount of time spent in the zones of the EPM by female or male mice. However, both female and male mice showed a preference for the closed arms of the EPM. Values are means +/- S.E.M.

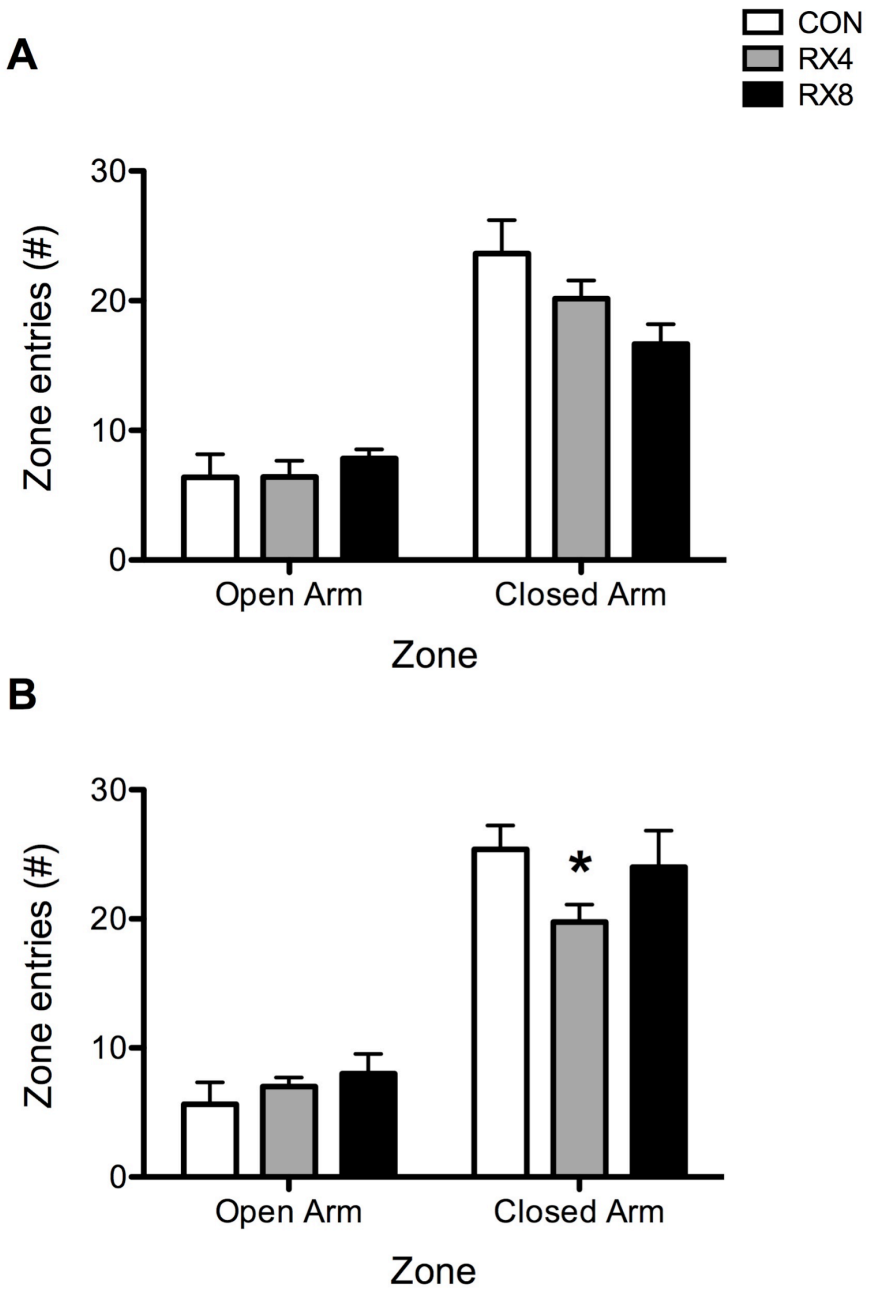

Figure 10. Number of entries into the open and closed arms of the elevated plus maze (EPM) by female (A) and male (B) mice were measured. Both female and male mice entered the closed arms of the EPM more than the open arms. No effect of treatment was observed on the number of entries into the open arms of the EPM by female or male mice. Male Rx4 mice entered the closed arms fewer times compared to sex matched controls (*). Values are means +/- S.E.M., *$p<0.05$.

Female (Fig. 11A) and male (Fig. 11B) mice showed a significant effect of zone on distance travelled in the open and closed arms of the EPM ($F_{1,42}$=223.4, $p<0.0005$; Fig. 11). No significant effect of sex or treatment was observed on distance travelled in the EPM.

3.5 Auditory Fear Conditioning

Following the first 135 s of the auditory fear conditoning test mice were presented with a tone for the duration of 30 s. During the last 2 s of the tone presentation, a 0.5 mA floor grid shock was administered for the duration of 2 s. Figure 12 depicts total distance travelled by female (A) and male (B) mice throughout the total period for which the behaviour test was run. Data is presented at 15 s intervals over the 300 s test period. In all treatment groups, both female and male mice exhibited normal freezing behaviour upon administration of floor grid shock as seen by the decrease in distance travelled throughout the remainder of the behaviour test period. The contextual and cued trial of the auditory fear conditioning test is depicted in Figure 13 as freezing behaviour. No differences were observed between treatment groups and no effect sex was observed.

3.6 Flow Cytometric Analysis of Spleens

Analysis of mice spleens by flow cytometric analysis determined percentage levels of immune cells, T and B cells, and macrophages in spleens of control and antibiotic-treated mice (Fig. 14). The distribution of immune cell type revealed few

Figure 11. Total distance travelled in the open and closed arms of the elevated plus maze (EPM) by female (A) and male (B) mice were measured. No effect of treatment was observed on the total distance travelled in the zones of the EPM by female or male mice. However, both female and male mice travelled farther in the closed arms of the EPM compared to the open arms. Values are means +/- S.E.M.

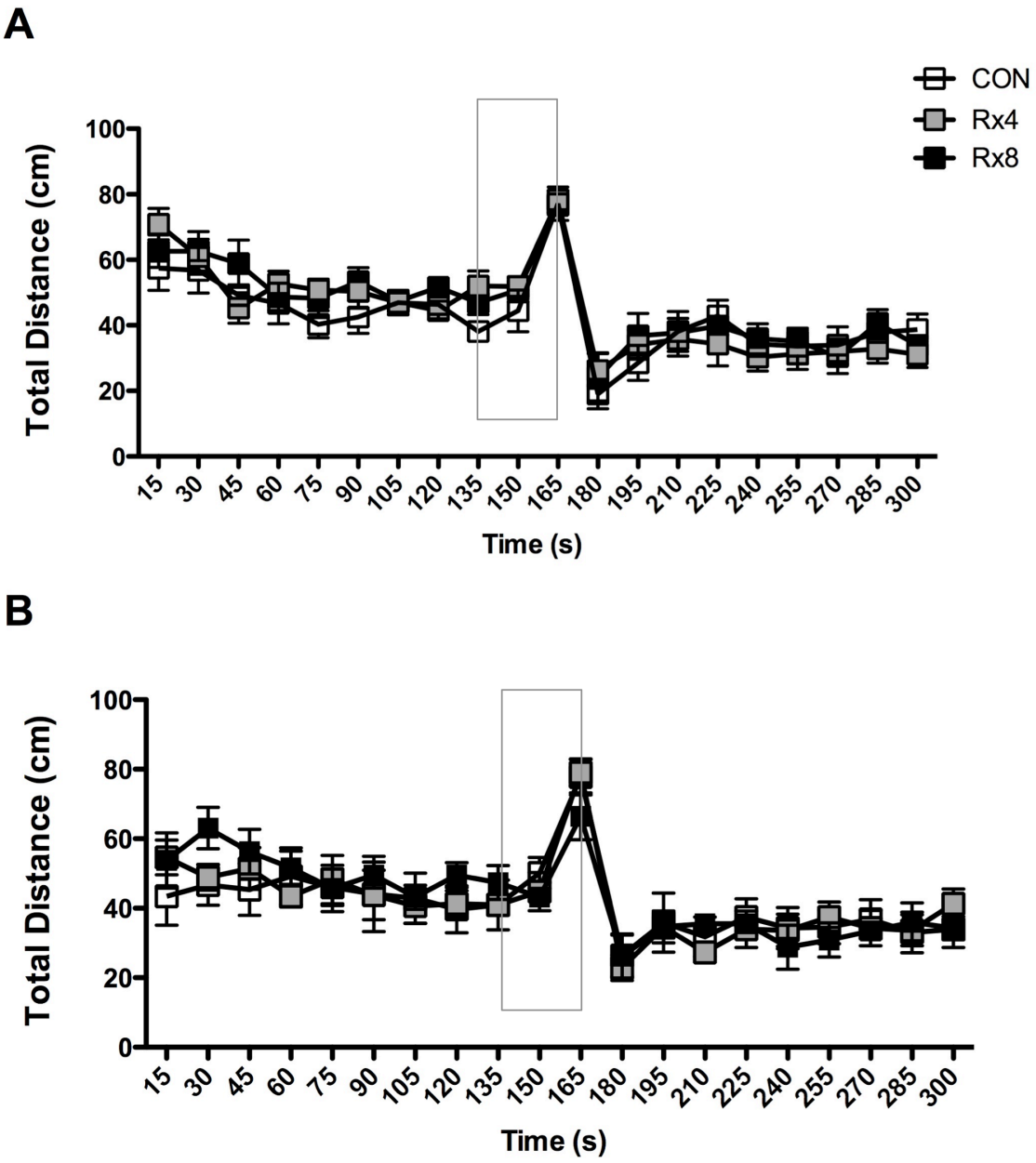

Figure 12. Total distance travelled by female (A) and male (B) mice in the activity chambers of the auditory fear conditioning test is presented at 15 s intervals over the 300 s test period. Both female and male mice show decreased distance travelled after the floor grid shock at 165 s. Neither females nor males exhibited differences between treatment between treatment groups in distance travelled in the activity chambers. Values are means +/- S.E.M.

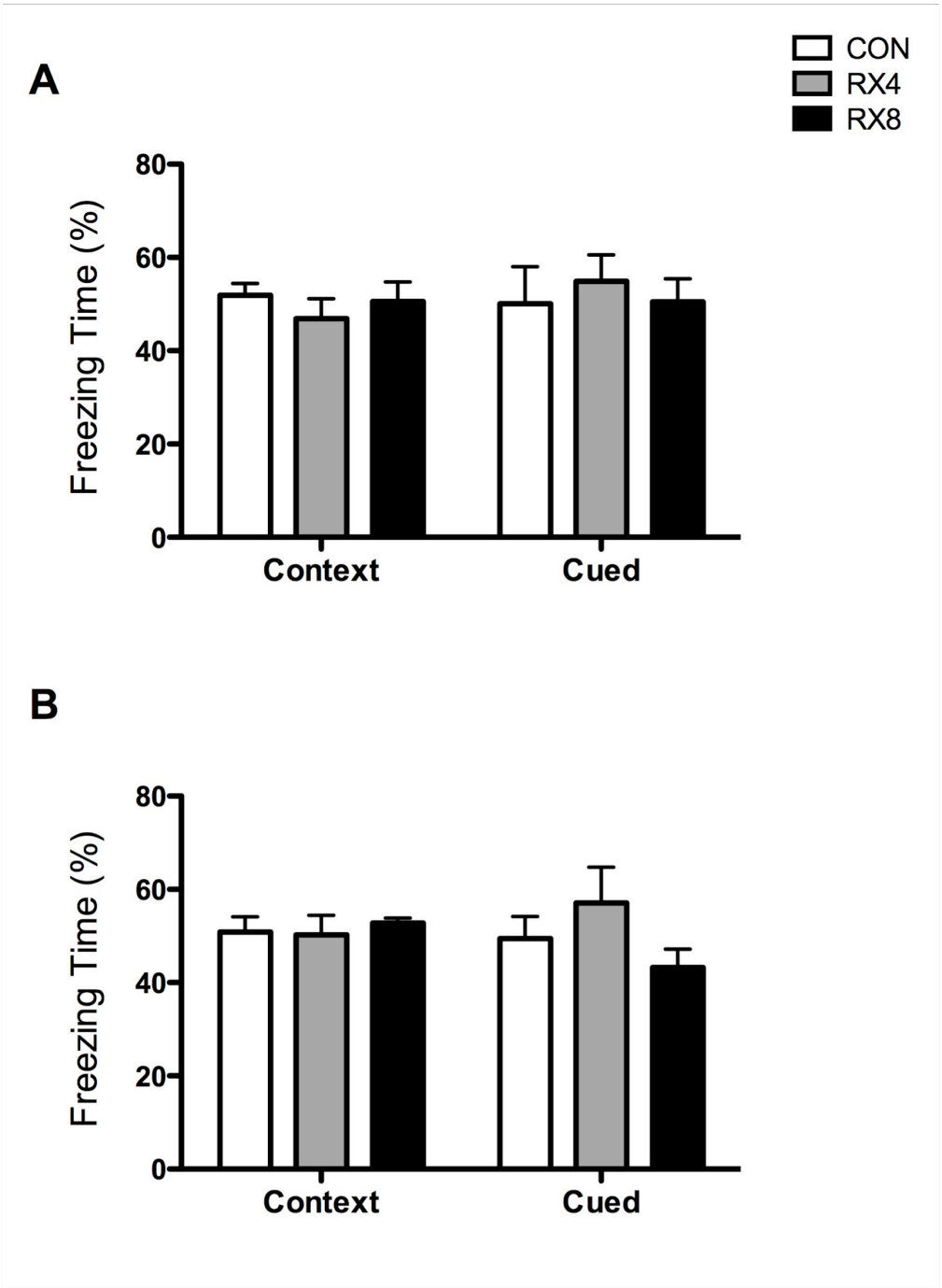

Figure 13. Percentage of time spent in freezing behaviour by female (A) and male (B) mice in the activity chambers of the auditory fear conditioning test are presented for both the contextual and cued part of the test. For both female and male mice show no effect of treatment was observed in freezing behaviour in either the contextual or cued parts of the auditory fear conditioning test. Values are means +/- S.E.M.

macrophages but high numbers of T cells and B cells in spleen. No treatment differences were observed in immune cell numbers in female (Fig. 14A) or male (Fig. 14B) mice.

Study #2 – Results

3.7 Body Weight + Water and Food Consumption

Body weight measurements of mice were recorded biweekly from the start of treatment administration until tissue collection age. Figure 15 displays body weight measurements for Balb/C (A) and C57Bl/6 (B) mice. Repeated measures ANOVA revealed a significant effect of within-subject factor, experimental day ($F_{2.7,94}$=81.6, $p<0.0005$, Fig. 15) and significant interactions between day and strain ($F_{2.7,94}$=19.8, $p<0.0005$, Fig. 15), day and treatment ($F_{8,94}$=10.6, $p<0.0005$, Fig. 15), and a significant three way interaction between day, strain, and treatment ($F_{5.4,94}$=2.7, p=0.022, Fig. 15). In addition, a significant effect of between subject factors strain ($F_{1,35}$=63.7, $p<0.0005$, Fig. 15) and treatment ($F_{3,35}$=4.5, p=0.009, Fig. 15) and a significant interaction between strain and treatment ($F_{2,35}$=7.6, p=0.002, Fig. 15) were found. Treatment differences in body weight were determined by independent t-test (strain and age matched groups). Balb/C mice of Tx1 group showed weight loss compared to CON group at day 4 (t=10.4, $p<0.0005$) and 7 (t=10.2, $p<0.0005$). Balb/C mice of Tx2 group showed weight loss compared to CON group at day 4 (t=3.2, p=0.01), 7 (t=3.5, p=0.005), 11 (t=3.6, p=0.007 and 14 (t=4, p=0.005). No differences were revealed between Balb/C mice of Tx3 and CON groups. C57Bl/6 mice of Tx1 group compared to CON group showed higher body

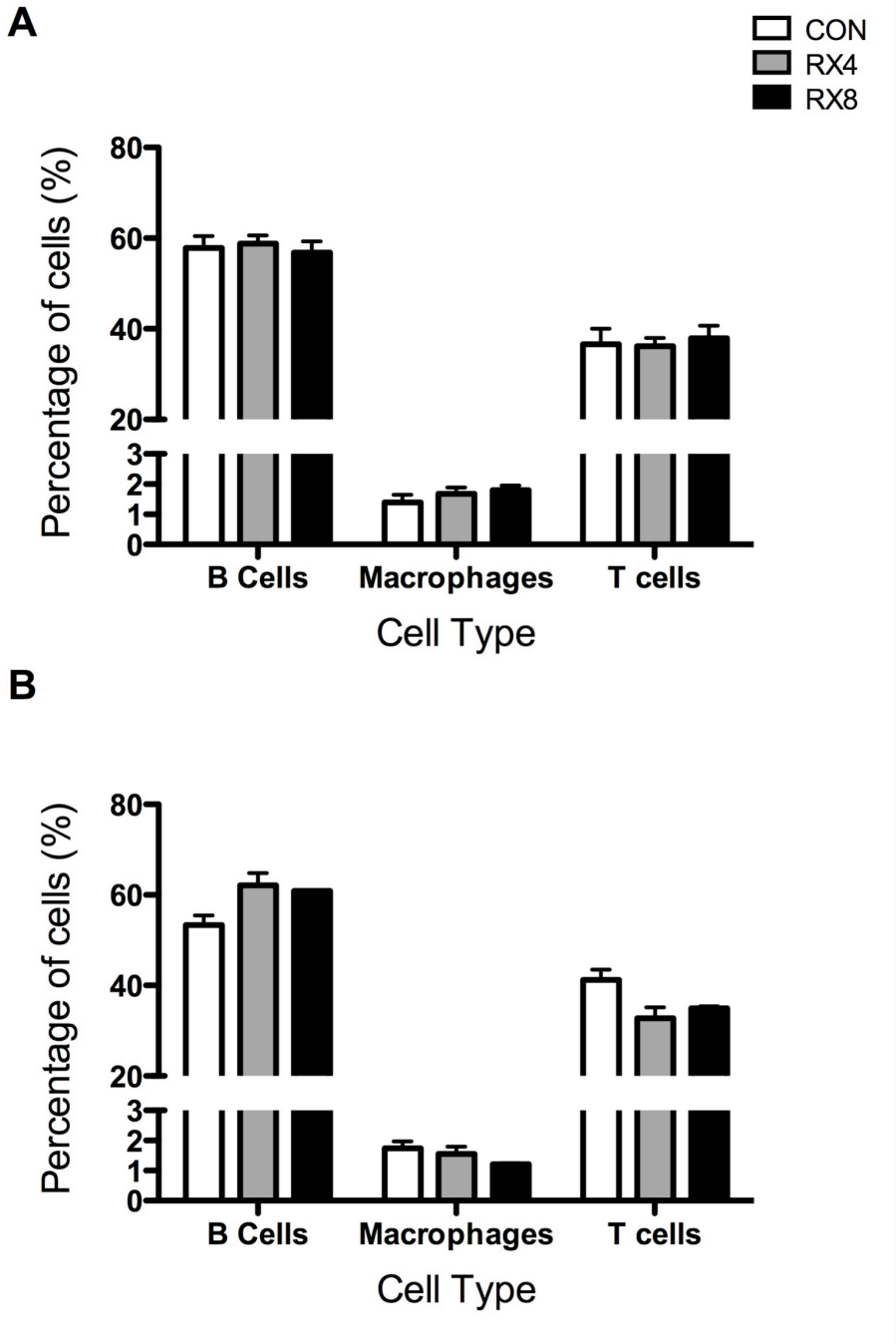

Figure 14. Percentage of immune cells for female (A) and male (B) mice were measured in spleens. The distribution of immunes cell type revealed few macrophages, but high numbers of T and B cells in both sexes. For both female and male mice show no effect of treatment was observed in number of B and T cells, and macrophages. Values are means +/- S.E.M.

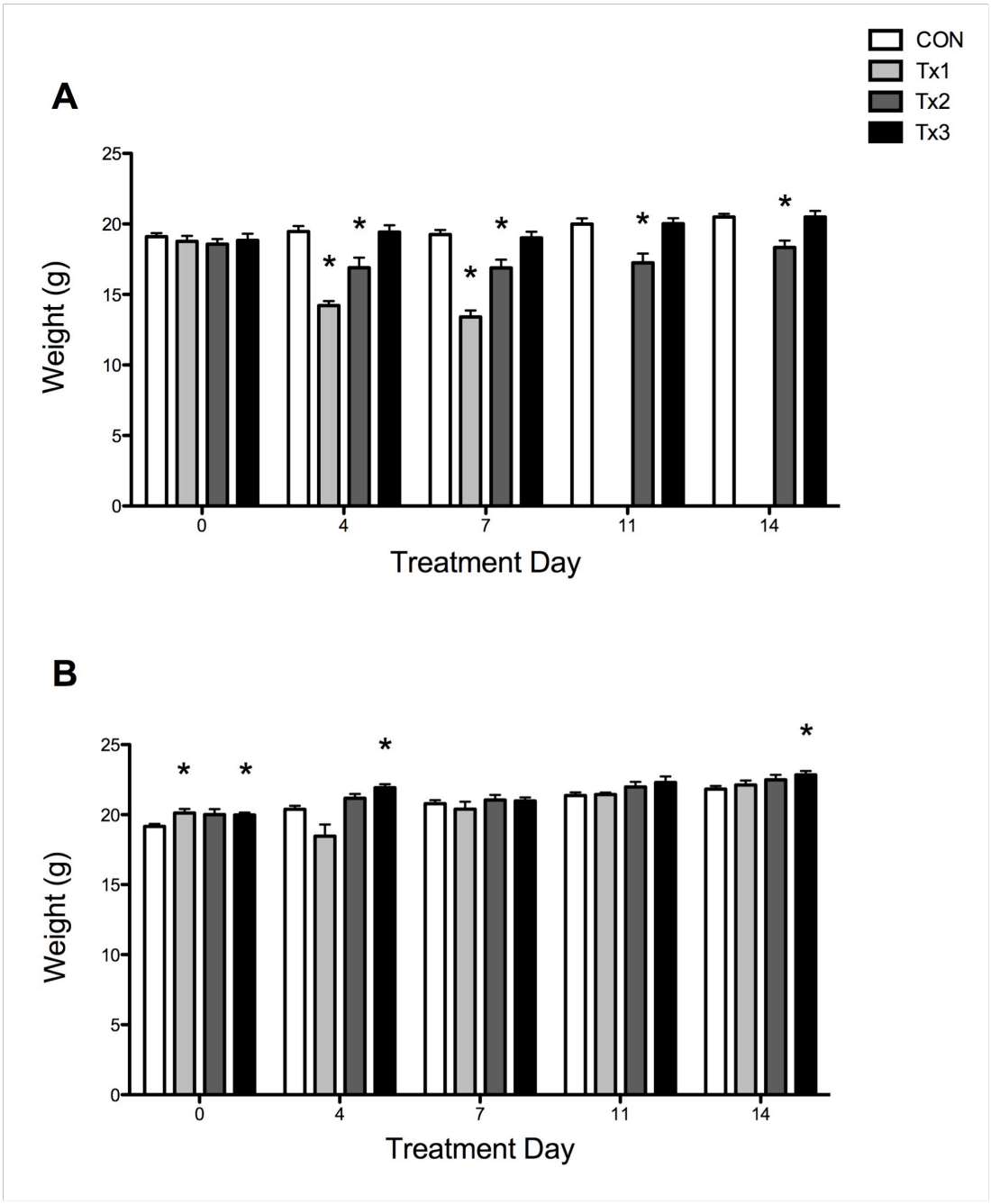

Figure 15. Body weights were measured for Balb/C (A) and C57Bl/6 (B) mice biweekly from the start of the antibiotic treatment administration until tissue collection. A. Balb/C mice of treatment 1 and 2 groups showed loss in body weight relative to controls starting on treatment day 4 (*). B. C57Bl/6 mice of treatment group 1 showed higher body weight compared to controls on treatment day 0. C57Bl/6 mice of treatment 3 group showed higher body weight relative to controls on treatment day 0, 4 and 14 (*). Data is presented as mean +/- S.E.M., *$p<0.05$.

weight on day 0 (t=2.7, p=0.023). No differences were revealed between C57Bl/6 mice of Tx2 and CON groups. C57Bl/6 mice of Tx3 group were higher in body weight compared to CON group on day 0 (t=3.4, p=0.007), 4 (t=4.4, p=0.001) and day 14 (t=2.8, p=0.02). Furthermore, an independent t-test comparison for body weight between Balb/C CON and C57Bl/6 CON groups revealed differences at day 7 (t=3.7, p=0.004), 11 (t=3.0, p=0.013) and 14 (t=4.3, p=0.002).

Water and food measurements were recorded biweekly in order to observe consumption trends for both Balb/C and C57Bl/6 mice from the start of treatment administration until tissue collection age. Values for water and food consumption per day were generated by dividing the biweekly measurements recorded for each cage of mice by the number of days since the last measurement. Figure 16 displays water consumption per day for Balb/C (A) and C57Bl/6 (B) mice. Repeated measures ANOVA revealed a significant effect of within-subject factor, experimental day ($F_{1.4,19.4}$=11.1, p=0.002, Fig. 16); with significant interactions between day and strain ($F_{1.4,19.4}$=4.2, p=0.043, Fig. 16), and day and treatment ($F_{4.2,19.4}$=4.4, p=0.01, Fig. 16). In addition, a significant effect of between subject factors strain ($F_{1,14}$=31.2, p<0.0005, Fig. 16) and treatment ($F_{3,14}$=3.9, p=0.033, Fig. 16) were found. Treatment differences in water consumption were determined by independent t-test (strain and age matched groups). No differences were revealed in Balb/C mice of Tx1 and Tx3 groups compared to the CON group. However, Balb/C mice of Tx2 group had decreased water consumption at day 4 of treatment (t=3.7, p=0.021). C57Bl/6 mice of Tx1 group compared to CON group had lower water consumption on day 4 (t=6.4, p=0.003) and 11 (t=3.2, p=0.033). No differences were

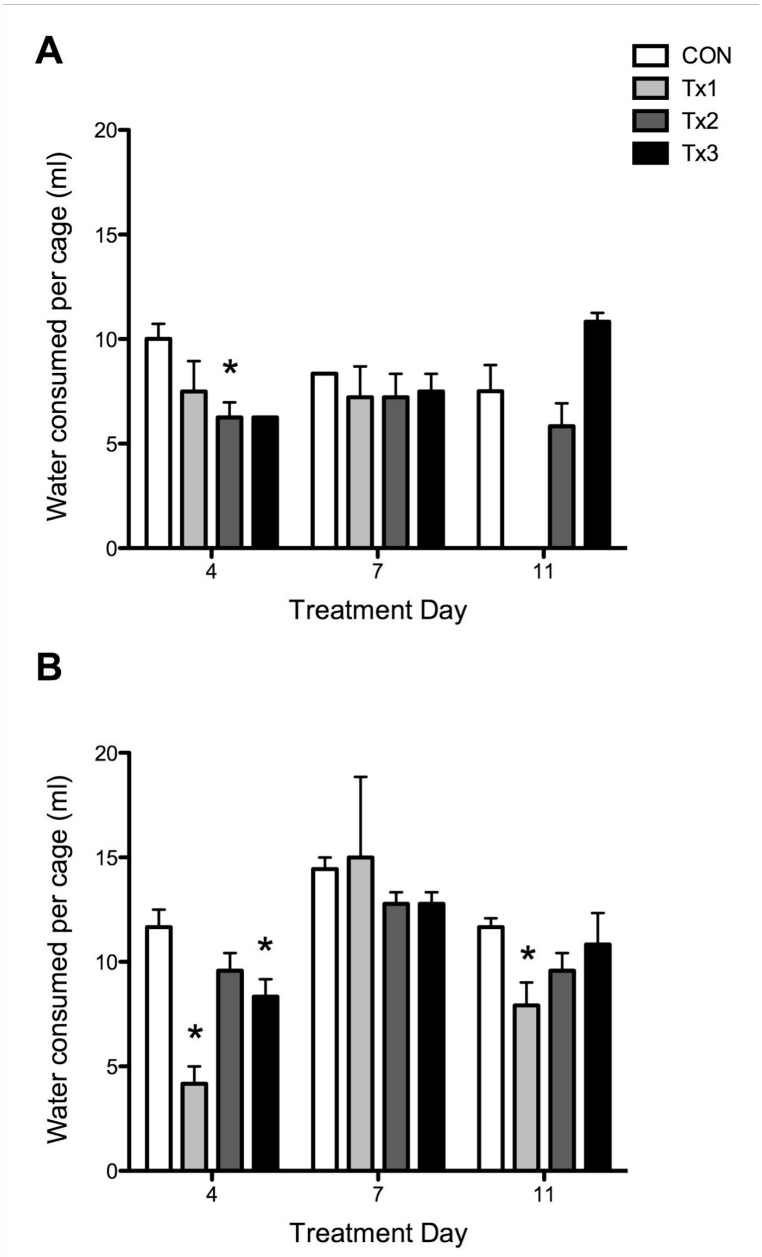

Figure 16. Water consumption was measured for Balb/C (A) and C57Bl/6 (B) mice biweekly from weaning age until tissue collection. Values for water consumption per day were generated by dividing the biweekly measurements recorded for each cage of mice by the number of days since the last measurement. A. Balb/C mice of treatment 2 group showed reduced water consumption compared to controls on treatment day 4 (*). B. C57Bl/6 mice of treatment 1 group showed reduced water consumption compared to controls on treatment day 4 and 11. C57Bl/6 mice of treatment 3 group showed reduced water consumption compared to controls on treatment day 4 (*). Data is presented as mean +/- S.E.M., *$p<0.05$.

revealed between C57Bl/6 mice of Tx2 and CON groups. C57Bl/6 mice of Tx3 group also had lower water consumption compared to CON group on day 4 (t=2.8, p=0.047). Furthermore, an independent t-test comparison for water consumption between Balb/C and C57Bl/6 CON groups revealed differences at day 7 (t=11, p=0.008) and 11 (t=3.2, p=0.034).

Figure 17 displays food consumption per day for Balb/C (A) and C57Bl/6 (B) mice. Repeated measures ANOVA revealed a significant effect of within-subject factor, experimental day ($F_{3,42}$=115.1, p<0.0005, Fig. 17); with significant interactions between day and strain ($F_{3,42}$=29.4, p<0.0005, Fig. 17), and day and treatment ($F_{9,42}$=7.3, p<0.0005, Fig. 17). A three way interaction between day, strain and treatment was also observed ($F_{6,42}$=6.7, p<0.0005, Fig. 17). In addition, a significant effect of between subject factors strain ($F_{1,14}$=83.1 , p<0.0005, Fig. 17) and treatment ($F_{3,14}$=3.9, p=0.033, Fig. 17) were found. Treatment differences in food consumption were determined by independent t-test (strain and age matched groups). Balb/C mice of Tx1 group compared to controls had decreased food consumption at treatment day 4 (t=10.5, p=0.009) and 7 (t=5.3, p=0.006). Balb/C mice of Tx2 group compared to controls had decreased food consumption at treatment day 4 (t=3.0, p=0.04) and 11 (t=3.0, p=0.04). Also, Balb/C mice of Tx3 group compared to controls had decreased food consumption at treatment day 4 (t=5.4, p=0.006). C57Bl/6 mice of Tx1 group compared to controls had decreased food consumption at treatment day 4 (t=4.9, p=0.008) and 7 (t=3.3, p=0.03). C57Bl/6 mice of Tx2 group compared to controls had decreased food consumption at treatment day 7 (t=2.9, p=0.042). C57Bl/6 mice of Tx3 group compared to controls had decreased food consumption at

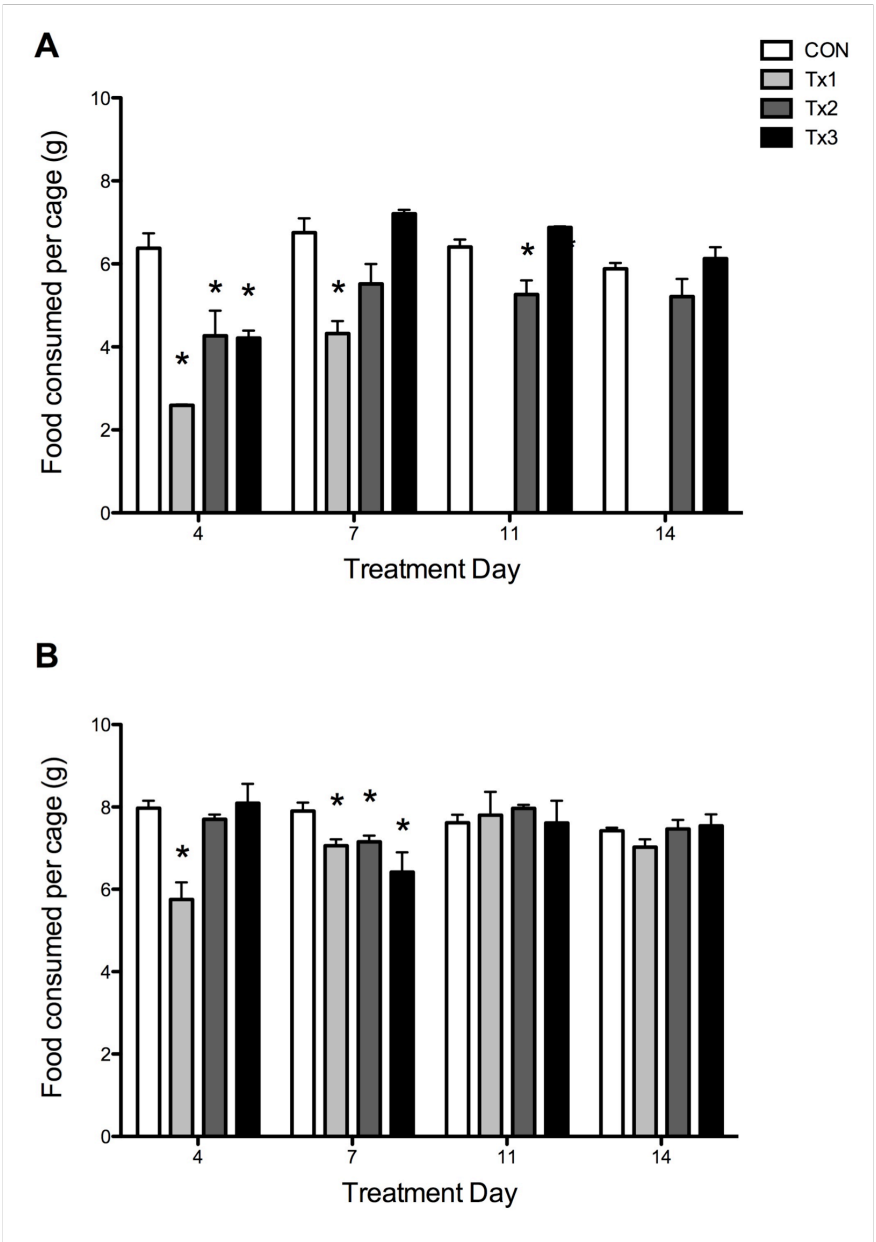

Figure 17. Food consumption was measured for Balb/C (A) and C57Bl/6 (B) mice biweekly from weaning age until tissue collection. Values for food consumption per day were generated by dividing the biweekly measurements recorded for each cage of mice by the number of days since the last measurement. A. Balb/C mice of treatment 1, 2 and 3 groups showed reduced food consumption compared to controls on treatment day 4, and also on day 7 for treatment 1 group and day 11 for treatment 2 group (*). B. C57Bl/6 mice of treatment 1 group showed reduced food consumption compared to controls on treatment day 4 (*). C57Bl/6 mice of treatment 1, 2 and 3 groups showed reduced food consumption compared to controls on treatment day 7 (*). Data is presented as mean +/- S.E.M., *$p<0.05$.

treatment day 7 (t=2.8, p=0.047). Furthermore, an independent t-test comparison for food consumption between Balb/C and C57Bl/6 control groups revealed differences at treatment day 4 (t=4.0, p=0.016), 7 (t=2.8, p=0.047), 11 (t=4.6, p=0.01) and 14 (t=9.7, p=0.001).

3.8 Elevated Plus Maze

Animals were tested in the elevated plus maze (EPM), which measured the following: amount of time spent in the open arms, intersection and closed arms of the apparatus, number of entries into the open and closed arms, and distance travelled in open and closed arms. Fig. 18 shows the time spent in each of the EPM zones for Balb/C (A) and C57Bl/6 (B) mice. Repeated measures ANOVA with zone as a within-subject factor and strain and treatment as between subject factors showed a significant effect of zone location ($F_{1.2,48.6}$=185.5, p<0.0005, Fig. 18). Interactions between location and strain ($F_{1.2,48.6}$=21.6, p<0.0005, Fig. 18), and location and treatment ($F_{3.6,48.6}$=4.4, p=0.005, Fig. 18) were observed. A three-way interaction among location, strain and treatment was also observed ($F_{3.6,48.6}$=5.1, p=0.002, Fig. 18). All mice spent the most time in the closed arms and the least time in the open arms, with a mid-level time spent in the intersection of the maze (Fig. 18). A significant effect of strain on time spent in the different EPM zones was observed ($F_{1,40}$=20.1, p<0.0005, Fig. 18). Balb/C mice of Tx1 group spent less time in the intersection of the EPM (Fig. 18A, t=2.7, p=0.033), but more time in the closed arms of the EPM (Fig. 18A, t=2.7, p=0.035), compared to Balb/C control mice. No differences

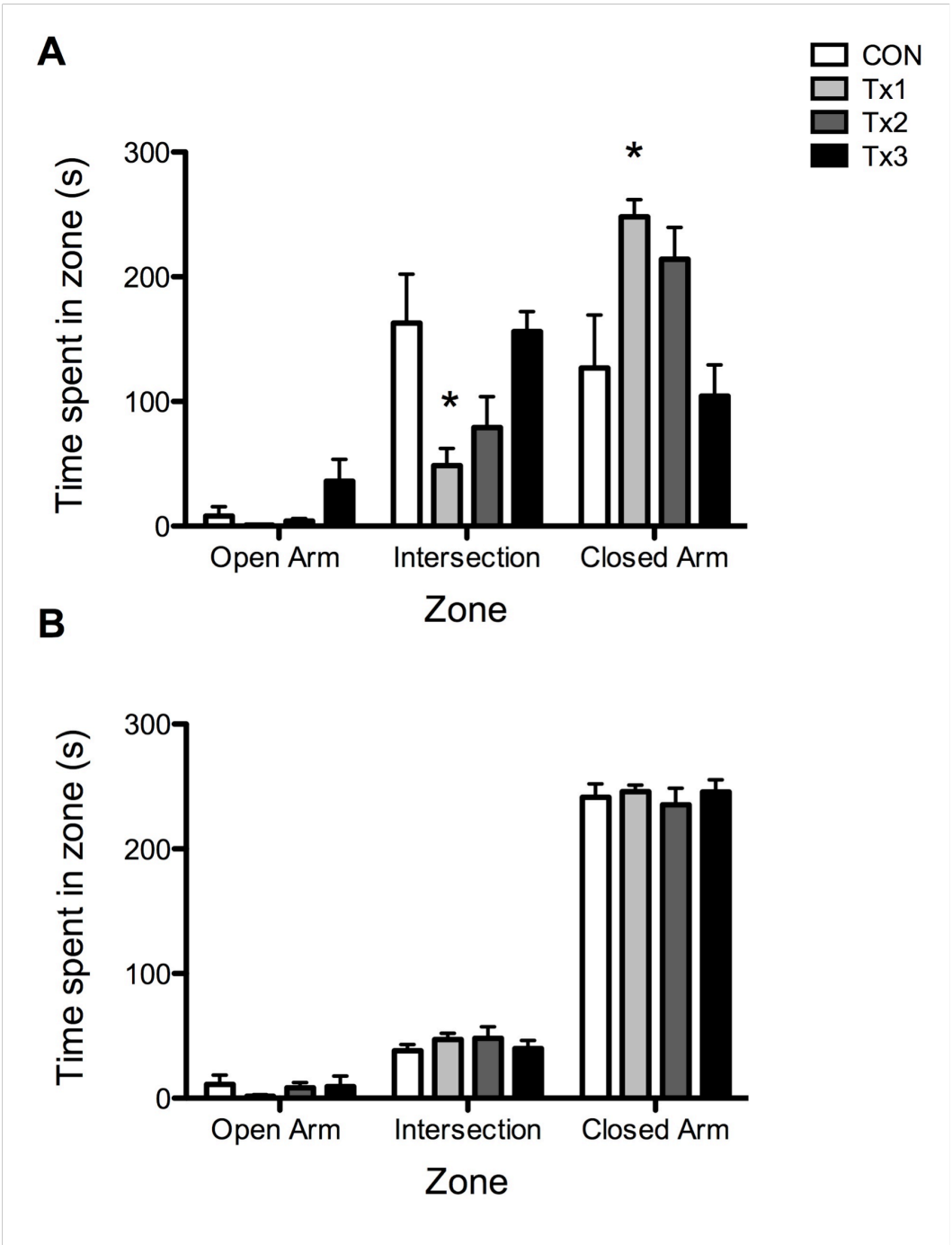

Figure 18. Amount of time spent in the different zones of the elevated plus maze (EPM) by Balb/C (A) and C57Bl/6 (B) mice were measured. Balb/C mice of treatment 1 group spent less time in the intersection and more time in the closed arms of the EPM compared to controls (*). No effect of treatment was observed on the amount of time spent in the zones of the EPM by C57Bl/6 mice. Values are means +/- S.E.M., *$p<0.05$.

were observed in amount of time spent in the zones of EPM for the other treatment groups in either Balb/C or C57Bl/6 mice.

Both Balb/C (Fig. 19A) and C57Bl/6 (Fig. 19B) mice had more entries into the closed arms compared to the open arms of the maze ($F_{1,40}$=232.8, p<0.0005, Fig. 19). An interaction between location and strain was observed ($F_{1,40}$=36.5, p<0.0005, Fig. 19). A significant effect of strain was observed on zone entries ($F_{1,40}$=32.7, p<0.0005, Fig. 19), but no effect of treatment or interaction between strain and treatment were revealed. No differences between treatment groups were observed for open and closed arm entries in either Balb/C or C57Bl/6 mice.

Balb/C (Fig. 20A) and C57Bl/6 (Fig. 20B) mice travelled a greater distance in the closed arm compared to the open arm of the maze ($F_{1,40}$=467.1, p<0.0005; Fig. 20). Interactions between location and strain ($F_{1,40}$=51.4, p<0.0005, Fig. 20), and location and treatment ($F_{3,40}$=3.6, p=0.022, Fig. 20) were observed. A significant effect of strain on distance travelled in the different EPM zones was observed ($F_{1,40}$=72.7, p<0.0005, Fig. 20). No differences between treatment groups were observed for distance traveled in the open and closed arms of EPM in either Balb/C or C57Bl/6 mice.

3.9 Intestinal Permeability

Figure 21 displays the intestinal permeability of Balb/C and C57Bl/6 mice measured as serum fluorescent isothiocyanate (FITC) recovery. An ANOVA test revealed an effect of treatment on intestinal permeability ($F_{3, 40}$=7.8, p<0.0005, Fig. 21) and also an interaction between strain and treatment ($F_{3, 40}$=4, p=0.015, Fig. 21). Treatment

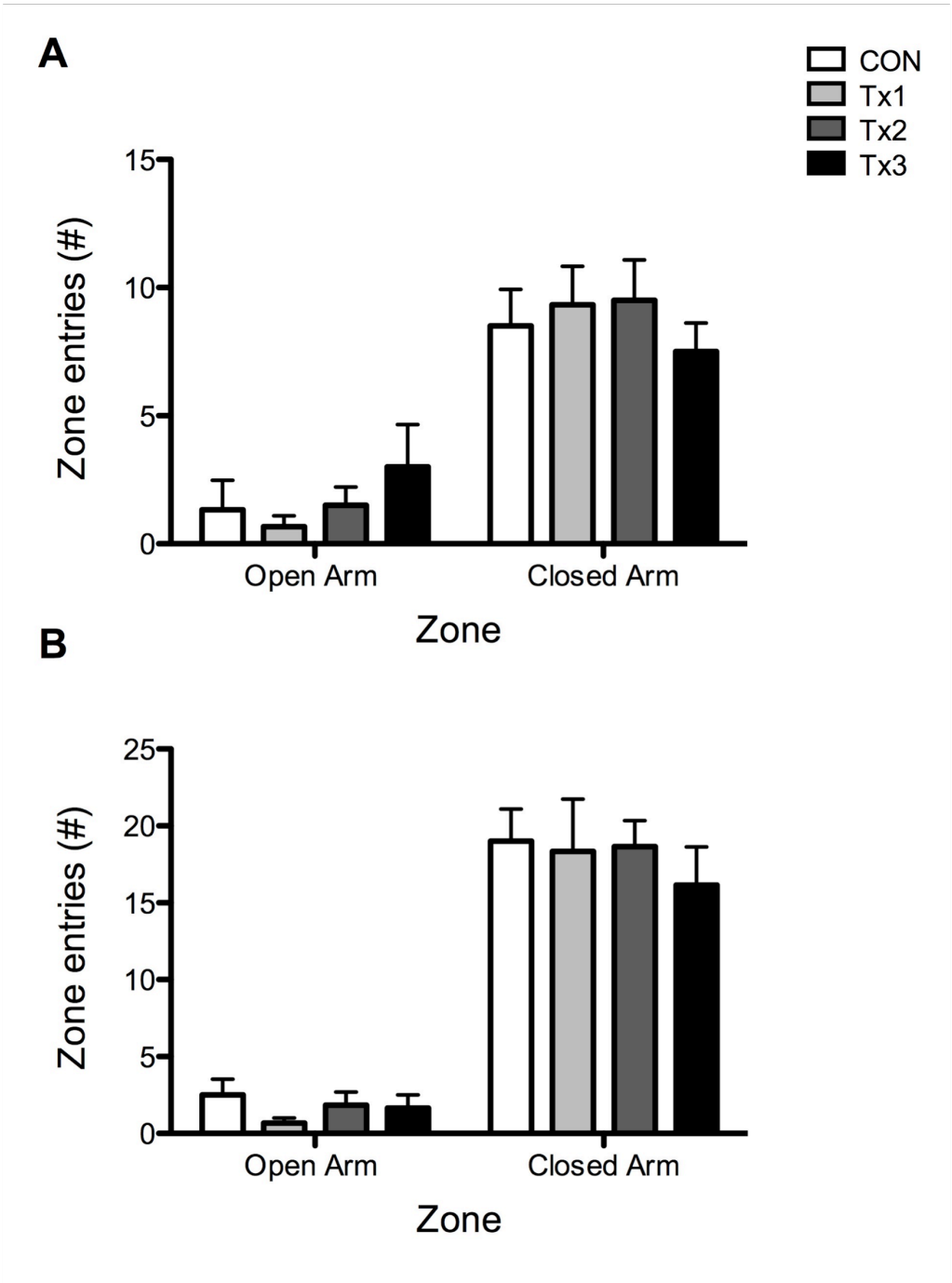

Figure 19. Number of entries into the open and closed arms of the elevated plus maze (EPM) by Balb/C (A) and C57Bl/6 (B) mice were measured. Both Balb/C and C57Bl/6 mice entered the closed arms of the EPM more than the open arms. No effect of treatment was observed on the number of entries into the open or closed arms of the EPM by Balb/C or C57Bl/6 mice. Values are means +/- S.E.M.

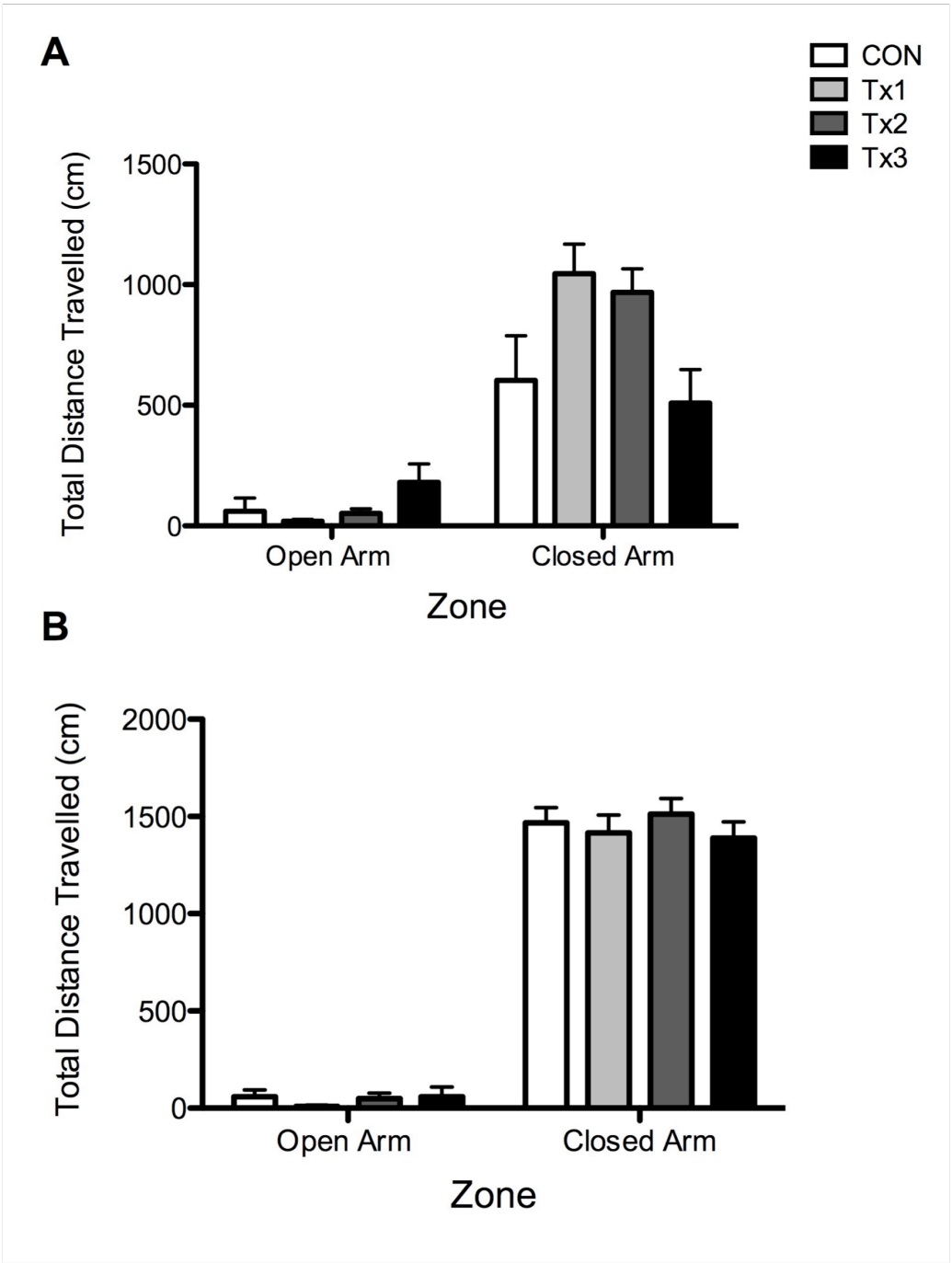

Figure 20. Total distance travelled in the open and closed arms of the elevated plus maze (EPM) by Balb/C (A) and C57Bl/6 (B) mice were measured. No effect of treatment was observed on the total distance travelled in the zones of the EPM by Balb/C or C57Bl/6 mice. However, both Balb/C and C57Bl/6 mice travelled farther in the closed arms of the EPM compared to the open arms. Values are means +/- S.E.M.

differences in intestinal permeability were determined by independent t-test (strain matched groups). Balb/C mice of Tx1 ($t=7.7$, $p<0.0005$) and Tx2 ($t=4.7$, $p=0.004$) groups had reduced intestinal permeability as observed in the lower amount of serum FITC recovery compared to controls. No difference was observed in intestinal permeability between Balb/C mice of Tx3 group and controls. Furthermore, no differences in intestinal permeability were observed between C57Bl/6 mice administered antibiotic treatment compared to controls. An independent t-test comparison for intestinal permeability between Balb/C and C57Bl/6 control groups did not revealed differences.

3.10 Insulin and Leptin Analysis

Figure 22 displays insulin (A) and leptin (B) levels measured in serum of Balb/C and C57Bl/6 experimental mice. An ANOVA test revealed a significant effect of within-subject factor, gut hormone ($F_{1,30}=22.4$, $p<0.0005$, Fig. 22); but no interactions were observed between gut hormone and strain, gut hormone and treatment, or a three way interaction between gut hormone, strain and treatment. In addition, an effect of between subject factors strain and treatment were not observed, and neither an interaction between strain and treatment was revealed. Treatment differences in insulin and leptin levels were determined by independent t-test (strain matched groups). It was revealed that Balb/C mice of Tx2 group had a lower concentration of serum insulin ($t=3.2$, $p=0.009$) compared to controls, however no differences were observed in serum leptin concentration. No differences in serum insulin and leptin levels were observed in Balb/C mice of treatment 3 group compared to controls. Also, no differences in serum insulin and leptin levels were

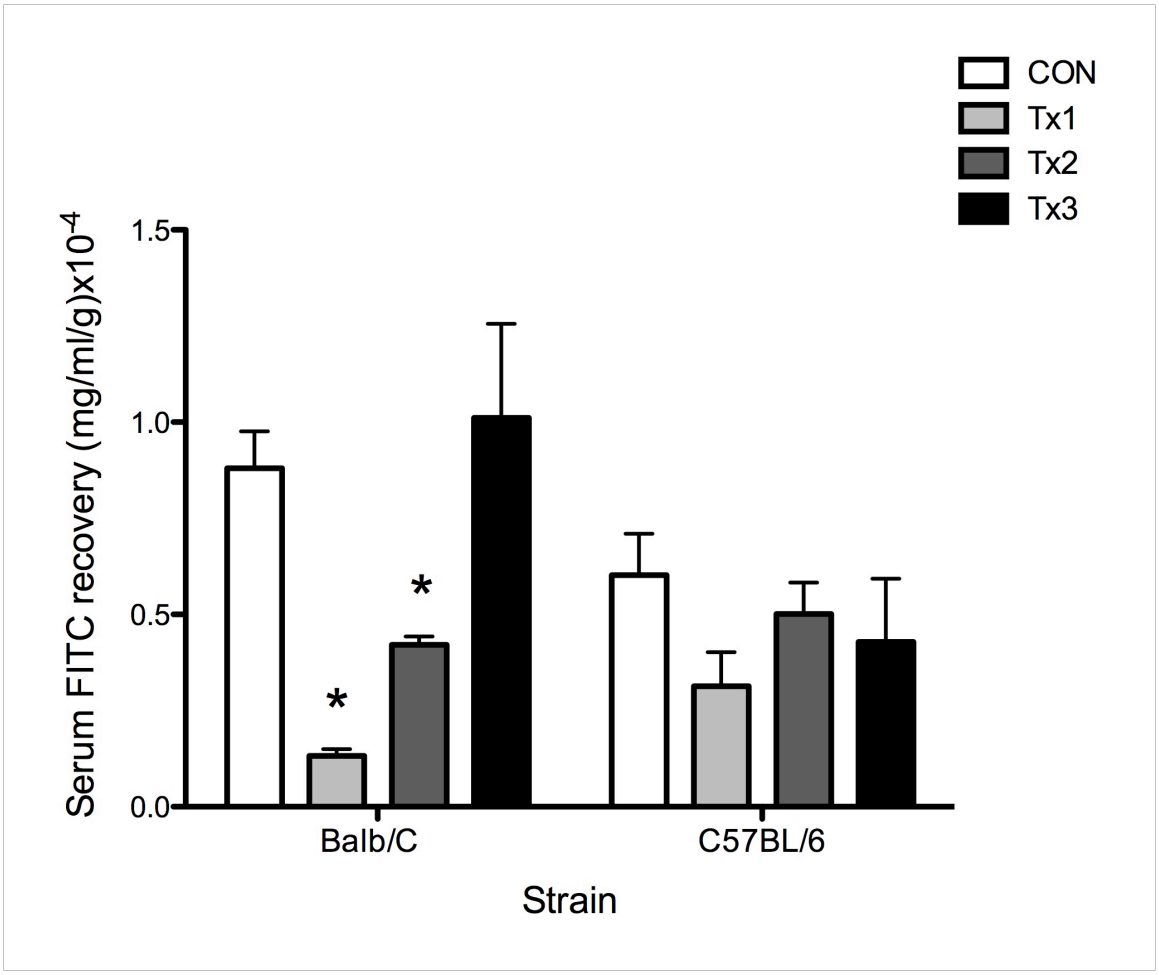

Figure 21. Serum FITC recovery was assessed as a measurement of intestinal permeability for Balb/C and C57Bl/6 mice. Balb/C mice of treatment 1 and 2 groups showed reduced intestinal permeability as displayed by the low values of serum FITC recovery relative to corresponding control mice (*). No differences in intestinal permeability were observed in C57Bl/6 mice administered antibiotic treatment relative to corresponding control mice. Values are means +/- S.E.M., *$p<0.05$.

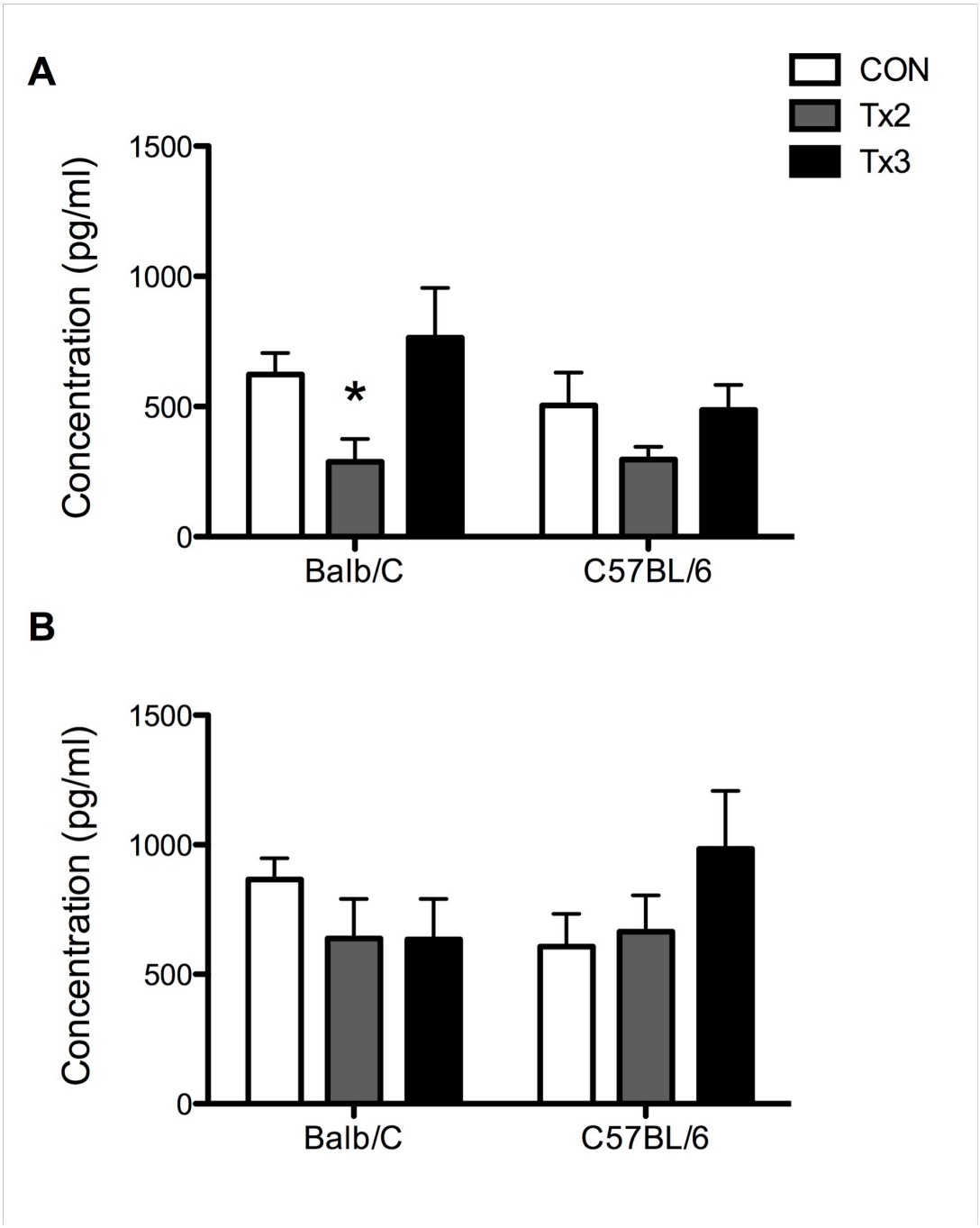

Figure 22. Serum insulin (A) and leptin (B) levels were measured for Balb/C and C57Bl/6 mice. A. Balb/C mice of treatment 2 group showed reduced serum insulin levels relative to corresponding control mice (*). No differences observed in serum insulin levels of C57Bl/6 mice. B. No differences in serum leptin levels were observed in either Balb/C or C57Bl/6 mice administered antibiotic treatment relative to corresponding control mice. Values are means +/- S.E.M., *$p<0.05$.

observed in C57Bl/6 mice of Tx2 and Tx3 groups compared to controls. Independent t-test comparisons for insulin and leptin levels between Balb/C and C57Bl/6 control groups did not revealed differences.

3.11 Cytokine Analysis

Figure 23 displays IL-1α (A), IL-1β (B), IL-6 (C), IL-10 (D) and TNF-α (E) levels measured in serum of Balb/C and C57Bl/6 experimental mice. An ANOVA test revealed a significant effect of within-subject factor, cytokines ($F_{1.2,35.7}$=95.5, p<0.0005, Fig. 23). Treatment differences in cytokine levels were determined by independent t-test (strain matched groups). It was revealed that Balb/C mice of Tx2 group had a lower concentration of serum IL-10 (t=2.9, p=0.028) compared to controls, however no differences were observed in serum IL-1α, IL-1β, IL-6 and TNF-α concentrations. No differences in serum IL-1α, IL-1β, IL-6, IL-10 and TNF-α levels were observed in Balb/C mice of Tx3 group compared to controls. A lower concentration in serum IL-1β was observed in C57Bl/6 mice of Tx2 group compared to controls (t=2.4, p=0.043), however no differences were observed in serum IL-1α, IL-6, IL-10 and TNF-α concentrations. No differences in serum IL-1α, IL-1β, IL-6, IL-10 and TNF-α levels were observed in C57Bl/6 mice of Tx3 group compared to controls. Furthermore, an independent t-test comparison for serum cytokine levels between Balb/C and C57Bl/6 control groups did not revealed differences.

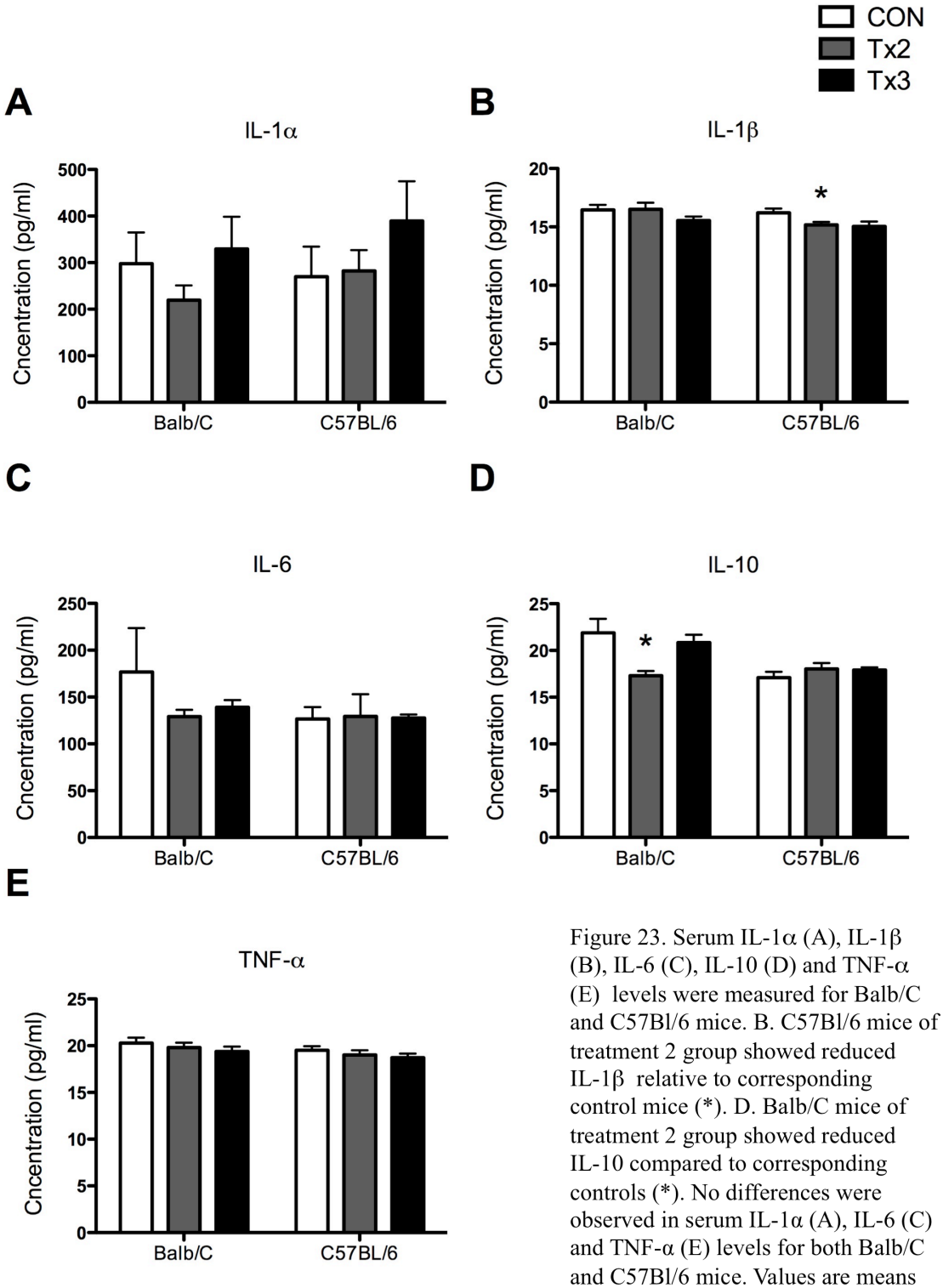

Figure 23. Serum IL-1α (A), IL-1β (B), IL-6 (C), IL-10 (D) and TNF-α (E) levels were measured for Balb/C and C57Bl/6 mice. B. C57Bl/6 mice of treatment 2 group showed reduced IL-1β relative to corresponding control mice (*). D. Balb/C mice of treatment 2 group showed reduced IL-10 compared to corresponding controls (*). No differences were observed in serum IL-1α (A), IL-6 (C) and TNF-α (E) levels for both Balb/C and C57Bl/6 mice. Values are means +/- S.E.M., *$p<0.05$.

4.0 Discussion

4.1 Thesis Impact and Summary of Findings

Currently, the use of antibiotics to treat disease has become widespread starting in early infancy and continuing into adulthood. The average child in the United States now receives approximately one antibiotic treatment per year (Cho *et al.*, 2012). A study looking at 193,412 children aged 6 to 7 years of age from 29 countries around the world showed that antibiotic treatment during the first year of life is associated with a significantly increased risk of immune related allergic disorders (Foliaki *et al.*, 2009). Therefore, an interest has grown in understanding the effects of antibiotic treatment on bodily homeostasis throughout a person's lifetime.

Research shows that administration of antibiotics in early life has been associated with immune system dysregulation and associated disorders, such as celiac disease, asthma, rhinitis, and eczema (Pozo-Rubio *et al.*, 2013; Muc, Padez and Pinto, 2013; Tsakok *et al.*, 2013). It was found by Pozo-Rubio and colleagues (2013) that antibiotic intake during the first four months of life is one of the early environmental factors associated with celiac disease development in infancy. Antibiotics have modulatory impacts on the gut microbiota composition and immune system development as seen through lymphocyte subpopulation profiles (Pozo-Rubio *et al.*, 2013). Furthermore, Muc, Padez and Pinto (2013) found a significant correlation between paracetamol, a pain killer, and antibiotic intake during the first year of life and the prevalence and severity of both asthma and rhinitis symptoms in children between the ages of five and nine. Tsakok and

colleagues (2013) also found that exposure to antibiotics during the first year of life is associated with the development of eczema in children.

Various distinct dose-dependent findings have been made regarding antibiotic use during early life and its effects on metabolism, with a focus on obesity and diabetes development. Most of this research was conducted in murine models, and has yet to be translated into human studies. The common practice in the farming industry of administering low dose antimicrobials to animals in order to induce weight gain has stirred great interest in the impact of antibiotics on metabolism (Cho *et al.*, 2012). Cho and colleagues (2012) found that intake of subtherapeutic levels (below levels required to treat disease) of antibiotics caused increased adiposity and an increase in metabolism related hormone levels in young mice. These findings were primarily associated with changes in the gut microbiome profile, increased colonic content of short-chain fatty acids, and changes in the hepatic metabolism of lipids and cholesterol (Cho *et al.*, 2012). However, research shows that therapeutic doses of antibiotics have positive effects on glucose tolerance and insulin sensitivity. Membrez and colleagues (2008) found that modulation of the gut microbiota with norfloxacin and ampicillin ameliorated glucose tolerance of mice via changes in metabolic, inflammatory and hormonal pathways.

While it has been established that antimicrobials induce dysbiosis in the gut, which further disrupts immune and metabolic homeostasis, limited research on brain and behaviour development has been conducted. The gut and brain are involved in a bi-directional communication system. Therefore, alterations via antibiotics at the level of the gut microbiota will impact the gut-brain axis. In this thesis project it is primarily of

interest to understand the effects that antibiotics have on brain and behaviour development in conjunction with changes in the immune system and metabolism using the antibiotic mouse model. Furthermore, because all humans contain a gut microbiota specific to themselves and which varies in profile in relation to other individuals, it was important to avoid the use of the germ free (GF) mouse model. While this tool is of great benefit in mechanistic research it is not directly relatable to human condition as all humans live in a bacteria filled environment. Therefore, even though this is a pilot study, the use of the antibiotic mouse model gives this research a high pre-clinical impact as it can be directly translated to what is observed in human diseases and disorders.

Minimal differences were observed in behaviour testing in the open field apparatus, elevated plus maze (EPM), three-chamber social behaviour apparatus and auditory fear conditionings enclosures in mice treated with antibiotics. Firstly, it was observed that female CD1 mice administered ampicillin (1 mg/ml) starting at 8 weeks of age (RX8) showed reduced anxiety-like behaviour in the open field test. Secondly, it was observed that male CD1 mice administered ampicillin (1 mg/ml) starting at 4 weeks of age (RX4) showed increased sociability in the three-chamber social behaviour apparatus. Thirdly, female Balb/C mice administered a combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) showed a decrease in the amount of time spent in the intersection, and an increased duration of time in the closed arms of the EPM, without any differences in time spent in the open arms of the EPM, compared to strain matched controls. No other differences were observed during behavioural testing indicative of alterations in anxiety-like behaviour due to antibiotic intake.

With regards to immune system condition, fluorescent-activated cell sorting (FACS) analysis revealed no effect of treatment on immune cell profiles in both female and male CD1 mice. Furthermore, it was observed that female Balb/C mice administered a combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) and another group administered a combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) showed reduced intestinal permeability. Differences in serum cytokine levels were also few. A decrease in IL-1β serum concentration was observed in female C57Bl/6 mice administered ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml), and a decrease in IL-10 serum concentration was observed in female Balb/C mice administered ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml).

Ampicillin treatment in female and male CD1 mice did not alter body weight or food and water consumption in mice. Furthermore, weight loss differences were observed in two groups of female Balb/C mice, with the first group administered bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) and the second group administered ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml). No differences were observed in female and male CD1 mice administered ampicillin treatment. Female Balb/C and C57Bl/6 mice administered ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) and another group of female Balb/C mice administered erythromycin (1 mg/ml) and primaricin (1.2 μg/ ml) showed reduction in water consumption during the first four days of treatment. No impact on food consumption was observed for female and male CD1 mice as a result of ampicillin

treatment. However differences were observed in food consumption as a result of the three different treatments administered to Balb/C and C57Bl/6 mice. Serum insulin and leptin level investigation revealed that Balb/C mice administered ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) had reduced serum insulin levels compared to strain matched controls. No other differences were observed in insulin and leptin levels in either strain of mice.

4.2 The Gut Microbiota and the Brain

Recent studies have shown the impact of gut microbiota on brain development and behaviour. Both the GF mouse and antibiotic mouse models have been utilized to demonstrate these findings. As previously mentioned, Neufeld *et al.* (2011) have shown that GF mice exhibit reduced anxiety-like behaviour compared to SPF mice in the EPM, along with neurochemical differences. Furthermore, a study by Bercik *et al.* (2011) using the antibiotic mouse model has also demonstrated that alterations in the gut microbiota profile led to increased exploratory behaviour in mice as seen in the light/dark test and step-down test. The findings of the first study conducted for this thesis project have shown that administration of the antibiotic ampicillin starting at 8 weeks of age (RX8) induced reduced anxiety-like behaviours in female CD1 mice, as observed in the open field test. These changes were not observed in female CD1 mice administered ampicillin starting at 4 weeks of age (RX4) or in male CD1 mice administered ampicillin starting at 4 (RX4) and 8 (RX8) weeks of age. However, in the EPM, which is a tool used to measure anxiety-like behaviour, neither female nor male CD1 mice showed any differences due to ampicillin treatment intake.

Contrary to the findings made by Bercik *et al.* (2011), in the open field test neither female nor male CD1 mice exhibited any differences in locomotor or exploratory behaviour as a result of ampicillin treatment. Similarly, no differences were observed in exploratory behaviour in the EPM for either female or male CD1 mice, as a result of antibiotic treatment. In addition to the established behavioural changes observed in anxiety-like behaviour and exploratory behaviour, we also decided to evaluate social and learning behaviour (Neufeld *et al.*, 2011; Bercik *et al.*, 2011). Male CD1 mice starting ampicillin treatment at 4 weeks of age (RX4) exhibited increased sociability in the three chamber social behaviour apparatus. Male CD1 mice administered ampicllin starting at 8 weeks of age (RX8) did not exhibit any differences and neither did female CD1 mice as result of antibiotic treatment.

The reason for the observed incongruity between our results and those seen in the literature can be attributed to the antibiotic used. Ampicillin primarily targets Gram-positive and some Gram-negative bacteria. It can be speculated that impacting the populations of Gram-positive bacteria in the gut might not be conducive to changes in behaviour. Furthermore, even though in previous studies ampicillin was administered to mice in a concentration of 1mg/ml, it is the strain of mice that differs in our work and this could impact the way that mice respond to the treatment. Lastly, even though there was no sex or treatment effect on water consumption observed, a better alternative to deliver an exact amount of the antibiotic into the animals would be by means of gavage. This suggestion is in order to be able to better regulate the amount of antibiotic treatment that is delivered and which could possibly elicit a change in behaviour.

According to Heijtz *et al.* (2011) conventionalization of GF mice from birth normalized behaviour in the EPM, but not in the light/ dark test. Furthermore, Heijtz and colleagues (2011) explained that the conventionalization of GF mice in adulthood does not lead to behaviour normalization of GF mice in the open field test. A second study by Clarke *et al.* (2012) showed that conventionalization of GF mice at 3 weeks of age (weaning age) normalized anxiety-like behaviour in the light/dark box test. These observations together with previous findings from our lab led Foster and McVey Neufeld (2013) to postulate that adolescence may be the critical window during development when the gut microbiota influences CNS wiring pertaining to stress-associated behaviour. We were unable to show in this thesis that adolescence is the critical time frame for stress-associated behaviour programming by the gut microbiota. However, we do not dismiss the possibility that adolescence represents a critical time window in stress-associated behaviour as this experiment contained several limitations. In the current study we did not examine the microbiota profile, however it is possible that ampicillin treatment did not lead to microbiota dysbiosis and therefore had a limited impact on behaviour. We propose that understanding gut-brain interactions during adolescence is an avenue that requires further exploration.

Observing the limitations presented by the first experiment, we conducted a second in which we explored a combination of different antibiotic treatments and focused on anxiety-like behaviours measured in the EPM. The Bercik *et al.* study (2011) mentioned above used the same treatment, a combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml), as we did in one of our experimental

groups. The antibiotic was similarly administered in drinking water. Bercik and colleagues (2011) used male Balb/C mice and administered the treatment for one week. For the purposes of this thesis, our study was performed on female Balb/C mice that were supposed to be administered the treatment for a duration of two weeks. However, this was not possible as they developed sickness behaviour and exhibited severe weight loss. Behavioural testing in the EPM and endpoint were rescheduled to 10 and 11 days from start of treatment, respectively. Bercik and colleagues (2011) showed that treatment with the following combination of antibiotics and antifungals, bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml), disrupted the gut microbiota of the mice and led to increases in hippocampal BDNF and an increase in exploratory behaviour, measured in the light/dark test and the step-down test. Our findings showed that female Balb/C mice administered the combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) showed a decrease in amount of time spent in the intersection, and an increased duration of time in the closed arms of the EPM, without any differences in time spent in the open arms of the EPM, compared to strain matched controls. It is possible that behavioural changes may have been associated with weight loss in response to treatment, however, there were no differences in closed arm entries across treatments which is a behavioural measure that is often used to indicate activity. In addition, there were no differences in closed arm distance travelled (an activity measure) between groups suggesting that the activity of the different treatment groups was not affected and therefore the EPM changes in behaviour were not likely a result of sickness from treatment. With respect to the other antibiotic and antifungal combinations, ampicillin (1

mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml), and the combination of erythromycin (1 mg/ml) and primaricin (1.2 μg/ ml) no differences were observed during behavioural testing indicative of alterations in anxiety-like behaviour.

4.3 The Gut Microbiota and the Immune System

The relationship between the gut microbiota and the development of a properly functioning immune system is pivotal in bodily homeostasis. Studies performed on the GF mouse model have shown that a lack of gut microbiota changes the development of the structure and composition of the spleen and lymph nodes, as seen through poorly developed B- and T-cell areas (Macpherson & Harris, 2004). This study explored spleen cell profiles in mice treated with ampicillin (1 mg/ml) and observed no differences in B and T cells or macrophages in either mice starting treatment at 4 weeks of age, during adolescence, or at adulthood starting at 8 weeks of age. These results do not indicate that gut microbiota alterations by means of antibiotic treatment influence the immune system.

Furthermore, the first line of defense at the mucosal sites of the GI tract is the intestinal epithelial barrier, which ensures a clear separation between the peritoneal tissues and the intestinal lumen. The mucosal epithelial barrier prevents the crossing of pathogenic bacteria and their products, which can have negative effects on immune and metabolic functions (Honda and Littman, 2012; Carvalho and Saad, 2013). A recent study by Cani and colleagues (2008) showed that administration of a high-fat diet modulated the gut microbiota profile and resulted in strongly increased intestinal permeability as seen in the reduced expression of tight junction proteins, ZO-1 and occludin. Interestingly,

upon administration of antibiotic treatment we only observed differences in Balb/C mice and not in C57Bl/6 mice. The combination of antibiotics and antifungal, bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) and another combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) resulted in reduced intestinal permeability. Both of these treatment combinations target both Gram-positive and Gram-negative bacteria, plus existing fungi that compose the gut microflora. Therefore, these combinations can be considered to target a broad-spectrum and can be speculated to have eliminated a large variety of bacteria and fungi as seen through the effects on body weight. It is of interest to verify the expression of tight junction proteins in the intestinal epithelial barrier in order to further confirm the source of the reduced intestinal permeability observed in the Balb/C mice.

Intestinal permeability has also been linked to low-grade inflammation—metabolic endotoxemia due to the increased plasma concentration of LPS—produced by Gram-negative bacteria. Increased plasma LPS levels cause the immune system to elicit a systemic inflammatory response, which can be identified by looking at plasma pro-inflammatory cytokines (Honda and Littman, 2012; Carvalho and Saad, 2013). In 2004, Sudo and colleagues showed that gnotobiotic mice containing the Gram-negative bacteria enteropathogenic *Escherichia coli* (EPEC) showed increased plasma levels of IL-1β and IL-6. Furthermore, gnotobiotic mice containing an EPEC mutant strain, ΔTir (Gram-negative bacteria) or *Bifidobacterium infantis* (Gram-positive bacteria) showed increased plasma levels of only IL-6, and not of IL-1β. Another study by Cani *et al.* (2008) showed that antibiotic treatment (ampicillin and neomycin) had beneficial effects as it returned

high levels of plasma IL-1 and TNF-α, caused by a high-fat diet, to normal values. However, most recently a study by Bercik and colleagues (2011) showed that administration of antibiotic treatment (bacitracin, neomycin and primaricin) did not change intestinal morphology and did not cause inflammatory responses as shown through normal levels of TNF-α, IL-1β, IL-4, IL-6, IL-12, interferon (IF)-γ, transforming growth factor (TGF)-β, IL-10, and IL-17. Similarly to the findings of Bercik *et al.* (2011), we showed that the same treatment, bacitracin, neomycin and primaricin, did not cause any alterations in plasma cytokine levels in either Balb/C or C57Bl/6 mice. However, we observed some minor reductions in cytokine levels caused by the ampicillin, neomycin and primaricin treatment combination. In Balb/C mice this treatment caused a decrease in IL-10 plasma levels, while in C57Bl/6 mice it caused a decrease in IL-1β plasma levels. These changes, however, were minor and overall it can be concluded that inflammatory responses were not elicited as a result of gut microbiota modulation by means of antibiotic treatment.

4.4 The Gut Microbiota and Metabolism

Several studies have shown the direct impact of the gut microbiota on metabolism and furthermore, the indirect impact on metabolism due to alterations in immune function. Diet and antibiotic intake are among the primary environmental factors that influence the condition of the gut microbiota profile and have been shown to have an impact on metabolic function (Cani *et al.*, 2008; Membrez *et al.*, 2008; Bech-Nielsen *et al.*, 2011; Greiner and Backhed, 2011). It has been shown that antibiotic induced gut microbiota

dysbiosis is not correlated to weight development in mice, and furthermore is not correlated to obesity (Membrez *et al.*, 2008; Bech-Nielsen *et al.*, 2011). In this thesis, similar findings were observed in female and male CD1 mice administered a long-term ampicillin (1 mg/ml) treatment, as these mice did not experience changes in body weight or diet. Also, in female C57Bl/6 mice administered a variety of combinations of antibiotics with an antifungal it was shown that these treatments overall do no impact body weight greatly. However, in female Balb/C mice administered the combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml) and another combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) significant weight loss was observed. This can be attributed to a significant eradication of gut microbiota, which in turn negatively impacts harvesting and storing of energy. Water and food consumption were also decreased in the two experimental groups that experienced significant weight loss. These findings remain to be further explored as in fact sickness behaviour was observed in female Balb/C mice treated with the combination of bacitracin (5 mg/ml), neomycin (5 mg/ml) and primaricin (1.2 μg/ ml). Some reductions were observed in water and food consumption by female C57Bl/6 mice, however as mentioned above these changes did not cause body weight alterations in these mice.

Membrez and colleagues (2008) found through an antibiotic mouse model study that the gut microbiota is a contributing factor to whole body insulin sensitivity. A study by Rabot *et al.* (2010) confirmed this finding as they observed that GF C57Bl/6 mice were resistant to high-fat-diet-induced insulin resistance. In 2011, Bech-Nielsen *et al.*

conducted an antibiotic mouse model study and showed that antibiotic induced gut microbiota modulation changed glucose metabolism. We observed a significant decrease in serum insulin concentration in female Balb/C mice administered a combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml). This confirms that glucose metabolism is negatively impacted by antibiotic induced gut microbiota modulation. This finding is further confirmed by the weight loss observed in this experimental group, since decreased levels of insulin impact the ability of mice to store glucose and thus gain weight. No other significant differences were observed in either female Balb/C or C57Bl/6 mice in serum insulin levels as a result of antibiotic treatment, which were also correlated to the absence of change observed in body weight.

Vehik and Dabelea (2011) proposed that increased gut permeability may affect the absorption of antigens that can attack and damage pancreatic beta-cells, leading to reduced insulin production. Our findings show contrasting evidence as we observed that a combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) caused reduced intestinal permeability and low levels of serum insulin. Therefore, the reduced insulin levels cannot be attributed to an immune attack on pancreatic beta-cells caused by intestinal antigen absorption.

Leptin plays an important role in energy metabolism as it regulates appetite (Halldén & Aponte, 1997). We did not observe any alterations in serum leptin levels in female Balb/C or C57Bl/6 mice treated with the treatment combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml), or the combination of erythromycin (1 mg/ml) and primaricin (1.2 μg/ ml). Therefore, we concluded that any

changes observed in the diet of female Balb/C mice administered the combination of ampicillin (1 mg/ml), neomycin (2 mg/ml) and primaricin (1.2 μg/ ml) were not caused by changes in serum leptin levels.

4.5 Limitations and Future Directions

We recognize that sample sizes in this study were small. In order to achieve more informative results we propose the study be carried out with a larger sample size. Furthermore, as observed in the second experiment the adult female Balb/C mice administered a combination of bacitracin (5mg/ml), neomycin (5mg/ml), primaricin (1.2μg/ml) in drinking water became sick (assessed as significant weight loss and reduced locomotor activity) therefore requiring an early endpoint. Moving forward, the concentrations of the antibiotics and antifungal found in this combination were modified when administered to adult female C57Bl/6 mice as follows: bacitracin (2mg/ml), neomycin (2mg/ml), and primaricin (1.2μg/ml). This event imposed several limitations on the study. In the EPM test measuring anxiety-like behaviour, the Balb/C mice of this particular treatment group showed significant changes in time spent in the different zones of the EPM. Furthermore, changing the antibiotic concentrations for the treatment mixture administered to the C57Bl/6 mice limits our ability to make accurate and reliable comparisons between strains. However, this event is particularly informative about mouse strain differences in response to antibiotic treatments. Therefore, we propose prior to conducting a large-scale study with a significantly larger sample size that a preliminary

dose determining study with a focus on mouse strain response be conducted in order to increase the validity of the results.

A very important step in order to observe whether antibiotics are a reliable method of research in this particular topic is to profile the gut microbiota, which can be achieved via metagenomic techniques on fecal samples obtained from the experimental mice (Blottiere *et al.*, 2013). Profiling the gut microbiota will indicate whether changes in behaviour, immune and metabolic functions can be attributed to changes in certain bacterial populations that form the microflora. For example, upon profiling the gut microbiota Cani *et al.* (2008) found that *Bifidobacteria* reduced plasma LPS levels and improved mucosal barrier function. Furthermore, Bercik *et al.* (2011) showed that gut microbiota modulation by administration of antibiotic treatment increased the proportion of *Firmicutes/ Lactobacilli* and *Actinobacteria* populations and decreased the *γ-proteobacteria* and *Bacteriodetes* populations.

Our previous work (Neufeld *et al.*, 2011a) has shown that GF mice showed increased levels of hippocampal brain-derived neurotrophic factor (BDNF) levels. Furthermore, a study by Bercik *et al.* (2011) also showed that mice administered antibiotic treatment showed increased hippocampal BDNF expression. BDNF is associated with learning and memory, and even though we did not observe any differences in the fear conditioning test for CD1 female and male mice administered ampicillin (1 mg/ml) we suggest that hippocampal BDNF mRNA expression be quantified via *in situ* hybridization.

A further method of measuring the effects of antibiotic use on intestinal permeability, a primary component of mucosal immune regulation, is the quantification of tight junction protein levels, which can be performed via real time quantitative polymerase chain reaction (Cani *et al.*, 2008). Histological evaluation of the intestinal lumen for structural integrity as well as measurements of inflammatory markers in the intestinal lumen will provide valuable insight on the effects of antibiotics on the mucosal immune system (Cani *et al.*, 2008).

In order to better understand the phases of development in which antibiotics causes changes at the level of brain and behaviour, the immune system, and metabolism we propose conducting a study including different time points for gut microbiota modulation. Administering antibiotic treatment to pregnant dams, breastfeeding dams, and to pups starting at birth will allow us to understand the impacts of antibiotics on brain and behaviour development, and immune and metabolic function prenatally and during early life.

Ultimately, the goal of this study is to be translated at a clinical level to observe how modulation of the gut microbiota impacts bodily homeostasis at the brain, immune, and metabolic levels in humans. Furthermore, we hope to potentially identify methods of preventing gut microbiota mediated pathology in these systems.

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