Sex-specific effects of a Mediterranean-based diet on behavioural and serotonin-related colonic and hippocampal changes in a mouse model of prenatal stress

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Abstract

Prenatal stress may increase the risk for depression in offspring and it has been suggested that this could be linked to alterations in tryptophan metabolism, leading to serotonergic changes. Dietary patterns based on the Mediterranean (Med) diet, which includes foods rich in nutrients involved in the tryptophan-serotonin pathway, have been linked to depressive symptom improvements when used as an intervention. This thesis examined, in a mouse model, whether a Med-based diet normalized depressive-like behaviour and changes in the serotonin system in the colon and hippocampus resulting from a repeated physical restraint stressor administered during the second trimester in adult C57BL/6N female and male offspring. The Med-based diet modulated behaviour and hippocampal serotonin receptors primarily in females and changed the enzyme involved in the colonic serotonergic pathway in males. These results suggest that a Med-based diet may help improve behavioural disturbances stemming from prenatal stress in a sex-specific way, perhaps through its actions on the gut-brain serotonin system.

Résumé

Le stress prénatal peut augmenter le risque de dépression chez la progéniture et ces effets pourraient être liés à des altérations du métabolisme du tryptophane, menant à des perturbations sérotoninergiques. Des études montrent que des interventions nutritionnelles basées sur la diète méditerranéenne (Med), comprenant des aliments riches en nutriments impliqués dans la voie métabolique tryptophane-sérotonine, ont mené à des améliorations des symptômes dépressifs. Cette thèse a examiné, au moyen d'un modèle murin C57BL/6N, si une intervention basée sur la diète Med a limité les comportements de type dépressif et les changements dans le système sérotoninergique du côlon et de l'hippocampe résultant d'une exposition répétée à un stresseur de contrainte physique pendant le deuxième trimestre de grossesse chez la progéniture adulte femelle et mâle. L'intervention basée sur la diète Med a modulé le comportement et les récepteurs de sérotonine dans l'hippocampe principalement chez les femelles et a modifié l'enzyme impliquée dans la voie sérotoninergique dans le côlon des mâles. Ces résultats suggèrent qu'une alimentation basée sur la diète Med pourrait aider à améliorer les changements comportementaux résultant du stress prénatal en fonction du sexe, possiblement par ses effets sur le système sérotoninergique intestin-cerveau.

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List of Abbreviations

CCAC	Canadian Council on Animal Care
MDD	Major Depressive Disorder
5-HT	5-Hydroxytryptamine
Med	Mediterranean
CANMAT	Canadian Network for Mood and Anxiety Treatments
SSRI	Selective Serotonin Reuptake Inhibitor
LPS	Lipopolysaccharides
SERT or 5-HTT	5-Hydroxytryptamine or serotonin Transporter
E	Embryonic Day
TPH	Tryptophan Hydroxylase
TDO	Tryptophan 2,3-Dioxygenase
BBB	Blood-Brain Barrier
CNS	Central Nervous System
BH_4	Tetrahydrobiopterin
IDO	Indoleamine 2-3 Dioxygenase
5-HIAA	5-Hydroxyindoleacetic Acid
P	Postnatal Day
mRNA	Messenger Ribonucleic Acid
RNA	Ribonucleic Acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
cDNA	Complementary Deoxyribonucleic Acid
ANOVA	Analysis of Variance

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1. Introduction

The 2019 Global Burden of Diseases report found that since 1990, the prevalence of mental disorders increased by 48.1% (GBD 2019 Mental Disorders Collaborator, 2022). Amongst these disorders, major depressive disorder (MDD) was one of the two most common and was more prevalent in females compared to males (GBD 2019 Mental Disorders Collaborator, 2022). Given that MDD leads to significant disability and the rate of its diagnosis is increasing, there is a global movement calling for the prioritization and more investment in mental health services (GBD 2019 Mental Disorders Collaborator, 2022; Patel et al., 2011). In hopes to improve prevention and treatment options for depression in particular, focus is being placed on the identification of risk factors for the disorder and the development of strategies to mitigate them.

It has been demonstrated that environmental stressors increase the risk and contribute to the onset and maintenance of mental health disorders such as MDD (Heim et al., 2008; Park et al., 2019; Slavich & Irwin, 2014). Specifically, exposure to a stressor during the prenatal period increases the risk of depression in humans and promotes depressive-like behaviour in rodents (Van den Bergh et al., 2018; Watson et al., 1999). The demonstration that a prenatal stressor in rodents led to alterations of serotonin or 5-hydroxytryptamine (5-HT), a neurotransmitter synthesized from the essential amino acid tryptophan (Comai et al., 2020), and of enzymes involved in tryptophan metabolic pathways in the colon and the hippocampus (Galley et al., 2021) has suggested that 5-HT dysregulations in the intestinal and brain environments resulting from prenatal stress could promote the risk of depression.

Considering that current available medications for depression are effective in only one-third of people living with MDD (Rush et al., 2006), other strategies have been sought out. In particular, the Mediterranean (Med) diet, which is rich in tryptophan as well as in cofactors

involved in 5-HT metabolism, such as vitamin B6 and other antioxidants (Dubost et al., 2015; Le Floc'h et al., 2011; Ruddick et al., 2006; Strasser et al., 2016), has been associated with a lower risk and incidence of depression (Lassale et al., 2019; Psaltopoulou et al., 2013). Importantly, a dietary intervention based on the Med diet has also been shown to decrease the severity of symptoms in individuals with moderate depression (Jacka et al., 2017). Given the sum of its nutrient composition and its effects on depressive symptoms, the Med diet appears favourable for optimal 5-HT synthesis and thus could help limit the gut-brain perturbations in the serotonergic system induced by a prenatal stressor and potentially protect offspring predisposed to depression by exposure to prenatal stress.

This thesis aims to examine if a Med-based diet could limit the effects of a prenatal stressor on depressive-like behaviour in a mouse model, specifically through its effects on tryptophan metabolism, the 5-HT transporter, and key 5-HT receptors in the colon and hippocampus. Given that MDD is more prevalent in women (GBD 2019 Mental Disorders Collaborator, 2022), that there are known sex differences in 5-HT synthesis (Nishizawa et al., 1997), and that sex differences in behavioural and 5-HT-related changes resulting from prenatal stress remain to be identified, these outcomes were evaluated in both prenatally stressed female and male offspring.

2. Literature Review

2.1 Major depressive disorder

2.1.1 Major depressive disorder symptoms and diagnosis

Major depressive disorder is a serious mental illness that was recognized as early as 460-377 AD by Hippocrates (Rosenberg & Pascual, 2015). Depression is defined as an illness that impairs how an individual is feeling, thinking, and acting, which can severely affect a person emotionally and physically, and lead to a diminished capability to function at home and in the

workplace (American Psychiatric Association, 2013). Depression is characterized by having a depressed mood or sadness and/or a decreased interest in activities that the individual once enjoyed, also known as anhedonia (American Psychiatric Association, 2013). Other symptoms include a change in appetite and/or weight, slower movements or more physical activity with no discernible purpose, sleep perturbations whether insomnia or hypersomnia, loss of energy, feeling worthless, a reduced ability to think, concentrate, or make decisions, and thinking of death or suicide (American Psychiatric Association, 2013). To receive a diagnosis, the individual must have a minimum of five symptoms listed above for two weeks, including having a depressed mood and/or anhedonia (American Psychiatric Association, 2013). Given the numerous symptoms, the presentation of this disorder is highly heterogeneous.

2.1.2 Current treatment options for major depressive disorder

Major depressive disorder is an illness that is treatable however, it can be complicated to achieve remission (Kverno & Mangano, 2021) as the disorder is highly heterogeneous, both in its etiology and pathophysiology, making it difficult for the healthcare provider to make an adequate treatment plan for their patients (Nemeroff, 2007). In the Canadian Network for Mood and Anxiety Treatments (CANMAT) 2016 depression guidelines, the scientific and educational organization outlined four main categories as current treatment options to optimize outcomes of MDD, notably pharmacological treatments, psychological treatments, neurostimulant treatments, and complementary and alternative medicine treatments (Lam et al., 2016).

Pharmacological treatments are indicated for individuals with moderate to severe depression, or those with mild symptoms that have previously responded to medications for depression (Kennedy et al., 2016). Amongst the first-line pharmaceutical options, there are selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors

(Kennedy et al., 2016). These medications are classes of pharmaceuticals that block the reuptake of serotonin and norepinephrine, respectively, at the synaptic cleft (Blier & De Montigny, 1983; Feighner, 1999; Frazer, 1997; Holtzheimer & Nemeroff, 2006). Other medications for depression that exist and act on the serotonergic system, amongst other systems, include tricyclic medications and monoamine oxidase inhibitors (Correia & Vale, 2022), and are classified as second- and third-line recommendations, respectively (Kennedy et al., 2016). As SSRIs have a strong impact on the serotonergic system, side effects associated with this class of medication include but are not limited to insomnia, gastrointestinal distress, and agitation (Holtzheimer & Nemeroff, 2006). Although these side effects may be tolerable for some individuals, they lead others to stop taking this medication (Holtzheimer & Nemeroff, 2006).

Current first-line pharmaceuticals lead to remission in only one-third of individuals upon a first trial (Rush et al., 2006), and thus there is a need for other treatment options. Psychological approaches include treating behavioural and psychiatric disorders by communicating and aims to alleviate depressive symptoms (Parikh et al., 2016). Currently, first-line psychological options include cognitive-behavioural therapy, interpersonal therapy, and behavioural activation (Parikh et al., 2016). These options are effective when it comes to treating MDD however, the costs associated make them not affordable for all individuals (Fortier et al., 2020). Neurostimulant use, which partly stems from the increase in knowledge of the neurocircuitry involved in depression (Lam et al., 2016; Milev et al., 2016), is separated into two sub-categories, which are non-invasive and invasive approaches (Milev et al., 2016). Non-invasive techniques include electroconvulsive therapy, transcranial direct current stimulation, and repetitive transcranial magnetic stimulation, whereas invasive approaches include surgical techniques such as vagus nerve stimulation and deep brain stimulation (Milev et al., 2016). These techniques are currently mostly used in treatment-

resistant individuals, meaning that they have not responded to other treatment options (Milev et al., 2016). The fourth category presented, complementary and alternative medicine treatments (Lam et al., 2016), includes light therapy, exercise, yoga, and dietary supplements such as St John's wort, Omega-3, and folate (Ravindran et al., 2018). Although not listed in the CANMAT review, healthy nutrition therapy has also been noted as a complementary and alternative treatment option (Qureshi & Al-Bedah, 2013). These practices are getting more popular mostly due to the belief that natural approaches are more self-directed as opposed to having a practitioner leading the intervention (Ravindran et al., 2018; Solomon & Adams, 2015).

Even with all the current treatment options for MDD, many individuals do not respond well (Nemeroff, 2007) and more research is being done to gain a better understanding of the biological processes involved in depression to find effective strategies to better prevent and/or alleviate symptoms, especially in individuals who do not respond well to available treatments.

2.1.3 Factors predisposing to major depressive disorder

As researchers are trying to gain a better understanding of the disorder, factors that could increase the risk to develop MDD have been noted, including adverse events during childhood, social isolation, and/or stressful events in the daily life amongst other factors (Nemeroff, 2020). The use of epidemiology has allowed to identify the link between stress as early as *in utero* and the risk for the offspring to develop mental health illnesses later on, such as MDD (Kofman, 2002; Weinstock, 2005). Based on these observations, a growing number of studies have looked at the effects of prenatal stress on the offspring to better understand the biological processes that could predispose an individual to MDD.

2.2 Prenatal stress

2.2.1 Fetal programming and depression

In 1989, David Barker noted an inverse relationship between birth weight and systolic blood pressure in adulthood (Barker et al., 1989). The concept that the prenatal environment could affect the offspring's development and health is now referred to as “fetal programming” and many studies since Barker et al. (1989) first noted this relationship have shown a link between various prenatal events and diseases in the offspring (Jansson & Powell, 2007). In particular, epidemiological research demonstrated a link between prenatal stress and mental health disorders in the offspring (Kofman, 2002; Weinstock, 2005). Specifically, exposure to a stressor during the prenatal period increased the incidence of depression in adult humans (Van den Bergh et al., 2018; Watson et al., 1999). A prospective population-based cohort assessed maternal exposure to stressful life events during the prenatal period and found high levels of depression in young adults aged 18 to 19 years-old (Kingsbury et al., 2016). Additionally, a mouse model of prenatal stress using a physical restraint stressor found that adult male offspring that were prenatally stressed had a decrease in sociability (Gur et al., 2019). Other work using a prenatal physical restraint stressor demonstrated that adult female offspring spent more time immobile during the tail suspension test, a feature of depressive-like behaviour (Moura et al., 2022).

2.2.2 Trimester-specific effects of prenatal stress in rodent models

Fetus organs and biological systems development entails a complex procedure that is vulnerable to environmental changes throughout the pregnancy (Rice & Barone, 2000; Semple et al., 2013). Exposure to a stressor during this critical developmental period can lead to adverse effects on the developing brain, later affecting behaviour and cognition (Lupien et al., 2009). It has been suggested that there may be trimester-specific and sex-specific effects of prenatal stress as reports in humans found that the prevalence of depression was higher in adult males compared to females who were exposed to a prenatal stressor during early pregnancy (Herbison et al., 2017;

Kleinhaus et al., 2013; Watson et al., 1999). Due to ethical implications, human literature examining the trimester-specific effects of prenatal stress on depression is sparse. Rodent models have been used to gain more knowledge of these effects but the findings have been inconsistent. A physical restraint stressor during the third trimester led to an increase in depressive-like behaviour in adult males (Szczesny et al., 2014) and females (Tamura et al., 2011), while some reported no difference (Bowman et al., 2004). Previous research in our lab demonstrated that a physical restraint stressor administered during the second trimester of gestation led to increased depressive-like behaviour in adult male offspring (Osborne, 2020). Similarly, the administration during the second trimester of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria known to induce immune activation in the mother, increased behavioural despair, a feature of depressive-like behaviour, in adult females and males (Depino, 2015; Lin & Wang, 2014). As research continues to highlight a link between prenatal stress and increased risk for MDD in the offspring (Lautarescu et al., 2020), researchers have also been looking at the potential processes involved, including the serotonergic system.

2.2.3 Prenatal stress leads to alterations of the serotonergic system in the hippocampus and colon

Researchers have linked the pathogenesis of depression to the mechanisms by which antidepressants act to reduce symptoms (Delgado, 2000). The 5-HT hypothesis of depression first suggested that the disorder was caused by low levels of the neurotransmitter, as SSRIs act by increasing 5-HT levels at the synaptic cleft by specifically binding to the 5-HT transporter (SERT or 5-HTT) and inhibiting 5-HT reuptake (Blier & de Montigny, 1994; Feighner, 1999; Frazer, 1997; Stahl, 1998). Based on the role of 5-HT in depression, the potential implications of the neurotransmitter in the predisposition to the disorder have been examined in rodent models of depression. In relation to prenatal experiences, adolescent offspring born to dams subjected to a

physical restraint stressor from Embryonic Days (E) 10 to 16 had a trend towards a decrease in the 5-HT rate-limiting enzyme tryptophan hydroxylase (TPH) 2 in the hippocampus (Galley et al., 2021), a brain region sensitive to stress and involved in depression (Belleau et al., 2019). In addition to increasing depressive-like behaviour, a physical restraint and noise exposure stressor from E3 to 20 reduced 5-HT levels in the hippocampus of adult rat offspring, suggesting that dysregulations of the neurotransmitter in this brain region may be linked to increased depressive-like behaviour in prenatally stressed adult offspring (Soares-Cunha et al., 2018).

Dams exposed to a physical restraint stressor also had higher concentrations of tryptophan and 5-HT in the placenta (Chen et al., 2020), which is the primary source of 5-HT for the developing fetal brain until E12-14 (Bonnin et al., 2011). This effect was not seen in mice kept in a completely sterile environment (thus lacking the presence of microbiota, also known as germ-free mice), suggesting that the presence of microbiota may influence placental 5-HT and tryptophan levels in the stressed mother (Chen et al., 2020) and thus potentially mediating disturbances in the offspring colonic environment. The gastrointestinal tract is dense with tryptophan and 5-HT metabolites, as tryptophan is exclusively found in food (Kałużna-Czaplińska et al., 2019). Previous work using a physical restraint prenatal stressor found that offspring born to a stressed dam had increased levels of the rate-limiting enzyme of the kynurenine pathway tryptophan 2,3-dioxygenase (TDO), which is the other metabolic pathway of tryptophan metabolism, in the colon (Galley et al., 2021). In addition, prenatally stressed offspring also had a reduction of bacterial communities in the colon known to metabolize tryptophan (Galley et al., 2021), suggesting that exposure to a prenatal stressor could affect 5-HT synthesis in the colon of the offspring.

2.3 The serotonergic system in major depressive disorder

The 5-HT compound was identified for the first time in 1948 by noting its presence in the plasma and its ability to induce vasocontraction (Rapport et al., 1948). Since then, 5-HT has been identified as a neurotransmitter, that can sometimes be considered a hormone, involved in the functioning of the brain (Roth, 1994) and of many other organs and systems, including the gastrointestinal system (Berger et al., 2009). Serotonergic signalling pathways have been identified as necessary in many processes such as motor activity, emotional and reward control, and cognition, including memory, perception, and attention (Berger et al., 2009; Roth, 1994).

2.3.1 5-HT transporter and receptors

The 5-HT transporter is a presynaptic protein that uptakes 5-HT, therefore removing the neurotransmitter from the extracellular space (Blakely et al., 1998; Rudnick & Clark, 1993). Many of the first-line medications for depression, such as SSRIs, specifically target SERT to inhibit 5-HT uptake and increase its concentration at the synaptic cleft (Frazer, 1997). In the last 20 years, researchers have gained insight on the different subtypes of 5-HT receptors, which are now classified in 7 families with subtypes according to their signalling mechanisms (Berger et al., 2009). The development of specific 5-HT receptor knockout mice has allowed to gain a better understanding of the function of each receptor (Berger et al., 2009).

The 5-HT_1 receptor family are predominantly inhibitory receptors that contribute to maintaining homeostasis of the serotonergic system (Artigas, 2013). Receptors 5-HT_{1a} and 5-HT_{1b} are two subtypes of the 5-HT_1 receptor family that have both been linked to MDD (Artigas, 2013; Garcia-Garcia et al., 2014). The 5-HT_{1a} auto-receptors, located on pre-synaptic neurons largely found in the raphe nucleus (Artigas, 2013), have a negative feedback mechanism meaning that when 5-HT release is increased following excessive excitatory input (for example after stress exposure), the receptor is activated to reduce 5-HT neuron firing rates (Adell et al., 1997; Celada

et al., 2001; Martín-Ruiz & Ugedo, 2001). This mechanism is involved in medications for depression such as SSRIs as they increase 5-HT concentrations (Adell & Artigas, 1991; Bel & Artigas, 1992; Celada & Artigas, 1993), which leads to the activation of the 5-HT_{1a} receptor and a decrease in 5-HT cell firing (Blier & de Montigny, 1994). This negative feedback loop gets less effective over time as the receptors get desensitized to the increase of 5-HT (Blier & De Montigny, 1983; El Mansari et al., 2005; Hervás, 2001), which has been suggested to explain the delay in the action of medications for depression as desensitization takes some time (Artigas et al., 1996, 2001). Stimulation of the 5-HT_{1a} receptor on post-synaptic neurons, which are largely expressed in the hippocampus, using agonists elicited antidepressant-like effects (Artigas, 2013; Cryan, Valentino, et al., 2005), suggesting that increased activity of these receptors may be involved in antidepressant effects. Human reports have identified that individuals with MDD had an increase of 5-HT_{1a} expression in many brain regions, including the hippocampus (Kaufman et al., 2015; Parsey et al., 2010; Stockmeier et al., 1998), suggesting that the expression level of the receptor in this brain region may play a role in the disorder (Albert et al., 2011).

The 5-HT_{1b} receptors are also located on both pre- and post-synaptic neurons, although predominantly found on pre-synaptic neurons (Boschert et al., 1994; Boulenguez et al., 1996; Riad et al., 2000; Sari, 2004; Sari et al., 1997, 1999). The 5-HT_{1b} autoreceptors are located on serotonergic neurons whereas the heteroreceptors are located on non-serotonergic neurons (Engel et al., 1986; Göthert et al., 1987; Sari, 2004). These receptors are inhibitory (Artigas, 2013) and depending on whether they are located on serotonergic or non-serotonergic neurons, they inhibit the release of 5-HT (autoreceptors) or of other neurotransmitters (heteroreceptors). In humans, polymorphism of the 5-HT_{1b} receptor gene was associated with a low density of the receptor in the brain (Huang, 1999) and with MDD as individuals with this disorder expressed more of this

polymorphism (Huang et al., 2003). A post-mortem analysis found lower 5-HT_{1b} expression in the hippocampus of females that had depression and died by suicide (Anisman et al., 2008). In the hippocampus of mice, stimulation of the 5-HT_{1b} receptor with an agonist led to a decreased release of 5-HT and an antagonist led to an increased release of 5-HT (Pineyro et al., 1994). Additionally, in 5-HT_{1b} knockout mice, 5-HT release was increased in the hippocampus (Rutz et al., 2006) and a decrease in depressive-like behaviour was observed (Nautiyal et al., 2016). In addition, rats that were chronically treated with SSRIs had desensitization of the 5-HT_{1b} receptor, in addition to a decrease in SERT expression in the raphe nucleus (Neumaier, 1996). When 5-HT_{1b} knockout mice were treated with SSRIs, there was an increase in 5-HT concentrations in the hippocampus 30 minutes after injection (Nautiyal et al., 2016), supporting the notion that this receptor may also be involved in the delay in response to SSRIs (Chaput et al., 1986).

The 5-HT_{2c} receptor is another subtype of receptor located in the hippocampus that has been implicated in mood and the mechanism of action of some medications for MDD (Artigas, 2013). Antagonists of the 5-HT_{2c} receptor enhanced the effects of SSRIs by increasing 5-HT concentrations in the hippocampus and decreasing depressive-like behaviour (Cremers et al., 2004, 2007). In addition to demonstrating more depressive-like behaviour, exposure to chronic stress in rats reduced the expression of 5-HT and the 5-HT_{2c} receptor in the hippocampus (Wankhar et al., 2020), suggesting that a decrease in the expression of this receptor as a result of the prenatal stressor could potentially mediate depressive-like behaviour.

Finally, the 5-HT_4 receptor is also expressed in the hippocampus (Artigas, 2013) and it has been shown that agonists of the receptor decreased immobility time in the forced swim test, a feature of depressive-like behaviour (Lucas et al., 2007), suggesting that stimulation of this receptor had antidepressant effects. Adult rat offspring subjected to maternal deprivation during

the first 13 days of life, a well-known early-life stress paradigm, had a decrease in 5-HT_4 receptor expression in the hippocampus compared to non-stressed offspring (Bai et al., 2014). Adult rats subjected to maternal deprivation also had a lower sucrose preference rate, a phenotype reflective of anhedonia (Bai et al., 2014), suggesting that the decrease in the 5-HT_4 receptor in the hippocampus may be linked to the increase in depressive-like behaviour in the stressed offspring. This class of receptor is also expressed in the colon, where it plays a role in gut motility and reducing visceral pain (Del Colle et al., 2020; Hoffman et al., 2012). Additionally, it has been shown to play a key role in neurogenesis and neuroprotection of the enteric nervous system (Li et al., 2011; Liu et al., 2009; Margolis et al., 2016), suggesting that the 5-HT_4 receptor is important for maintaining 5-HT homeostasis in the colon.

2.3.2 Tryptophan metabolism

Roughly 90% of the 5-HT found in the human body is located in the digestive tract (Vialli, 1966), where enterochromaffin cells, neurons of the enteric nervous system, and commensal gut bacteria can synthesize this neurotransmitter from tryptophan (Costa et al., 1982; Gershon, 1999; Gershon & Tack, 2007; Vialli, 1966). Tryptophan is an essential amino acid, meaning that the only source is through diet, and has a variety of roles including protein synthesis, being a precursor for various compounds including 5-HT, and acting as a coenzyme (Kałużna-Czaplińska et al., 2019). As dietary proteins are digested and tryptophan is released, the amino acid can pass through the intestinal epithelium layer into the bloodstream (Gao et al., 2020), where it can circulate in a free form, which can be transported across the blood-brain barrier (BBB) or bound to albumin, which prevents it from crossing the BBB (Ruddick et al., 2006). Free tryptophan can be metabolized in various pathways (Figure 1), including the 5-HT pathway and the kynurenine pathway, which account for less than 5% and an estimated 95% of free tryptophan metabolism, respectively (Gál

& Sherman, 1980; Oxenkrug, 2007). The LAT-1 transporter transports tryptophan and other large neutral amino acids, such as isoleucine, tyrosine, phenylalanine, valine, and leucine across the BBB, where cells from the central nervous system (CNS) can uptake amino acids, including tryptophan by serotonergic neurons (Ruddick et al., 2006).

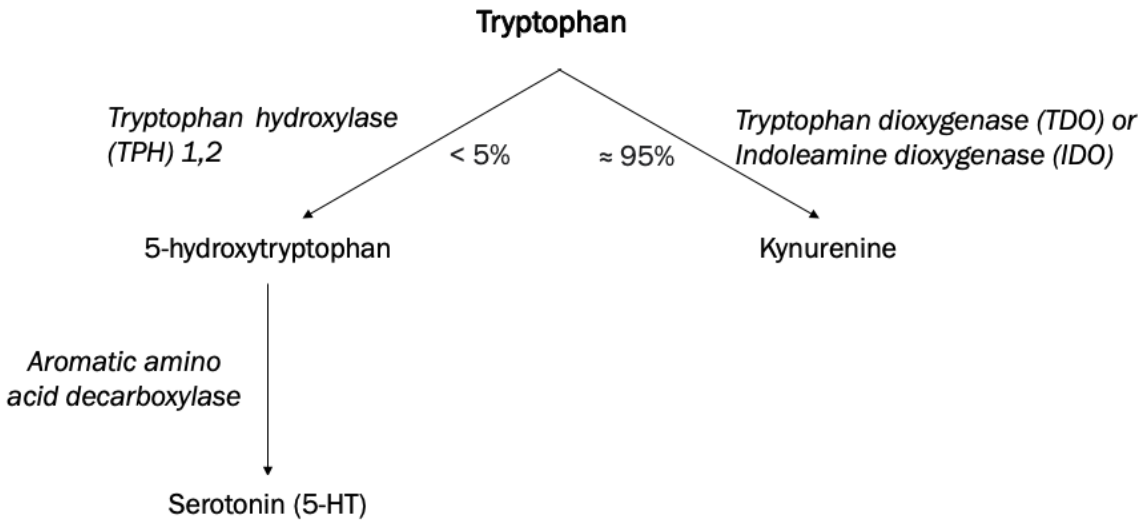


Figure 1. Summary of tryptophan metabolism in the kynurenine and 5-HT pathways.

In the 5-HT metabolic pathway, tryptophan is first converted to 5-hydroxytryptophan by the rate-limiting enzymes TPH 1 or 2 (Höglund et al., 2019). The isoform TPH 1 is mainly found in enterochromaffin cells (Le Floc'h et al., 2011), whereas the isoform TPH 2 is predominantly found in serotonergic neurons in the CNS and the enteric nervous system (Correia & Vale, 2022; Del Colle et al., 2020; Höglund et al., 2019). Both isoforms utilize tetrahydrobiopterin (BH_4) as a cofactor to convert tryptophan to 5-hydroxytryptophan (Ruddick et al., 2006). In the final step, 5-hydroxytryptophan is decarboxylated to form 5-HT (Höglund et al., 2019). The majority of tryptophan is metabolized in the kynurenine pathway (Höglund et al., 2019). This pathway is activated during inflammation and immune responses, through the stimulation of the enzymes

indoleamine 2-3 dioxygenase (IDO) 1 and 2 and TDO, which are the rate-limiting enzymes for this metabolic pathway (Höglund et al., 2019).

The two metabolic pathways of tryptophan have been involved in MDD (Correia & Vale, 2022). It has been suggested that a dysregulation between the two pathways, involving an increase of kynurenine and a decrease of 5-HT synthesis, may be a potential process altered in the disorder (Lapin & Oxenkrug, 1969). When rats were injected with LPS, this led to an increase in IDO expression in whole brain tissue and to depressive-like behaviour (O'Connor et al., 2009). The blockade of IDO activation prevented the LPS-induced increase in depressive-like behaviour (O'Connor et al., 2009), suggesting that the increase in this enzyme resulting from LPS injections may be a potential mediator in the increase of depressive-like behaviour. Rats that were subject to a restraint stressor had increased TDO in the cerebral cortex, increased kynurenine but decreased tryptophan in the plasma, in addition to increased depressive-like behaviour (Gibney et al., 2014). As for the serotonergic pathway, a rodent model of depression found that in the group exposed to various stressors, there was a decrease in TPH and 5-HT in whole brain tissue compared to the control group, who were left undisturbed (Chen et al., 2017). Tryptophan dysregulation in the brain may be a potential process by which prenatal stress increases the risk for depression in adult offspring. As the only source of tryptophan is through diet, dietary intake of the essential amino acid could modulate its metabolic pathways and increase 5-HT synthesis, thus improving mental health outcomes.

2.4 Dietary interventions are linked with mental health outcomes

Given that the available first-line medications improve depressive symptoms in a fraction of individuals (Rush et al., 2006), researchers have investigated additional treatment options for depression, including methods that could enhance 5-HT activity other than increasing its levels at

the synaptic cleft. It has been shown that dietary patterns not only have an impact on metabolism but can also influence mood and mental health (Strasser et al., 2016). The potential role of diet and nutrition in preventing and/or alleviating depressive symptoms has been explored.

2.4.1 Mediterranean diet and depression

Specific dietary patterns have been identified as a protective factor for mental health (Jacka et al., 2010; Lai et al., 2014; O'Neil et al., 2014). In particular, the Med diet, which is high in fruits, vegetables, whole grains, legumes, nuts, lean meat (e.g., fish, poultry), and unsaturated fats (e.g., olive and fish oil), and is low in red meats, processed foods, and saturated fats (European Food Safety Authority, 2011; Lai et al., 2014; Trichopoulou et al., 2003), has been associated with a lower risk as well as a lower incidence of depression (Lassale et al., 2019; Psaltopoulou et al., 2013). Importantly, a landmark study in this field using a modified version of the Med diet in a 12-week intervention showed that individuals with moderate depression had better improvements in depressive symptoms compared to a control group receiving social support sessions (Jacka et al., 2017). Together, these findings indicate that dietary patterns based on the Med diet have protective effects against MDD (Lassale et al., 2019; Psaltopoulou et al., 2013), in addition to alleviating symptoms in individuals who are already diagnosed with the disorder (Jacka et al., 2017), providing strong evidence to serve as a treatment option for depression.

2.4.2 Components of the Mediterranean diet and tryptophan metabolism

The Med diet is composed of foods such as nuts, legumes, wheat, and rice (Strasser et al., 2016) that are rich in tryptophan as well as in cofactors involved in its metabolism, such as vitamin B6, antioxidants, and folate (Dubost et al., 2015; Le Floc'h et al., 2011; Ruddick et al., 2006; Strasser et al., 2016). Specifically, the conversion of 5-hydroxytryptophan to 5-HT is dependent on vitamin B6, as this is the coenzyme in this reaction (Le Floc'h et al., 2011). Additionally,

antioxidants reduce the activity of IDO, thus allowing for more tryptophan to be synthesized in the 5-HT pathway as opposed to the kynurenine pathway (Strasser et al., 2016). With folate (Miller, 2008), antioxidants also protect the breakdown of BH_4, which is the cofactor of tryptophan hydroxylase, the rate-limiting enzyme of this metabolism pathway (Strasser et al., 2016), thus increasing the potential for 5-HT synthesis. The Med diet is also low in foods such as beef, which are known to be high in other large amino acids (Strasser et al., 2016; Government of Canada, 2021) that also use the LAT-1 transporter to get transported across the BBB (Ruddick et al., 2006), Based on these findings, it could be suggested that the Med diet is high in components that favour tryptophan metabolism in the 5-HT metabolic pathway and thus could help prevent the onset of depression.

2.4.3 Dietary modulation of tryptophan and depression

In healthy human volunteers, the consumption of a tryptophan-free amino acid drink decreased plasma tryptophan concentrations and increased depressed mood items on the Multiple Affect Adjective Checklist 5 hours after in comparison to participants who received an amino acid balanced drink or a tryptophan-supplemented amino acid drink (Young et al., 1985). Similarly, tryptophan depletion induced by a 24-hour low-tryptophan diet and consumption of a tryptophan-free drink in individuals with a history of depression who were currently in remission decreased blood tryptophan concentrations 5 hours after consuming the drink and increased depressive symptoms 26 hours after both in medicated (Delgado, 1990) and unmedicated (Moreno et al., 1999; Smith et al., 1997) individuals. In rats, a diet deficient in tryptophan led to a decrease in tryptophan concentrations in the blood and the whole brain, a decrease in 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the brain (Biggio et al., 1974), and an increase in depressive-like behaviour (Zhang et al., 2006), although these effects have been shown

to vary by strain (Jenkins et al., 2016). These findings provide insight into the modulation of tryptophan intake, the alteration of its metabolism, and its potential role in MDD (Bell et al., 2001).

The observation that tryptophan depletion in the diet led to depressive relapses in individuals in remission (Delgado, 1990; Moreno et al., 1999; Smith et al., 1997) led to further studies evaluating the effects of dietary tryptophan supplementation. In an American population, an inverse relationship was found between daily tryptophan intake and self-reported depression (Lieberman et al., 2016), suggesting that an increase in tryptophan intake could be beneficial. Increasing the ratio of free tryptophan to other amino acids by injecting tryptophan doses in rats aided its transportation to the brain and increased the 5-HT synthesis rate in brain tissue excluding the cerebellum (Grahame-Smith, 1971; Tagliamonte et al., 1973). As well, a diet high in tryptophan increased 5-HT levels in the hippocampus in rats (Musumeci et al., 2015) and reduced depressive-like behaviour in mice previously exposed to chronic unpredictable mild stress (Wang et al., 2022). This suggests that a dietary increase in foods rich in tryptophan intake could help protect against MDD.

2.4.4 Maternal diet during pregnancy influences offspring mental health

The importance of a healthy diet during pregnancy has been established for optimal health in the offspring, including mental health outcomes (Borge et al., 2017; Harding, 2001; Jacka et al., 2010; Sullivan, Grayson et al., 2010). The consumption of a high-fat diet during pregnancy decreased TPH 2 expression and 5-HT immunoreactivity while increasing anxiety-like behaviour in non-primate juvenile offspring (Thompson et al., 2017). High consumption of unhealthy foods such as sweet drinks, salty snacks, and processed meats during pregnancy also led to the child having increased internalizing behaviour compared to the consumption of healthy foods such as vegetables, fruits, and high-fibre cereals (Jacka et al., 2013), which are characteristic of the Med

diet. Conversely, a growing body of literature is showing the benefits of a maternal Med diet on many aspects of children's health (Biagi et al., 2019), including a lower risk of developing allergies (Netting et al., 2014), a healthy birth weight (Grieger & Clifton, 2014), and a lower risk of preterm birth (Amati et al., 2019; Chen et al., 2016). In addition, an inverse link between maternal adherence to a Med diet and behavioural outcomes reflective of depressive states in children has been noted (House et al., 2018). These findings together highlight the important role the maternal diet during pregnancy plays in the development of the offspring's serotonergic system, potentially predisposing versus protecting the offspring from mental health disorders, including MDD (Jacka et al., 2013; Sullivan et al., 2010).

2.5 Objectives and hypotheses

The literature shows that prenatal stress increases the risk for depression in adulthood (Depino, 2015; Lin & Wang, 2014) and disrupts the serotonergic system in the colon and hippocampus (Galley et al., 2021; Soares-Cunha et al., 2018). There is also strong evidence that the Med diet helps alleviate symptoms in individuals with MDD and also can be protective against the disorder (Jacka et al., 2017; Lassale et al., 2019; Psaltopoulou et al., 2013). Lastly, maternal dietary patterns during pregnancy were shown to influence mental health and the serotonergic system in the offspring (House et al., 2018; Jacka et al., 2013; Thompson et al., 2017).

2.5.1 Main objective, research questions, and aims

Based on these findings, and given that the Med diet is composed of foods rich in tryptophan and other nutrients that may favour its metabolism and its passage to the brain, the main objective of this research project was to examine if a dietary intervention based on this diet could limit the impact of a prenatal stressor on depressive-like behaviour through its actions on the serotonergic system in the intestinal and brain environments. While most work on the effects

of prenatal stress on behaviour and the serotonergic system has looked at males only (Akatsu et al., 2015; Drago et al., 1999; Guerrero et al., 2020; Gur et al., 2019), other reports that included both males and females found inconsistent findings in this regard (no sex differences or sex-specific outcomes) (Alonso et al., 1991; Galley et al., 2021). Considering these findings and that MDD is more prevalent in women than in men (GBD 2019 Mental Disorders Collaborator, 2022), this research project examined sex differences in depressive-like behaviour as well as in the expression of enzymes involved in tryptophan metabolism, the 5-HT transporter and 5-HT receptors in the colon and hippocampus as a result of the Med-based diet and prenatal stressor manipulations.

This research project aimed to answer three questions. First, can a Med-based diet in a mouse model limit depressive-like behaviour in adult offspring subjected to a prenatal stressor? Secondly, could the reductions in depressive-like behaviour in prenatally stressed mice fed a Med-based diet be related to changes in tryptophan metabolism, the 5-HT transporter, and/or 5-HT receptors in different parts of the gut-brain axis? Lastly, could the effects of the Med-based diet and prenatal stress on behaviour and the serotonergic system in the colon and hippocampus differ between sexes?

The first aim of this research project was to confirm the presence of depressive-like behaviour and alterations in tryptophan metabolism, the 5-HT transporter, and 5-HT receptors in the colon and hippocampus in mouse offspring subjected to a prenatal stressor. The second aim was to determine the capacity of a newly developed Med-based diet to limit the behavioural, tryptophan, and serotonergic alterations resulting from the prenatal stressor. Lastly, a third aim determined potential sex differences in these outcomes.

2.5.2 Hypotheses

It was first hypothesized that the offspring subjected to a prenatal stressor will have increased depressive-like behaviour and alterations in tryptophan metabolism, the 5-HT transporter, and 5-HT receptors in the colon and hippocampus in comparison to non-stressed offspring. Secondly, it was expected that the dietary intervention, which consists of a Med-based diet, will limit the behavioural as well as the tryptophan- and serotonergic alterations in the colon and hippocampus of offspring exposed to a prenatal stressor. Lastly, it is hypothesized that depressive-like behaviour, tryptophan metabolism, 5-HT transporter, and 5-HT receptors will be altered differently in males compared to females as a result of the prenatal stressor and the Med-based diet.

This research project will help fill the gaps in the literature in regard to preventing increased risk for MDD in adulthood. Findings from the present thesis will help understand the importance of maternal dietary patterns on the mental health outcomes of the offspring in the context of maternal distress. This research project will not only help to understand the effects that diets may have on different parts of the body, including the brain and intestinal systems, but also the effects they may have on behavioural perturbations caused by stressors. In addition, this project will explore the relation of a Med-based diet on tryptophan metabolism as well as on the transporter and the receptors of the 5-HT system at specific body sites. This field of research is very pertinent considering that the leading medications for MDD do not lead to complete remission in over half of individuals (Rush et al., 2006). This may offer a promising avenue in the prevention of mental illnesses and promote better treatment efficacy if combined for example with other medications.

3. Methodology

3.1 Animals

This study was conducted using female and male C57BL/6N mice as parent stocks (Charles River Laboratories, St-Constant, Québec, Canada). Naïve females were 6 to 8 weeks old upon their arrival and males were 7 to 9 weeks old. Mice were housed individually in 19 cm x 29 cm x 13 cm polycarbonate N10 mouse cages (Ancare) in a room on a 12h light-dark cycle, with the lights on from 0700 to 1900h, and kept between 21.0-23.0° Celsius and 30-50% humidity. Each cage was provided with a cotton nestlet, a cardboard house, standard woodchip bedding, and unlimited access to food and water. Cages were changed biweekly and water bottles were changed weekly. The University of Ottawa's Animal Care Committee approved all experimental procedures (AUP #3149), in compliance will the Canadian Council on Animal Care guidelines.

3.2 Summary of experimental procedures

After one week of acclimation on a diet that was later used as a control, female and male mice were randomly assigned to one of two dietary conditions, a Purified diet (used as the control diet) or a Med-based diet (used as the experimental diet). Females and males were placed on their respective diets for a minimum of 14 days before mating, where females were paired with a male fed the same diet. After the detection of a copulatory plug, pregnant mice were randomly assigned to a No stressor or a Stressor condition. Females in the Stressor condition were subjected to a physical restraint stressor from E7.5 to E12.5 whereas females in the No stressor condition were left undisturbed, other than standard handling procedures required for body weight measurements and the collection of fecal and blood samples. These collection procedures were conducted in all pregnant mice at key points during the dietary, pregnancy, and stressor procedures to determine biological markers as part of another study that will not be reported here.

After birth, which corresponded to Postnatal Day 1 (P1), pups were sexed and weighed, and litter sizes were standardized if needed. At P21, pups were weaned from their mother and

housed 2 per cage with a sex-matched sibling. Based on the litter size and sex distribution, 1 pup of each sex was euthanized at P7 and P28 for the purpose of another study, and only 2 females and 2 males per litter were brought to adulthood for the purpose of the current study. Body weights and food intake were determined in all pups at key points before and after weaning. In adulthood, all pups were subjected to five behavioural tests (open field, splash, elevated plus maze, three-chamber, and tail suspension tests) over a period of 3 days (P67-69). Three of these tests, the splash, the three-chamber, and the tail suspension tests are being reported in this thesis. On P70, approximately 20-24 hours after completing the last behavioural test, offspring were euthanized to collect their brain and gastrointestinal tract for subsequent organ dissections and tryptophan- and 5-HT-related analyses. A summary of the experimental procedures is provided in Figure 2.

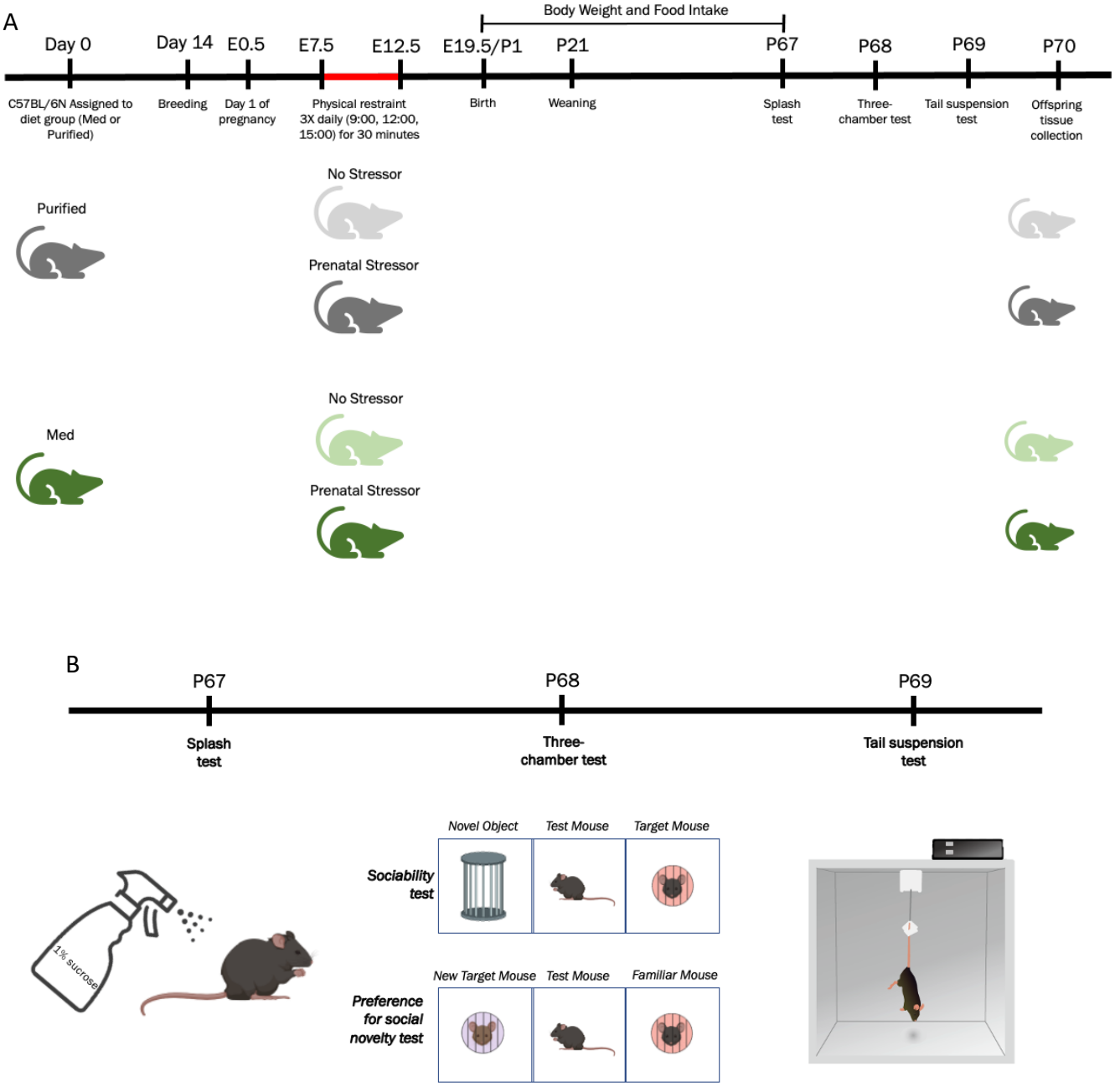


Figure 2. Summary of experimental procedures. (A) Summary of the animal portion of the research project **(B)** Visual description of the behavioural tests administered. Med-based (Med); Embryonic days (E); Postnatal day (P).

3.3 Dietary interventions

Dams maintained their respective diets for the entirety of their pregnancy and lactation periods, and offspring were fed the same diet from weaning onward. The two diets used in the present thesis (Research Diets Inc.) were previously developed in our laboratory, based on a published report (Barrington et al., 2018). The Purified control diet was developed from a previously modified version of the AIN-93G purified rodent diet (Barrington et al., 2018) and involved modifications to this diet to match the energy density of the Med-based diet, to bring the protein content to optimal levels for pregnancy, and to remove ingredients that could negatively impact the gut microbiota (e.g., food dye). Modifications included a 12% increase in casein, a 27% decrease in corn starch, the addition of maltodextrin, and the removal of inulin and food dye. The Med-based diet was designed by adding ingredients to the Purified control diet, based on human Med-style dietary patterns. Apart from low amounts of fish protein isolates, egg whites, and beef, the Med-based diet contains mostly plant-based ingredients, such as olive oil, chickpeas, lentils, oat fibres (beta-glucan), a blend of fruits and vegetables, and walnuts (Udechukwu et al., 2023). While the two diets have identical energy or kilocalorie profiles, their macronutrient composition varies to reflect the distribution of macronutrients in the human Med diet (Appendix 1) so that for the same amount of food, each diet provides an equal number of calories but not the same proportions of macronutrients. Because these two diets were newly developed, we previously confirmed that they were palatable and safe for pregnancy (Udechukwu et al., 2021).

3.4 Breeding procedures

Twenty-four hours before pairing females and males for mating, estrus was induced in females using nestlet and bedding from the cage of the male assigned to them. Twenty-four hours after estrus induction, two females were paired with a male from the same diet group overnight for

a period of 15 hours (from 1600 to 0700h the next morning). Upon separation from the male, females were observed for the detection of a copulation plug, which corresponded to E0.5, placed back in their respective cages, and monitored every three days to confirm pregnancy. If no pregnancy was confirmed by E10.5, non-pregnant mice underwent another round of breeding until pregnancy was confirmed.

3.5 Prenatal stressor procedure

The prenatal stressor used was physical restraint (Buynitsky & Mostofsky, 2009), based on previous studies demonstrating that it led to depressive-like behaviour in adult offspring (Akatsu et al., 2015; Alonso et al., 1991; Morley-Fletcher et al., 2004) and to altered tryptophan metabolism in the colon and hippocampus (Galley et al., 2021). To perform this stressor, dams were brought to a room separate from the housing room, where they were gently placed into a clear triangular plastic bag with the nose end containing a small hole that allowed the mouse to breathe, while the other end was closed and sealed with a piece of tape to restrict the mouse's ability to move. During the entirety of the stressor, dams were closely monitored to ensure that they were able to breathe properly and that their position stayed adequate. The stressor was performed during the entire second trimester (E7.5 to 12.5) for 30 minutes, three times per day at 0900, 1200 and 1500h, based on previous literature that found this led to depressive-like behaviour in the offspring (Izvolskaia et al., 2018; Osborne, 2020). Dams in the No Stressor condition were left undisturbed, other than body weight monitoring as well as fecal and blood collection.

3.6 Birth and offspring early life

3.6.1 Birth and litter standardization

Cages of pregnant dams were monitored on the day of expected birth (E19.5) for indication of birth (P1), which was determined by the presence of pups. All dams gave birth between E19.5

and 20.5. Upon birth, dams and their pups were weighed, and the sexes of the pups were noted. A cut-off of 10 mice was determined for litter standardization.

3.6.2 Offspring body weights and food intake

Pups and the dams were weighed daily from P1 to P21, after which pups were weighed at key time points until adulthood. The percentages of change in body weight from birth at P7, P14 and P21, representing the first 3 weeks of life before weaning, were calculated by subtracting the weight at P1 from the weight at a given time point (X), dividing this value by the weight at P1, and multiplying by 100 as follows: [(PX - P1)/P1] * 100. Postnatal Days 37 and 67 were chosen for the post-weaning time points as P37 was the halfway mark of their development into adulthood for this study and P67 was the endpoint in adulthood, before behavioural testing. The same calculation was done with post-weaning weights at P37 and P67, using P21 as the reference weight: [(PX - P21)/P21] * 100. The body weight at P21 was used as the reference weight for these calculations as pups were now without access to maternal breast milk, and thus their nutritional intake was different.

Food intake was measured after mice were weaned, as solid food pellets became their sole source of nutrition. Food intake was calculated at key time points by weighing the food pellets before being placed in the cage food basket and weighing the remainder food 48 to 72 hours later. A subtraction was done using these values to calculate how much food was eaten during this time (total food weight in cage – food weight left in cage). Food intake was calculated at P23 and P27, and then at P49 and P61. These time points were chosen as P23 reflects their first 48 hours separated from their mother, P27 is a week after separation from their mother and in the early stages of the puberty period (Ismail et al., 2011; Zhou et al., 2007), P49 represents young adulthood, after the surge of sex hormones (Cross et al., 2021), and P61 is near the endpoint of

this study in adulthood. As mice were housed in pairs, food intake measurements are representative of both animals in each cage.

3.7 Behavioural testing

Once the offspring reached adulthood (P67), they were subjected to five behavioural tests, over a period of three days. The open field and splash tests were conducted on P67, the elevated plus maze and three-chamber tests on P68, and the tail suspension test on P69. For this thesis, only the splash, three-chamber, and tail suspension tests were included as previous studies have shown that antidepressants have countered behaviours suggestive of depressive-like phenotypes in these three tests (Machado et al., 2012; M.-J. Park et al., 2018; Steru et al., 1985).

3.7.1 Splash test

The splash test analyzes grooming behaviour, using the knowledge that rodents groom themselves for stress reduction, and also measures the motivation to self-care, which is reflective of depressive-like states (Sachs, 1988; Smolinsky et al., 2009). Grooming behaviour during the splash test was negatively correlated with immobility time during the forced swim test, suggestive of depressive-like behaviour (Isingrini et al., 2010; Kalueff et al., 2016; Smolinsky et al., 2009). The splash test was conducted on P67, the first day of behavioural testing, one hour after completing the open field test, which is not reported in the current thesis. Mice were gently sprayed with 2 mists of a 1% sucrose solution on their back and placed individually into a clean cage identical to their home cage, but only containing woodchip bedding. A lid was placed on top of the cage, and mice were left undisturbed for 10 minutes while a digital camera recorded their grooming behaviour. For the entirety of the test, the room was set at 530 lux, determined with a lux meter. The total time spent grooming (s), the number of grooming sessions, and the average

duration of each grooming session (s) were manually scored by watching video software using the Boris software version 7 by an experimenter blind of treatment.

3.7.2 Three-chamber test

Depression can be characterized by a disruption of social interactions (Kaidanovich-Beilin et al., 2011). The three-chamber test analyzes social behaviour, in the form of sociability (social interest) and preference for social novelty (social recognition) (Lawande et al., 2020; Nadler et al., 2004; Yang et al., 2011). This test was administered on P68, 1-2 hours after completing the elevated-plus maze test, which is not reported in this thesis. The apparatus used for this test was composed of three chambers of equal size, each chamber measuring 39cm in length X 19cm in width x 22cm in height, placed side by side, and separated by removable doors (Sociability Cage, Noldus Information Technology). Mice were first placed, individually, in the center chamber (Chamber B) for 5 minutes without access to the other 2 chambers (A and C). After this habituation session, they were transferred individually into a clean cage identical to their home cage, but only containing woodchip bedding, for 5 minutes. For the first test session, an unfamiliar target mouse of the same sex was located under a wired cup in one extremity of Chamber A while an empty wired cup was at the extremity of Chamber C. The test mouse was then introduced to the centre of the middle chamber for 10 minutes, this time having access to all 3 chambers, and a ratio of the time spent sniffing the target mouse versus the target object, a measure of sociability, was calculated (Nadler et al., 2004). For the second test session, the target mouse in Chamber A was left under the wired cup, becoming a familiar mouse, and a new target mouse, again the same sex and having no previous interaction with the test mouse, was placed under the empty wired cup in Chamber C. The test mouse was introduced to the centre of the middle chamber having access to all 3 chambers for 10 minutes, and a ratio of the time spent with the new target mouse as opposed

to the familiar mouse, a measure of preference for social novelty, was determined (Nadler et al., 2004). A mouse will typically choose to spend more time interacting with another mouse instead of an object, which is a feature of sociability and, during the second part of the test, a new mouse rather than one they have already had interactions with, demonstrating a preference for social novelty (Nadler et al., 2004; Yang et al., 2011). During the entirety of the test, an automated system equipped with a camera detected the amount of time the centre point of the test mouse spent in each chamber, the number of transitions between chambers, as well as time spent at each wired cup (EthoVisionXT software, Noldus Information Technology), and thus behavioural scoring remained blind of treatment. For the entirety of the test, the room was set at 100 lux. Cleaning of the chambers was done before and after a mouse completed the 3 sessions of the test.

3.7.3 Tail suspension test

The tail suspension test is a well-validated test based on the observation that mice eventually become immobile after trying to escape the uncomfortable position of being hung upside down by the tail (Cryan, Mombereau, et al., 2005). A mouse that spends more time immobile, also known as passive coping during a stressful situation, is suggestive of more depressive-like behaviour than a mouse that is moving and attempting to escape, also known as active coping (Cryan, Mombereau, et al., 2005). Mice were subjected to this test on the third and last day of testing (P69), roughly 18 to 22 hours after the last test and was the only behavioural test of the day. Surgical tape was used to place the tail of the mice on an elevated aluminum bar that was attached to a strain gauge that detected the mouse's movement for a total of 6 minutes (Tail Suspension Test Cubicle [SOF-821], Med Associates Inc). For the entirety of the test, the room was set at 100 lux, determined with a lux meter. To prevent the mouse from climbing its tail, a small plastic tube was placed so that it would cover the portion that was closer to the body. The

total time spent immobile (s) during the 6 minutes was calculated for each mouse using the Tail Suspension system by Med Associates Inc software and was blind to treatment.

3.8 Tissue collection and analysis

3.8.1 Tissue collection

On P70, approximately 20 to 24 hours after completing the last behavioural test, mice were euthanized by rapid decapitation. Whole brains were immediately collected and put on a piece of Parafilm M placed on dry ice. Once frozen, whole brains were wrapped in the Parafilm M piece and placed in the -80ºC freezer until microdissection. In parallel, the gastrointestinal tract was removed, placed on a nuclease-free frozen surface, and separated into sections including the colon as well as the jejunum and ileum, which will not be presented in this thesis. The intestinal content was then collected from the colon by gently pressing on the tissue with a metal spatula. The whole colonic tissue was then placed in nuclease-free cryotubes and immediately in liquid nitrogen. Once the tissue collection was completed, whole brains and cryotubes containing the colonic tissue were stored in a -80ºC freezer until further mRNA expression analysis of enzymes involved in tryptophan metabolic pathways, as well as of the 5-HT transporter and selected 5-HT receptors.

3.8.2 Brain microdissection

Using the cryochamber of a ThermoFisher HM525 NX cryostat set at -20˚C, each whole brain was placed in a stainless-steel brain matrix (2.5 X 3.75 X 2.0 cm; slots spaced approximately 500um apart). The matrix allows to guide the blade while slicing coronal brain sections. Once in the matrix, the dorsal hippocampus was dissected from one of the brain sections based on the Franklin and Paxinos mouse atlas (Paxinos & Franklin, 2019), placed in a nuclease-free tube in dry ice, and stored at -80ºC until further analysis of enzymes involved in the tryptophan metabolic pathway as well as 5-HT transporter, and selected 5-HT receptors mRNA expression.

3.8.3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

This procedure took place following protocols that were previously used in the laboratory (Szyszkowicz et al., 2017). The hippocampus and colon were homogenized with 500ul and 1ml of Trizol, respectively, and the total RNA was isolated using the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). For the colon, the frozen sample was first broken into smaller pieces in a mortar kept cold using liquid nitrogen, and 2-5 mm of tissue was collected to ensure both proximal and distal parts of the colon were being used. Once homogenization in Trizol was completed, chloroform, isopropyl alcohol, and linear acrylamide were added to the sample to extract the RNA and form a pellet, which was then diluted in a TE buffer. The NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific) was used to test RNA yields and purity. Samples with 260/80 and 260/230 ratios between 1.80 and 2.20 were included. The iScript™ Reverse Transcription Supermix for RT-qPCR and a T100™ Thermal Cycler (Bio-Rad, Canada) were used to reverse-transcribe the total RNA into complementary DNA (cDNA). Using the SsoAdvanced™ Universal SYBR® Green Supermix and a CFX96 Touch™ Real-time PCR Detection System (Bio-Rad, Canada), the cDNA aliquots were then analyzed in simultaneous quantitative polymerase chain reactions. This allowed for the determination of the gene expression of TPH1, TPH2, IDO1, TDO2, SERT (Slc6a4), 5-HT_{1a} (Htr1a), 5-HT_{1b} (Htr1b), 5-HT_{2c} (Htr2c) and 5-HT_4 (Htr4). The sequence used for each gene is found in Appendix 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta-Actin (B-Actin) were used as reference genes for both the colon and hippocampus analyses. Each gene of interest was then normalized against these reference genes. Fold changes for the mRNA expression for each gene of interest were calculated in reference to the control group, being females and males fed the Purified diet that had not been prenatally stressed.

3.9 Statistical analyses

Data was analyzed using SPSS version 28. As the number of pups at birth varied between litters, a 2-way analysis of variance (ANOVA) with Diet (Purified versus Med-based) and Stressor (No stressor versus Stressor) serving as the between-group factors was conducted to determine any potential impacts of these factors. Behaviours in the splash, three-chamber, and tail suspension tests, as well as the gene expression of TPH1, TPH2, IDO1, TDO2, Slc6a4, Htr1a, Htr1b, Htr2c, and Htr4, were analyzed using a series of 3-way ANOVAs with Sex (Females versus Males), Diet (Purified versus Med-based), and Stressor (No stressor versus Stressor) serving as the between-group factors. Follow-up comparisons of the simple effects of the significant interactions of these ANOVAs, comprised of t-tests with a Bonferroni correction to maintain the family-wise error rate at 0.05, were conducted. The alpha level was set to $p<0.05$ for all analyses.

4. Results

The litter sizes were analyzed using a series of 2 (Diet: Purified diet versus Med-based diet) x 2 (Stressor: No stressor versus Prenatal stressor) between-group ANOVAs. Body weights, food intake, social and depressive-like behaviour, as well as fold changes for the mRNA expression of tryptophan and 5-HT markers were analyzed using a series of 2 (Sex: Females versus Males) x 2 (Diet: Purified diet versus Med-based diet) x 2 (Stressor: No stressor versus Prenatal stressor) between-group ANOVAs. Follow-up comparisons of the simple effects of the significant ANOVAs interactions used t-tests with a Bonferroni correction to maintain the family-wise error rate at 0.05.

4.1 Size of litters at birth

Analysis of litter size was done to verify if there was a difference in litter size at birth between diet groups and stress conditions. Dams fed the Med-based diet gave birth to more pups

compared to those that were fed the Purified diet, irrespective of stress ($F_{(1,25)}$ = 6.701; p = 0.16; Supplementary Figure 1).

4.2 Body weight

To verify if the Med-based diet and/or the prenatal stressor would affect female and male pup's body weight at birth and weight gains throughout early development, body weights were taken at birth and every day until weaning (P21) as well as post-weaning at P37 and P67. The percentages of weight change were calculated weekly by subtracting the weight at P7, P14, and P21 from the birth weight, dividing these values by the birth weight, and multiplying these values by 100. For the data post-weaning, the weight at P37 and P67 was subtracted by the weight at P21, divided by the body weight at P21 and multiplied by 100. Sample sizes at each time point differed due to the euthanasia of a number of pups at P7 (for the purpose of another project) and to adult euthanasia due to humane intervention (e.g., over-grooming reflecting distress).

4.2.1 Body weight at birth and prior to weaning

At birth, pups born from a dam fed the Med-based diet weighed less than those born to a dam fed the Purified diet ($p < 0.0001$), irrespective of Sex or Stressor (p's > 0.05; Figure 3A). At P7, prenatally stressed pups had gained more weight than their non-stressed counterparts, $F_{(1,120)}$=9.426, p = 0.003. Although the interaction between Sex, Diet, and Stressor was not significant ($p > 0.05$), based on the *a priori* prediction that the Med-based diet would prevent the prenatal stressor effects in a sex-specific way, follow-up comparisons of the simple effects comprising this interaction were conducted. They revealed that both prenatally females (p = 0.029) and males (p = 0.048) fed the purified diet had gained more weight at P7, an effect that was not seen in prenatally stressed mice fed the Med-based diet (Figure 3B). At P14, the percentages of weight change did not differ between Sex, Diet, and Stressor groups and did not vary as a function

of the interactions between these factors (p's > 0.05; Figure 3C). At P21, which is the day pups were weaned from their mothers, pups fed the Med-based diet had gained more weight than those fed the Purified diet, $F_{(1,120)}$=28.287, $p < 0.0001$ (Figure 3D). In addition, the prenatal stressor increased weight gains in both females ($p = 0.035$) and males ($p = 0.047$) fed the Purified diet, but not in mice fed the Med-based diet (p's > 0.05), potentially due to the overall increased weight gains in mice fed this diet.

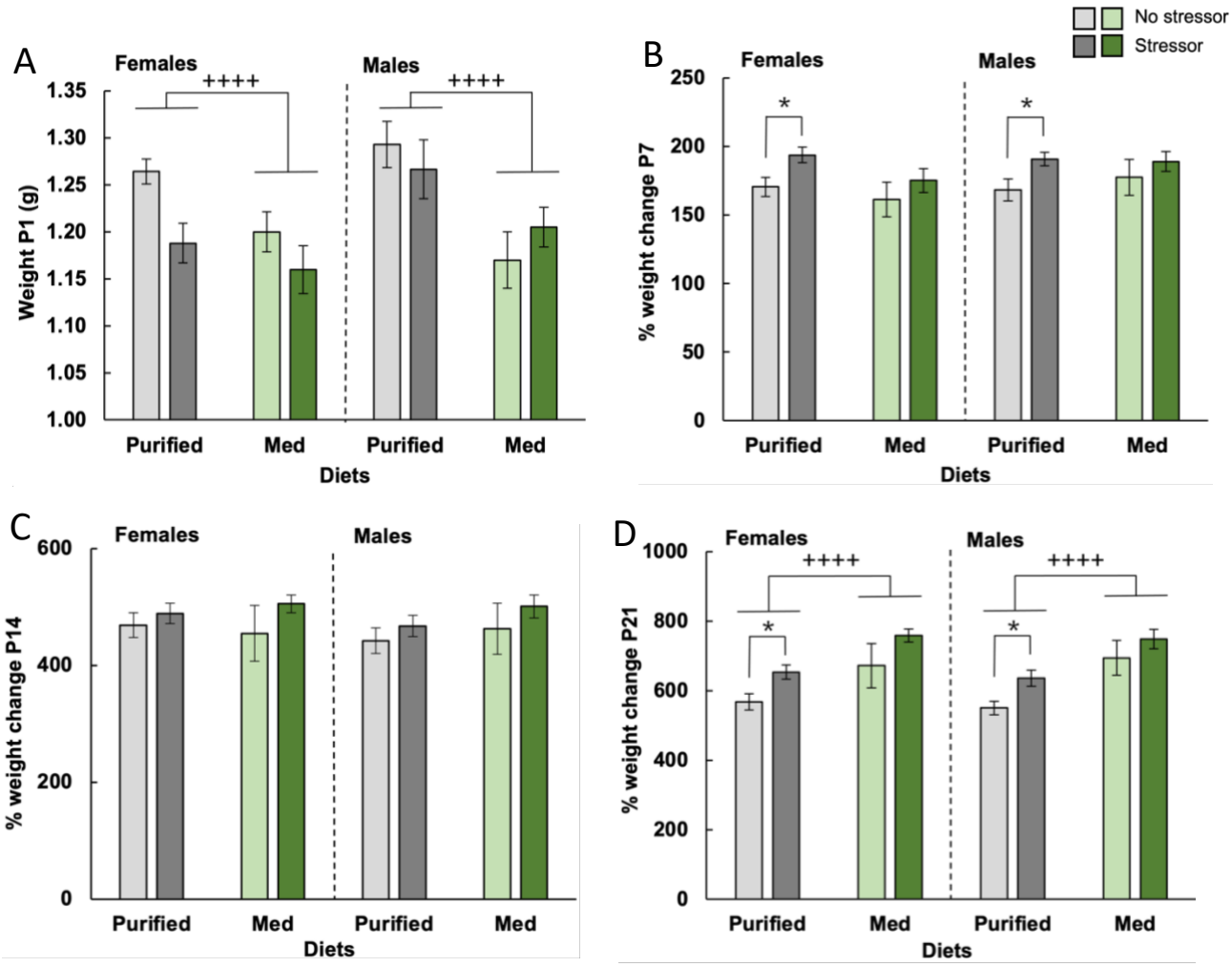


Figure 3. Offspring body weights and weight changes at various time points before weaning as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). (A) Body weight in grams (g) at birth (Postnatal Day [P] 1). **(B)** Percentages of weight change at P7 (%). **(C)** Percentages of weight change at P14 (%). **(D)** Percentages of weight change at P21 (%). Purified diet non-stressed mice (*n*=14M, 14F), Purified diet stressed mice (*n*=18M, 25F), Med-based diet non-stressed mice (*n*=10M, 12F) and Med-based diet stressed mice (*n*= 20M, 15F). Data represent average ± SEM. ++++$p < 0.001$ relative to pups fed the Purified diet. *$p < 0.05$ relative to non-stressed pups fed the Purified diet.

4.2.2 Body weight after weaning

At P21, where pups were weaned from their mothers and placed on their respective diets, pups fed the Med-based diet weighed more than those fed the Purified diet, $F_{(1, 98)}=15.412$, $p < 0.0001$, irrespective of Sex or Stressor (p's > 0.05; Figure 4A). At P37, which corresponds to the halfway mark of the pup adult development in this study, percentages of weight change differed between Sex, $F_{(1, 48)}=44.531$, $p < 0.0001$, Diet, $F_{(1, 48)}=4.556$, $p=0.038$, and Stressor, $F_{(1, 48)}=7.081$, $p=0.011$. Specifically, at P37 males had gained more weight than females, mice fed the Med-based diet had gained less weight than those fed the Purified diet, and prenatally stressed mice had gained less weight than their non-stressed counterparts (Figure 4B). At P67, which corresponds to adulthood on the first day of behavioural testing, males had gained more weight compared to females, $F_{(1, 46)}=75.048$, $p < 0.0001$, irrespective of Diet or Stressor that were not significant anymore (p's > 0.05; Figure 4C).

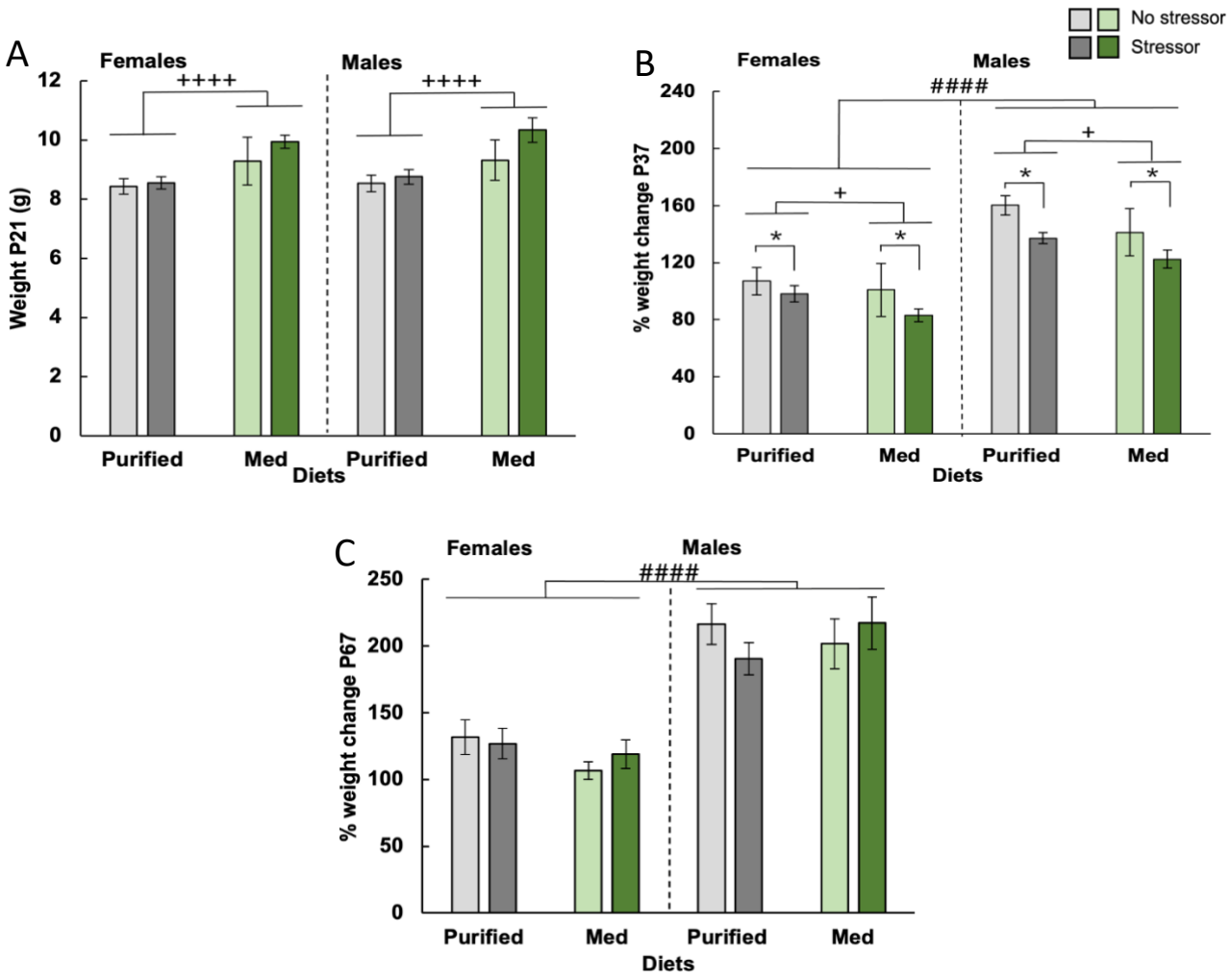


Figure 4. Offspring body weights and weight changes at various time points after weaning as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). (A) Body weights in grams (g) at Postnatal Day [P] 21. **(B)** Percentages of weight change at P37 (%). **(C)** Percentages of body change at P67 (%). Purified diet non-stressed mice (*n*=7M, 8F), Purified diet stressed mice (*n*=6M, 7F), Med-based diet non-stressed mice (*n*=6M, 6F) and Med-based diet stressed mice (*n*=6M, 8F). Data represent average ± SEM. ####$p < 0.0001$ relative to females. +$p < 0.05$ and ++++ $p < 0.0001$ relative to pups fed the Purified diet. *$p < 0.05$ relative to non-stressed pups.

4.3 Food intake

Food intake was analyzed from each cage, providing a value for two sibling mice of the same sex housed together, by weighing food pellets before placing them in the food basket and weighing the amount left 48 (P23 and P27) to 72 hours (P49 and P61) later. Sample sizes at each time point differed due to adult euthanasia because of humane intervention, as described above. Additionally, sample sizes for food intake are smaller than the body weight sample size as each measure is representative of two sibling mice housing together in one cage.

At P23, food intake over the 48 hours after weaning did not differ between Sex, Diet, and Stressor groups and did not vary as a function of the interactions between these factors (p's > 0.05; Figure 5A). At P27, roughly one week after the offspring were weaned, a 48-hour measure demonstrated an effect of the diet and of the stressor (Figure 5B). Specifically, mice fed the Med-based diet consumed less food than those fed the Purified diet (p = 0.023) and prenatally stressed mice ate more than non-stressed mice (p = 0.004). At P49, the effects of diet persisted, with the mice being fed the Med-based diet consuming less food than those fed the Purified (p = 0.002; Figure 5C). Lastly, at P61, males consumed more food than females (p = 0.007), irrespective of the diet or the stressor (p > 0.05; Figure 5D).

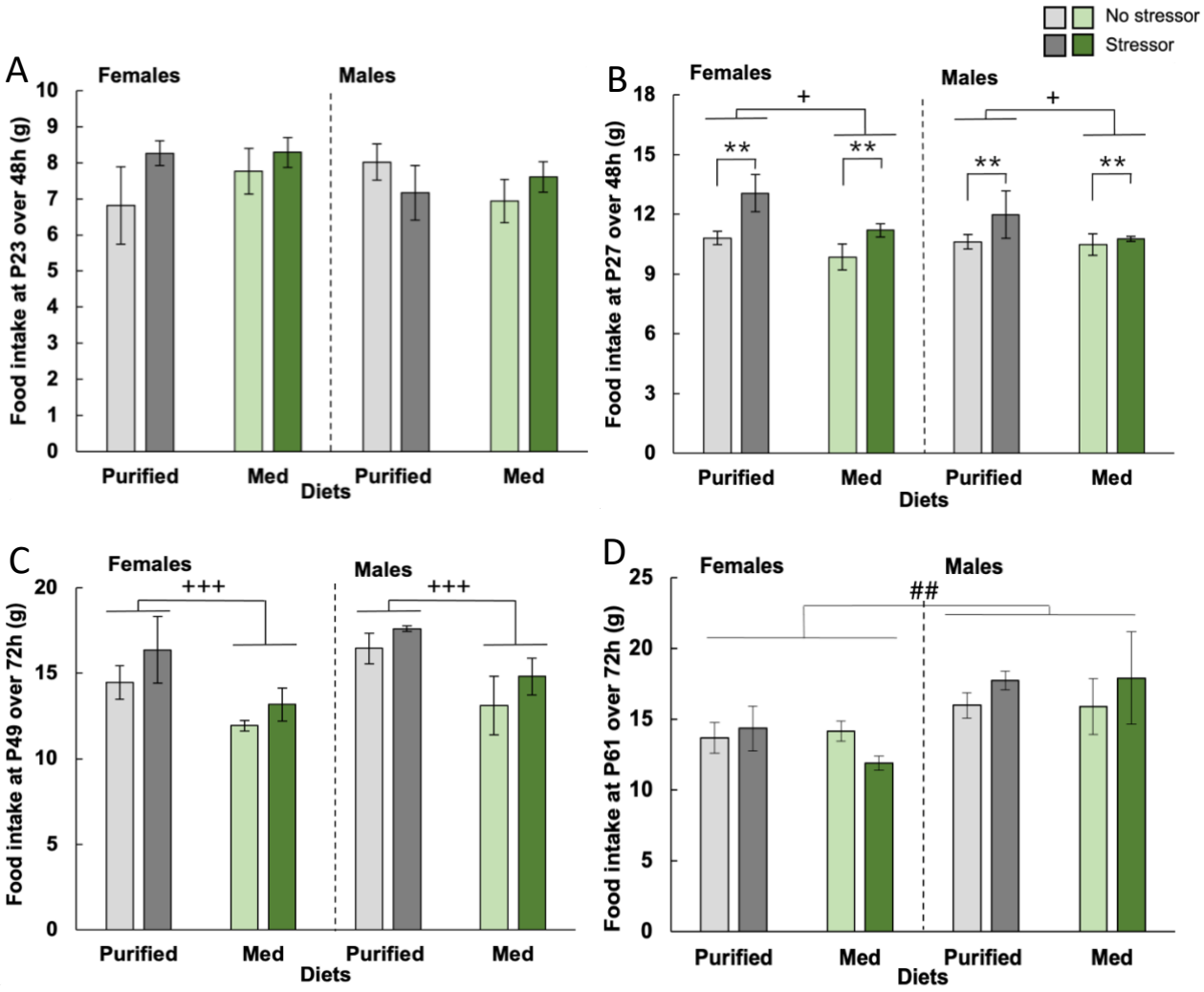


Figure 5. Offspring food intake at various time points as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). **(A)** 48-hour measure of food intake at Postnatal Day [P] 23 (in grams [g]). **(B)** 48-hour measure of food intake at P27 (in grams [g]). **(C)** 72-hour measure of food intake at P49 (in grams [g]). **(D)** 72-hour measure of food intake at P61 (in grams [g]). Purified diet non-stressed mice (*n*=4-6M, 4-7F), Purified diet stressed mice (*n*=3-6M, 4-6F), Med-based diet non-stressed mice (*n*=2-5M, 3-6F) and Med-based diet stressed mice (*n*=3-8M, 4-7F). Data represent average ± SEM. ##$p < 0.01$ relative to females. +$p < 0.05$ and +++$p < 0.005$ relative to mice fed the Purified diet. ***$p < 0.005$ relative to non-stressed mice.

4.4 Behavioural testing

4.4.1 Grooming behaviour: splash test

The splash test analyzes grooming behaviour, a measure of the motivation to self-care of mice, which is reflective of depressive-like states (Sachs, 1988; Smolinsky et al., 2009). A mouse that spends less time grooming is considered to have lower self-care and therefore more depressive-like behaviour (Isingrini et al., 2010; Kalueff et al., 2016; Smolinsky et al., 2009). In the current study, three measures were analyzed in this test: the total time spent grooming, the average length of grooming sessions, and the total number of grooming sessions. For the total time spent grooming and the average duration of grooming sessions, although the interactions between Sex, Diet, and Stressor were not significant ($p > 0.05$), based on the *a priori* prediction that the Med-based diet would prevent the behavioural effects of the prenatal stressor in a sex-specific way, follow-up comparisons of the simple effects comprising this interaction were conducted. These analyses revealed that prenatally stressed mice fed the Purified diet spent less time grooming than their non-stressed counterparts ($p = 0.009$), an effect that was not apparent in mice fed the Med-based diet ($p > 0.05$; Figure 6A). As well, males spent less time grooming than females ($p = 0.039$), irrespective of Diet or Stressor ($p > 0.05$; Figure 6A). Additionally, prenatally stressed mice had shorter grooming sessions on average ($p = 0.050$), an effect that was not seen in the offspring fed the Med-based diet ($p > 0.05$; Figure 6B). Finally, the total number of grooming sessions did not differ between Sex, Diet, and Stressor groups and did not vary as a function of the interactions between these factors (p's > 0.05; Figure 6C).

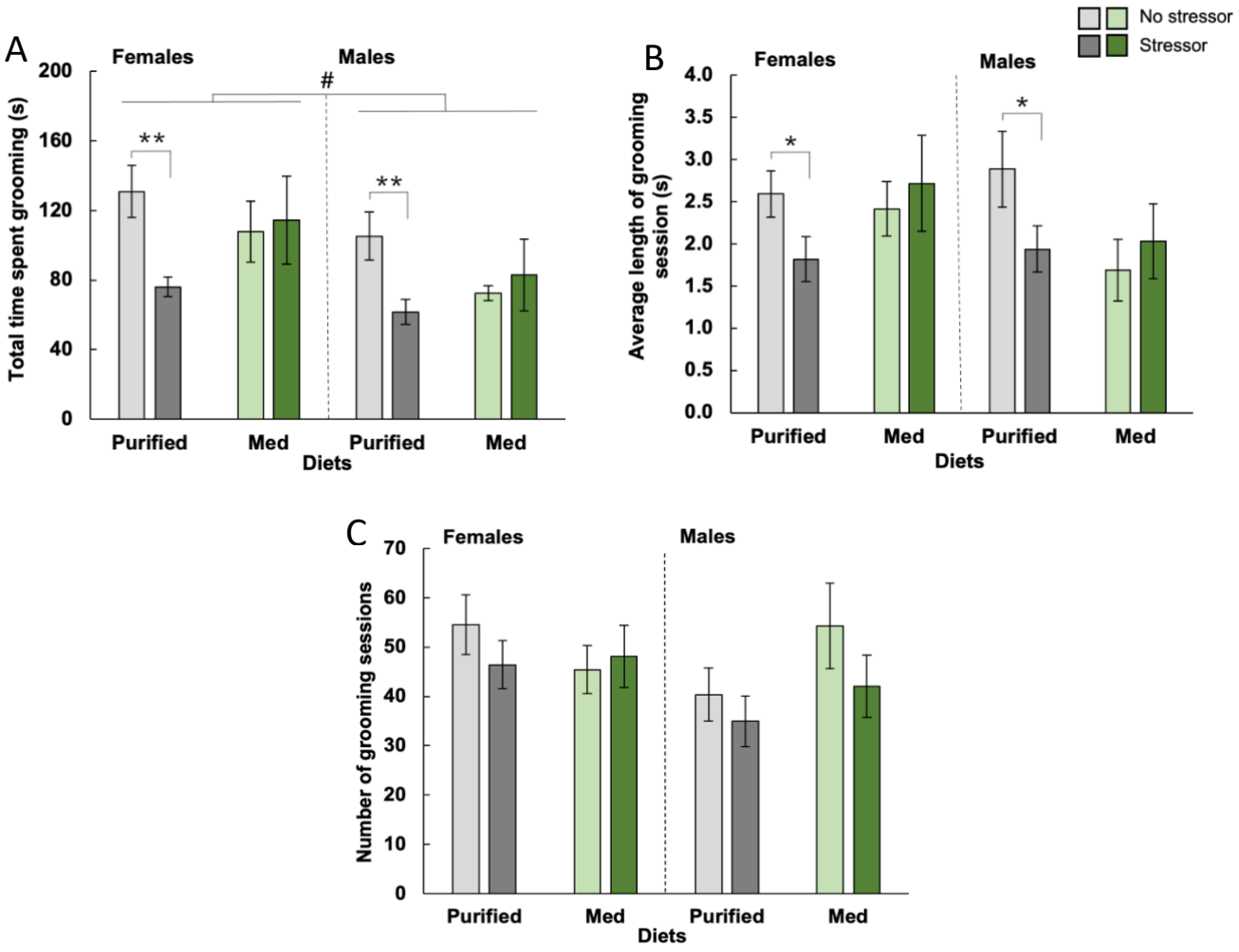


Figure 6. Measures of grooming behaviour in the splash test as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). (A) Total time spent grooming in seconds (s). **(B)** Average length of grooming sessions in seconds (s). **(C)** Total number of grooming sessions. Purified diet non-stressed mice (*n*=12M, 12F), Purified diet stressed mice (*n*=8M, 9F), Med-based diet non-stressed mice (*n*=8M, 10F) and Med-based diet stressed mice (*n*=11M, 10F). Data represent average ± SEM. #$p < 0.05$ relative to females **$p < 0.01$ and *$p \leq 0.05$ relative to non-stressed females.

4.4.2 Social behaviour: three-chamber test

The three-chamber test includes two sections, one evaluating the sociability of the mouse and the second evaluating its preference for social novelty. Mice that exhibit social deficits spend less time interacting with a mouse versus an inanimate object during the first part of the test, and less time interacting with a novel mouse versus a familiar mouse in the second part of the test (Nadler et al., 2004; Yang et al., 2011). In the current study, although the interactions between Sex, Diet, and Stressor were not significant (p's > 0.05), based on the *a priori* prediction that the Med-based diet would prevent the behavioural effects of the prenatal stressor in a sex-specific way, follow-up comparisons of the simple effects comprising this interaction were conducted. They revealed that the prenatal stressor increased the Sociability index (time spent with a target mouse/time spent with an object) in females fed the Purified diet ($p = 0.019$; Figure 7A), an effect that was not seen in prenatally stressed females fed the Med-based diet ($p > 0.05$). The Social Novelty index (time spent with a novel mouse/time spent with a familiar mouse) did not differ between Sex, Diet, and Stressor groups and did not vary as a function of the interactions between these factors (p's > 0.05; Figure 7B).

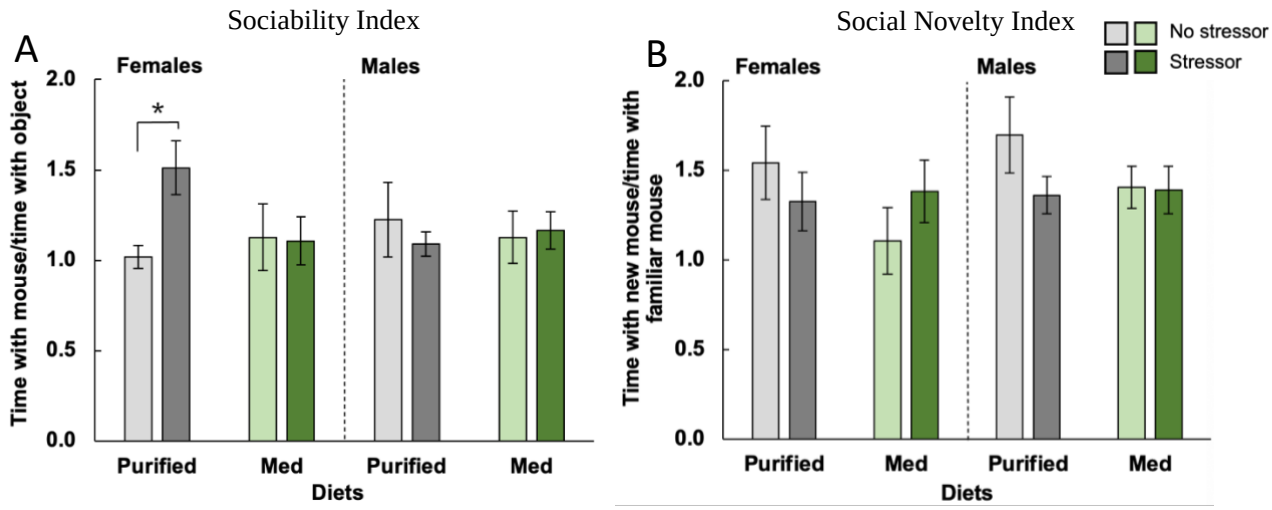


Figure 7. Ratio of time spent in each chamber during the three-chamber test as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). (A) Ratio of time spent with a target mouse versus a novel object (Sociability index). **(B)** Ratio of time spent with a new target mouse versus a familiar mouse (Social novelty index). Purified diet non-stressed mice (*n*=12M, 12F), Purified diet stressed mice (*n*=8M, 9F), Med-based diet non-stressed mice (*n*=8M, 10F) and Med-based diet stressed mice (*n*=11M, 10F). Data represent average ± SEM. **p* < 0.05 relative to non-stressed females fed the Purified diet.

4.4.3 Passive coping behaviour: tail suspension test

To measure active versus passive coping behaviour in the face of a stressful situation, the tail suspension test was used (Cryan, Mombereau, et al., 2005). Mice that spend more time immobile during this test are exhibiting more passive coping behaviour, which is suggestive of depressive-like behaviour (Cryan, Mombereau, et al., 2005). The data indicated that the time spent immobile in the tail suspension test differed between Sex and Diet groups (Figure 8). Males spent less time immobile than females, $F_{(1,69)}=31.780$, $p < 0.0001$, irrespective of Stressor and Diet. Although the interaction Sex x Diet x Stressor was not significant ($p > 0.05$), based on the *a priori* prediction that the Med-based diet would prevent the behavioural effects of the prenatal stressor in a sex-specific way, follow-up comparisons of the simple effects comprising this interaction were conducted. They revealed that the Med-based diet reduced the time spent immobile in prenatally stressed females compared to their counterparts fed the Purified diet ($p = 0.025$).

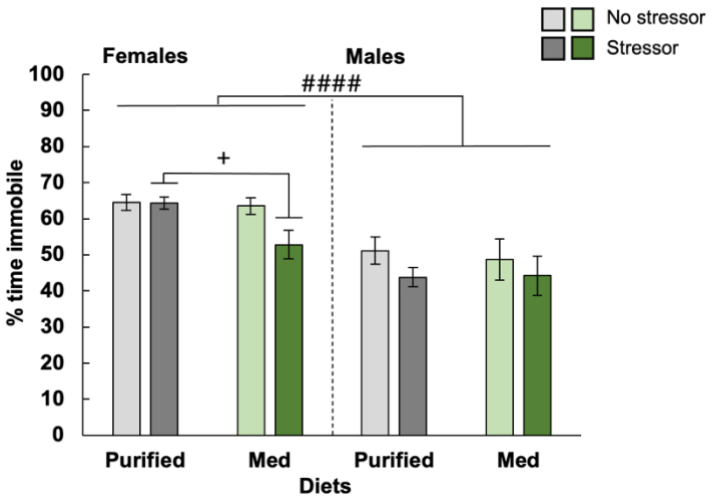


Figure 8. Percentage of time spent immobile during the tail suspension test as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). Purified diet non-stressed mice (*n*=12M, 12F), Purified diet stressed mice (*n*=8M, 9F), Med-based diet non-stressed mice (*n*=8M, 10F) and Med-based diet stressed mice (*n*=11M, 10F). Data represent average ± SEM. +$p < 0.05$ relative to females fed the Purified diet. ####$p < 0.0001$ relative to females.

4.5 Tryptophan metabolism and the 5-HT$_4$ receptor in the colon

Tryptophan can be metabolized via two distinct pathways, the kynurenine or the 5-HT pathways. To analyze the two tryptophan metabolism pathways in the colon, the mRNA expression of TPH1 and IDO1 was determined. In addition, the mRNA expression of the 5-HT transporter and the 5-HT$_4$ receptor, which is largely expressed in the colon (Hoffman et al., 2012), was examined to analyze 5-HT uptake and neurotransmission using the genes Slc6a4 and Htr4, respectively.

The expression of TPH1 in the colon was affected by Diet, $F_{(1,62)}$=7.829, p = 0.007, and varied as a function of the interaction between Sex and Diet, $F_{(1,62)}$=4.713, p = 0.034. Follow-up comparisons of the simple effects comprising this interaction showed that the Med-based diet increased colonic expression of TPH1 in males only (p = 0.010), irrespective of whether they had been stressed prenatally (p > 0.05; Figure 9A). In contrast, the expression of IDO1 in the colon was affected by Sex, $F_{(1,62)}$=4.299, p = 0.042, and varied as a function of the interaction between Sex and Stressor, $F_{(1,62)}$=6.767, p = 0.012. Follow-up comparisons of the simple effects comprising this interaction showed that the prenatal stressor increased colonic IDO1 expression in males only (p = 0.014), irrespective of their diet (p > 0.05; Figure 9B). With respect to the 5-HT transporter, colonic Slc6a4 expression was affected by the Stressor, $F_{(1,66)}$=8.344, p = 0.005, and varied as a function of the interaction between Sex, Diet, and Stressor, $F_{(1,66)}$=4.497, p = 0.038. Follow-up comparisons of the simple effects comprising this interaction showed that the Med-based diet increased the expression of Slc6a4 in the colon in non-stressed males (p = 0.029) but not in those that were prenatally stressed (p > 0.05; Figure 9C). Finally, the colonic expression of Htr4 varied as a function of the interaction between Sex and Stressor, $F_{(1,65)}$=4.381, p = 0.040. Although the interaction between Sex, Diet, and Stressor was not significant (p > 0.05), based on the *a priori*

prediction that the Med-based diet would prevent the prenatal stressor effects on colonic markers in a sex-specific way, follow-up comparisons of the simple effects comprising this interaction were conducted. They revealed that the Med-based diet decreased colonic Htr4 expression in non-stressed males ($p = 0.031$) but not in those that were prenatally stressed or in females (p's > 0.05; Figure 9D).

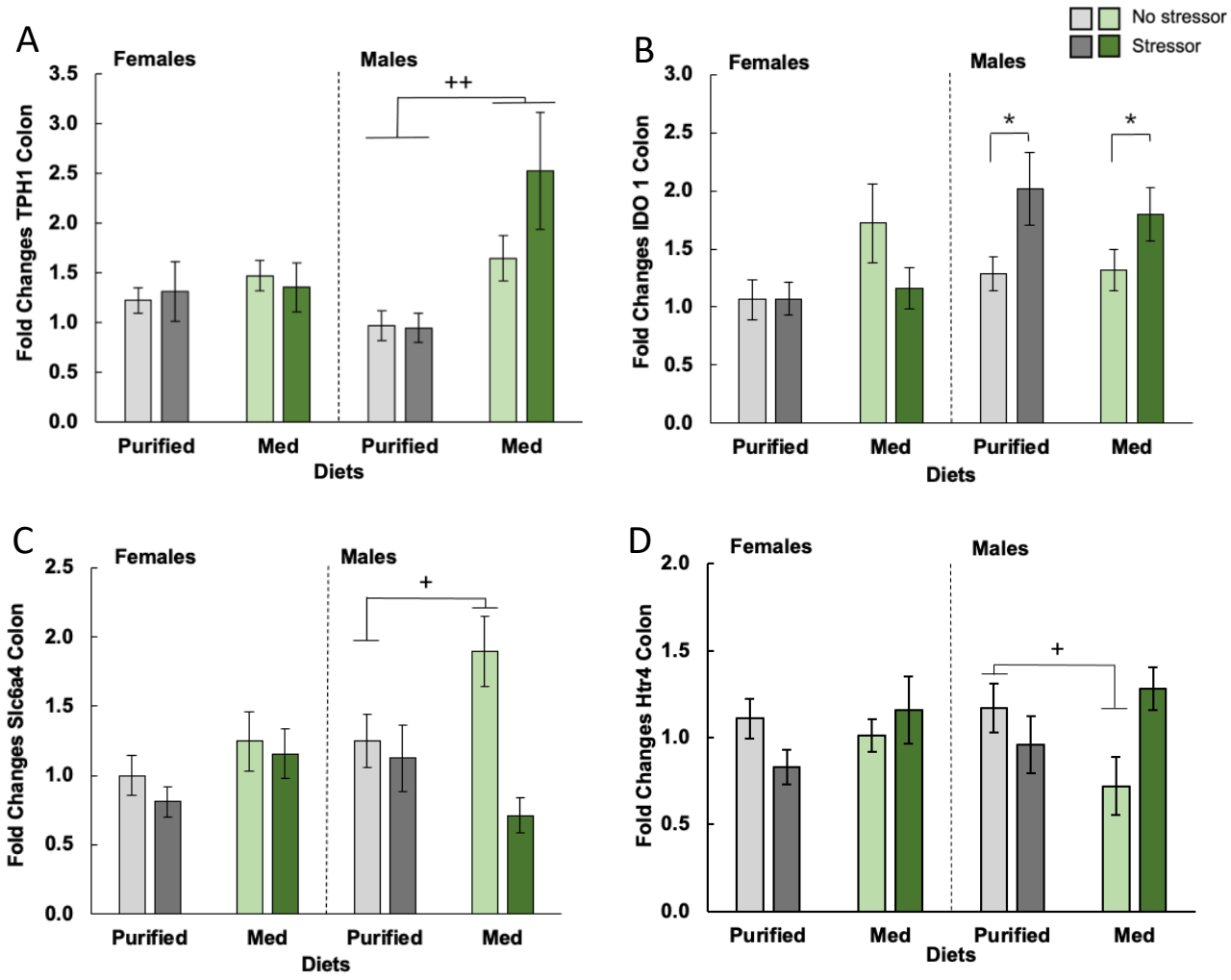
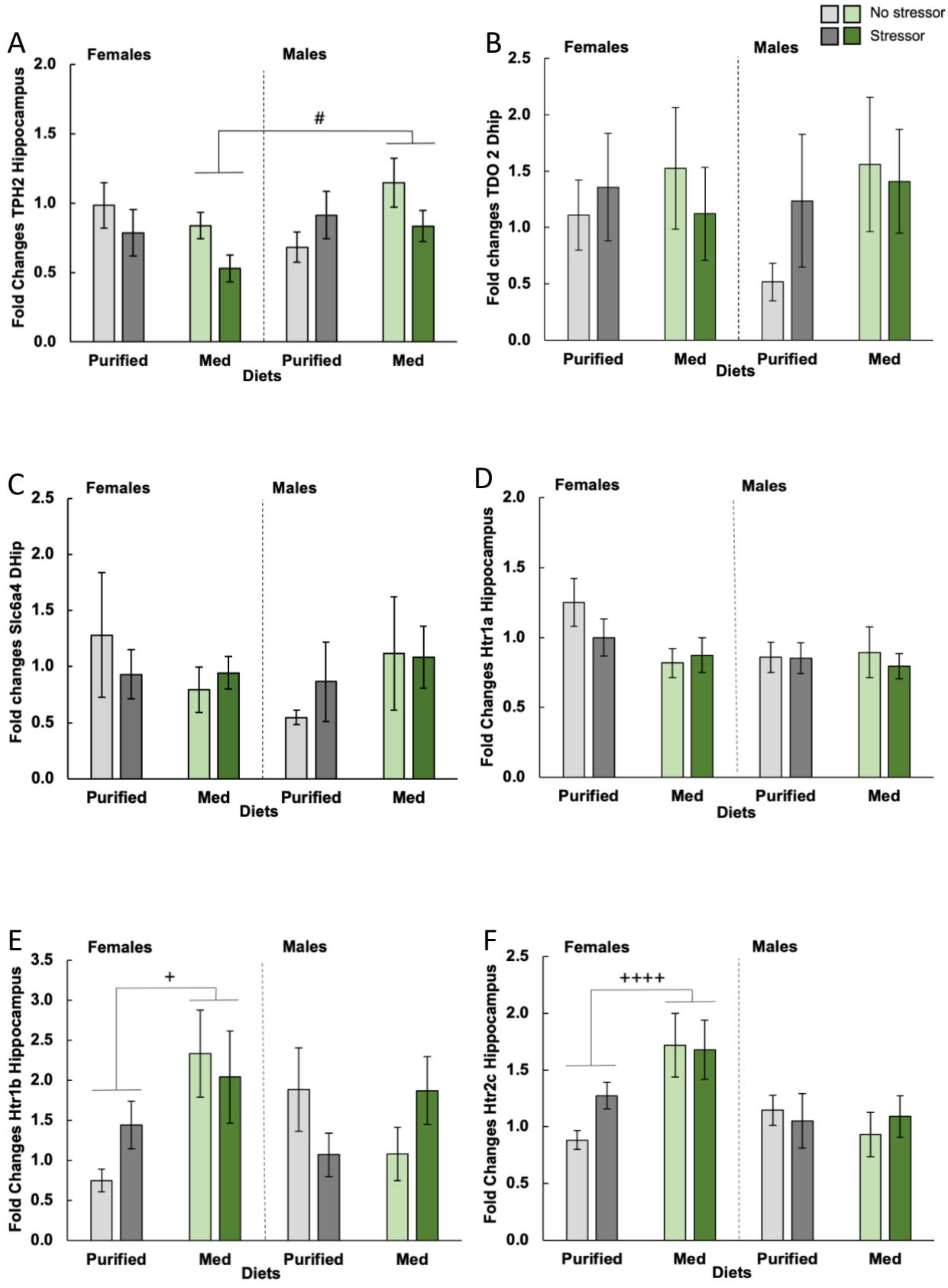


Figure 9. Fold changes for the mRNA expression of tryptophan metabolism rate-limiting enzymes, the 5-HT transporter, and the 5-HT$_4$ receptor in the colon as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). Colonic gene expression of **(A)** Serotonergic rate-limiting enzyme tryptophan hydroxylase 1 (TPH1), **(B)** Kynurenine rate-limiting enzyme indoleamine 2,3-dioxygenase 1 (IDO1), **(C)** Gene encoding 5-HT transporter (Slc6a4), and **(D)** Gene encoding 5-HT$_4$ receptor (Htr4). Purified diet non-stressed mice (n=12M, 10F), Purified diet stressed mice (n=8M, 9F), Med-based diet non-stressed mice (n=8M, 10F) and Med-based diet stressed mice (n=11M, 10F). Data represent fold changes ± SEM. +$p < 0.05$ and ++$p < 0.01$ relative to males fed the Purified diet. *$p < 0.05$ relative to non-stressed males.

4.6 Tryptophan metabolism and 5-HT receptors in the hippocampus

To analyze the two tryptophan metabolism pathways in the hippocampus, the mRNA expression of the rate-limiting enzymes TPH2 and TDO2 in this brain region was evaluated. In addition, the mRNA expression of the Slc6a4 gene, which codes for the 5-HT transporter, and of the 5-HT receptors 1A (Htr1a), 1B (Htr1b), 2C (Htr2c), and 4 (Htr4), that are expressed in the hippocampus and have been implicated in the stress response and MDD (Yohn et al., 2017), was determined.

The hippocampal expression of the rate-limiting enzyme TPH2 varied as a function of the interaction between Sex and Diet, $F_{(1,46)}= 4.096$, $p = 0.049$. Follow-up comparisons of the simple effects comprising this interaction revealed that males fed the Med-based diet had a higher expression of TPH2 compared to their female counterparts ($p = 0.026$; Figure 10A). With respect to the 5-HT receptor 1b, its expression in the hippocampus varied as a function of the interaction between Sex, Diet, and Stressor, $F_{(1,60)}= 4.390$, $p = 0.04$. Follow-up comparisons of the simple effects comprising this interaction showed that females fed the Med-based diet had increased expression of Htr1b, $F_{(1,60)} = 7.643$, $p = 0.029$, an effect that was not seen in males ($p > 0.05$; Figure 10E). Lastly, the expression of Htr2c varied as a function of Sex and Diet, $F_{(1,58)}= 6.494$, $p = 0.013$. Females fed the Med-based diet had increased expression of Htr2c ($p = 0.001$), regardless of the stressor, an effect that was not seen in males ($p > 0.05$; Figure 10F). The data indicate that the expression of TDO2 (Figure 10B), Slc6a4 (Figure 10C), Htr1a (Figure 10D), and Htr4 (Figure 10G) did not differ between Sex, Diet, and Stressor groups and did not vary as a function of the interactions between these factors (p's > 0.05).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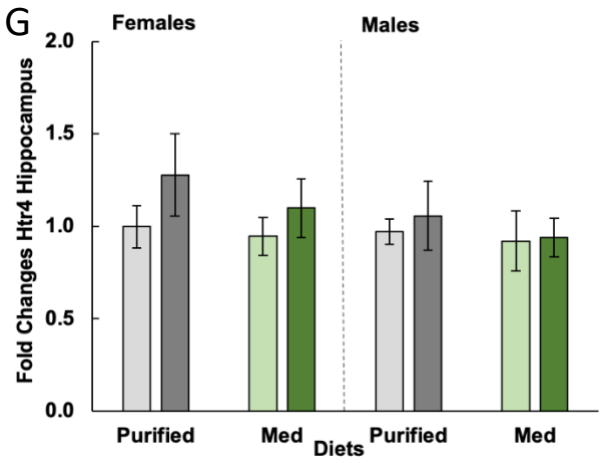


Figure 10. Fold changes for the mRNA expression of tryptophan metabolism rate-limiting enzymes, the 5-HT transporter, and 5-HT receptors in the hippocampus as a function of Sex (Females versus Males), Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). (A) Serotonergic rate-limiting enzyme tryptophan hydroxylase 2 (TPH2), **(B)** Kynurenine rate-limiting enzyme tryptophan 2,3-dioxygenase 2 (TDO2), **(C)** Gene encoding 5-HT transporter (Slc6a4), **(D)** Gene encoding 5-HT_{1a} receptor (Htr1a), **(E)** Gene encoding 5-HT_{1b} receptor (Htr1b), **(F)** Gene encoding 5-HT2c receptor (Htr2c), and **(G)** Gene encoding 5-HT4 receptor (Htr4). Data represent fold changes ± SEM. Purified diet non-stressed (*n*=6-11M, 6-11F), Purified diet stressed (*n*=6M, 6-9F), Med diet non-stressed (*n*=4-6M, 6-9F) and Med diet stressed (*n*= 9-11M, 7-9F). #$p < 0.05$ relative to females. + $p < 0.05$ relative to non-prenatally stressed females fed the Purified control diet. ++++ $p \leq 0.001$ relative to females fed the Purified control diet.

5. Discussion

The main objective of this study was to determine the capacity of a rodent Med-based diet to limit the effects of a prenatal stressor, specifically on depressive-like behaviour and on tryptophan- and 5-HT-related markers in the colon and hippocampus. A secondary objective of this research project was to examine the impact of sex on these outcomes. The Med-based diet limited reductions in grooming seen in both prenatally stressed females and males. In prenatally stressed females, the Med-based diet normalized an increase in sociability and decreased passive coping behaviour. The increase in sociability was not seen in prenatally stressed males or in mice fed the Med-based diet. Curiously, males had less passive coping behaviour than females, although the Med-based diet or the stressor did not affect males in this outcome, demonstrating a sex effect independent of the diet and stressor. In contrast, the effects of the diet and of the prenatal stressor on colonic enzymes of tryptophan metabolism, SERT, and the 5-HT_4 receptor were only seen in males. Specifically, the Med-based diet increased TPH1 and SERT and decreased the 5-HT_4 receptor whereas the prenatal stressor increased IDO1 in mice of this sex. The diet effects seen on the 5-HT transporter and receptor were blocked by the prenatal stressor. In the hippocampus, TPH2 expression was lower in females fed the Med-based diet compared to males. Additionally, only females fed the Med-based diet expressed more of the 5-HT_{1b} and 5-HT_{2c} receptors than their counterparts fed the Purified diet. The findings in this thesis highlight the sex differences in the behavioural, tryptophan- and 5-HT-related colonic and hippocampal outcomes as a result of the Med-based diet and the prenatal stressor. Importantly, they demonstrate the importance of evaluating both sexes when developing potential prevention or treatment options when considering the predisposition to MDD in individuals exposed to a stressor early in development.

5.1 Offspring body weight throughout development was affected by sex, diet, and stressor

As the Purified control and Med-based diets were newly developed, body weight and food intake were assessed to ensure adequate physical growth and consumption of food, respectively. At birth, pups born to a dam fed the Med-based diet weighed less than those fed the Purified diet. One potential explanation for this could be that dams fed the Med-based diet gave birth to significantly larger litters compared to those fed the Purified diet, and thus the pups born in larger litters weighed less. During the offspring's early development period at P7, while the dams' breast milk was the primary source of nutrition for the offspring, the Med-based diet normalized the higher weight gain induced by the prenatal stressor. By P21, although the prenatal stressor still induced a higher weight gain in those fed the Purified control diet, the pups fed the Med-based diet had gained more than their counterparts and the stressor did not have an effect on their body weight. This suggests that the breast milk from dams fed the Med-based diet allowed for a higher weight gain than the Purified control. Studies looking at the effects of a prenatal stressor on offspring body weight demonstrate variable results. In addition, these reports administered a stressor at different time points in the pregnancy than the current thesis. While some demonstrate that a prenatal stress administered late in the pregnancy led to a lower birth weight and body weight throughout development (Barlow et al., 1978; Jeje & Raji, 2017), some have found that a prenatal stressor late in gestation led to a higher birth weight (Mueller & Bale, 2006) and others have found that the prenatal stressor did not lead to significant alterations in body weight at birth (Dmello & Liu, 2006). As some of these results vary from the ones found in this research project, it may be due to differences in rodent strains, in the type of prenatal stressor or in the timing of the prenatal stressor, as the differences in these variables, such as the type of prenatal stressor used, have all been shown to lead to different results (Freide & Weinstock, 1984; Mueller & Bale, 2006; Stöhr et al., 1998). Numerous studies have reported the benefits of the Mediterranean diet during

pregnancy on offspring development (Zaragoza-Martí et al., 2022), such as the reduced risk of low birth weight, which is associated with neonatal complications (Assaf-Balut et al., 2018), decreased adiposity levels, and an overall better metabolic function during development (Chatzi et al., 2017). These findings could potentially explain how the Med-based diet normalized the increased weight gain induced by prenatal stress at P7, especially during early development, highlighting a potential protective effect of maternal breast milk from dams fed the Med-based diet against increased weight gain in prenatally stressed offspring.

After weaning, when the offspring were separated from their mother and were able to feed on their own, the sex, diet and stressor all influenced the offspring's weight gain. On the day of weaning, P21, pups from dams fed the Med-based diet weighed more than those fed the Purified control diet. At P37, which corresponds to early adulthood and near the halfway mark of development of this research project, offspring that were prenatally stressed gained less weight than those that were not prenatally stressed, which is consistent with previous reports examining the same time of development in rat offspring (Barlow et al., 1978; Jeje & Raji, 2017). In addition, at P37, the offspring fed the Med-based diet gained less weight than those fed the Purified diet. This could potentially reflect a stabilization of their weight in adulthood. During young adult development, at P27 and P49 offspring fed the Med-based diet ate significantly less than their counterparts, which may have contributed to the smaller weight gain at P37. At P37 and P67, there is also a sex difference as males are gaining more weight than females, which is consistent with the food intake data as males ate more food than females at P61. This difference could also be due to the age of the offspring, as P37 is after the onset of puberty (Cross et al., 2021; Edwards, 1970; Gore et al., 1999; Ismail et al., 2011; Zhou et al., 2007), meaning that sex hormones are potentially influencing the offspring body weight. Previous literature identified differences in hormones

between males and females which leads to a difference in the regulation of body weight (Shi & Clegg, 2009). Leptin, a hormone that inhibits hunger, was found to be higher in females (Demerath et al., 1999) and interacted with estrogen, a female sex hormone (Quinton et al., 1999). This could explain why females consumed less food than males in adulthood.

5.2 Behavioural outcomes were altered by sex, diet, and stressor

It has previously been demonstrated that exposure to a stressor *in utero*, specifically during the second trimester, increased depressive-like behaviour in adulthood (Depino, 2015; Lin & Wang, 2014; Osborne, 2020) and that a tryptophan-rich diet attenuated depressive-like behaviour resulting from chronic unpredictable mild stress (Wang et al., 2022), leading us to think that a Med-based diet, which is high in foods containing tryptophan and components involved in its metabolism, could prevent the behavioural effects of a prenatal stressor. In the current research project, the Med-based diet limited the increase in depressive-like behaviour resulting from the prenatal stressor, mostly in females.

In the splash test, the prenatal stressor decreased the average duration of grooming sessions, and the total time spent grooming in males and females. This means that, although they relatively had the same number of grooming sessions, mice that were prenatally stressed spent less time cleaning themselves in the 10-minute period, and each grooming session was shorter. This could potentially represent unorganized grooming sessions, although this was not analyzed. The decrease in time spent grooming was normalized by the Med-based diet in both males and females. In addition, males spent overall less time grooming than females, irrespective of diet or stressor. Interestingly, previous reports found that adult male C57BL/6N mice spent more time grooming than their female counterparts (Pitzer et al., 2022; Sens et al., 2017). As studies have reported that a decrease in grooming is suggestive of lower motivation to self-care, which is a feature of

depressive-like behaviour (Isingrini et al., 2010; Kalueff et al., 2016; Smolinsky et al., 2009), the results from the splash test are indicative that the Med-based did normalize the increase in depressive-like behaviour induced by the prenatal stressor in both female and male offspring.

Results from the three-chamber test were not what was hypothesized, as it was previously shown that, in adult male offspring, exposure to a prenatal stressor during the third trimester decreased sociability (Gur et al., 2019). Another study using a chronic model of stress by injecting mice with corticosterone found that stressed females also had a decrease in sociability (Berger et al., 2019). Conversely, in the current thesis, the prenatal stressor did not have an effect in males and increased sociability in females. Additionally, mice typically spend more time with a new mouse than one they have had previous interactions (Moy et al., 2004), but no difference was seen in this project. Depressive symptoms are typically associated with social isolation (Elmer & Stadtfeld, 2020) and thus it is uncertain if the increase in sociability as a result of the prenatal stressor in females is a positive or negative outcome. However, a report found that social support improved cognitive tasks in mice exposed to stressors (Kim et al., 2018) and could reduce the stress of isolation (Denommé & Mason, 2022). As the mice were separated from their housing partner during this test, the prenatally stressed offspring may have needed more social support, an effect that was only seen in females, suggesting that the stressor increased the need for social support in this sex only. The Med-based did normalize this effect, possibly suggesting that the females fed this diet required less social support during this test. The variance in experimental procedures may explain the differences in sociability and social novelty outcomes. For example, previous studies did not indicate the strength of the lighting (Lux) used (Berger et al., 2019; Elmer & Stadtfeld, 2020), while others indicated a much higher Lux than the current study (Kaidanovich-Beilin et al., 2011). A recent review highlighted the need for standardization of Lux used while

conducting behavioural testing as typically, the three-chamber test is done with less lighting to increase sociability (Neuwirth et al., 2022).

During the tail suspension test, although the prenatal stressor did not increase the time spent immobile in either sex as was predicted, the Med-based diet decreased the time spent immobile in prenatally stressed mice and this effect was seen only in females. This suggests that the dietary intervention used in this research project limited the depressive-like behaviour in this group. Additionally, an overall sex effect was noted as males spent less time immobile, irrespective of diet or stressor. Considering that women are twice as likely to be diagnosed with MDD than men (GBD 2019 Mental Disorders Collaborator, 2022), the lower depressive-like behaviour in males compared to females appears to be consistent with these statistics. Previous studies found that a prenatal stressor administered during the second trimester increased immobility in males only (Majidi-Zolbanin et al., 2015), in both males and females (Lin & Wang, 2014), in neither sex (Mueller & Bale, 2008) or chose to exclude females altogether and found increased depressive-like behaviour in males (Depino, 2015).

The behavioural findings of this research project further highlight the need for studies to consider both sexes when examining animal models of depression and potential treatments. The outcomes listed above revealed that both the prenatal stressor and the diet affected the offspring in a sex-specific manner, suggesting that biology such as sex hormones may be interacting with these factors. As the Med-based diet normalized a decrease in time spent grooming in the splash test and an increase in sociability in females resulting from the stressor, and decreased immobility time during the tail suspension test in prenatally stressed females, this suggests that the Med-based diet improves behaviour in adult females. There was an overall sex effect as males spent overall less time grooming in the splash test, and less time immobile during the tail suspension test, irrespective

of stressor. These results are rather contradicting as the decrease in time spent grooming suggests less motivation to self-care (Isingrini et al., 2010; Kalueff et al., 2016; Smolinsky et al., 2009) and less time immobile suggests more active coping, therefore less depressive-like behaviour (Cryan, Mombereau, et al., 2005). However, previous work that demonstrated males spent more time grooming compared to females used a 10% sucrose solution (Pitzer et al., 2022; Sens et al., 2017), whereas a 1% sucrose solution was used in the present thesis. The difference in the administration of the splash test could potentially explain the different outcomes.

5.3 Modulation of the colonic tryptophan metabolic pathways and 5-HT neurotransmission by the diet and stressor was seen only in males

Prior studies have found that tryptophan metabolism was altered in the colon as a result of prenatal stress (Galley et al., 2021) and that a tryptophan-rich diet prevented alterations to tryptophan metabolism induced by chronic unpredictable mild stress (Wang et al., 2022). Considering these findings, the capacity of the Med-based diet, which is high in foods containing tryptophan and other nutrients involved in 5-HT metabolism, to prevent alterations in the tryptophan metabolism in the colon was assessed. In contrast to the results from Galley et al. (2021), results from this thesis show that the prenatal stressor and the Med-based diet had altered enzymes of the tryptophan metabolic pathway, 5-HT transporter and the 5-HT_4 receptor only in male offspring, demonstrating a sex-dependant effect on tryptophan metabolism and 5-HT system.

The rate-limiting enzyme for 5-HT synthesis, TPH1, was increased in the colon of males fed the Med-based diet, irrespective of the stressor. The observation that stressor did not alter the expression of TPH1 is consistent with previous work looking at the effects of prenatal stress in the colon of adult offspring (Galley et al., 2021). Considering that this enzyme is necessary for the conversion of tryptophan to 5-HT (Höglund et al., 2019), the findings suggest that the Med-based

diet increased the potential for colonic 5-HT synthesis in males, and that this effect was maintained in the prenatally stressed group. This could also suggest that the Med-based diet in males promoted the 5-HT metabolic pathway as opposed to the kynurenine pathway in the colon but this seems unlikely, given that the diet had no effects on colonic IDO1 expression. IDO1 is an enzyme that is increased by the presence of pro-inflammatory cytokines and chronic exposure to stress, thereby increasing tryptophan metabolism into the kynurenine pathway and decreasing 5-HT synthesis in the face of inflammation and/or stress (Höglund et al., 2019). Although an alteration in the offspring colonic expression of TDO2 as a result of a prenatal stressor was previously demonstrated (Galley et al., 2021), and an increase in IDO1 in the colon of mice subject to chronic restraint stress has been noted (Deng et al., 2021), IDO1 expression in the colon of offspring subject to a prenatal stressor has not been examined. Seeing as the expression of this enzyme was elevated in the placenta of dams subject to a prenatal stressor (Galley et al., 2021), the findings from this thesis demonstrate that the increase of this enzyme persists in the colon of the offspring until adulthood, and the Med-based diet did not normalize this effect. The increase in TPH1 seen in the colon of males fed the Med-based diet could be due to the higher intake of the essential amino acid in the diet, thus increasing the tryptophan metabolic rate.

As for the 5-HT transporter and the 5-HT_4 receptor, a diet effect was seen in their colonic expression. SERT was increased in the colon of males fed the Med-based diet while the 5-HT_4 receptor was decreased however, this effect was not seen in the males that were prenatally stressed, suggesting a potential interaction. A study using dextran sodium sulfate to increase permeability in the colon and create an inflammatory response found that this procedure decreased SERT and the 5-HT_4 receptor expression in the colon and that a supplement of tryptophan attenuated these effects (Wang et al., 2020). Interestingly, in the present thesis, the prenatal stressor did not alter

the expression of either of these genes but may have interacted with the diet, thus limiting the increase in transporter and decrease in receptor expression. The 5-HT transporter plays an important role in keeping homeostasis of 5-HT in the colon as it reuptakes the neurotransmitter into the epithelium cells, therefore removing it from the intestinal lumen (Mawe & Hoffman, 2013). A decreased expression of SERT led to an increased concentration of 5-HT in the colon (Bian et al., 2007; Linden et al., 2003), thus the increase in SERT may be a result of an increase of 5-HT synthesis, to maintain the colonic serotonergic concentration in homeostasis. The 5-HT_4 receptor is largely expressed in the colon and notably plays a role in gut motility (Mawe & Hoffman, 2013). In the present thesis, the expression of this receptor was decreased in males that were fed the Med-based diet. Interestingly, it was demonstrated that, in a rat model of colonic irritation used to examine visceral hypersensitivity or pain, colonic irritation led to an increased expression of the 5-HT_4 receptor and a decrease in SERT expression (Yan et al., 2012). Considering these findings, this may suggest that the Med-diet reduced visceral pain for adult males, as an inverse effect was seen in this thesis. Unfortunately, this present thesis did not evaluate the binding capacity of the 5-HT_4 receptor, which could have given additional information on the activity of the receptor. Additionally, the concentration of 5-HT in the colon was not assessed. Whether the changes in the expression of SERT and the 5-HT_4 receptor resulting from the Med-based diet reported in this thesis are linked directly to 5-HT concentration changes remains to be explored.

The results from the colonic data in this present thesis demonstrate that the Med-based diet and the prenatal stressor altered tryptophan and serotonergic system in the colon of adult males only. An increase in IDO1 expression in colonic tissue of prenatally stressed offspring was noted, although the Med-based diet could not mitigate this increase. However, the findings demonstrate

that the diet potentially increased 5-HT synthesis as an increase in TPH1, as well as in SERT, was noted. These changes could be related to an increase in 5-HT synthesis and homeostasis as SERT works to remove the neurotransmitter from the lumen. Given the fact that 5-HT concentrations were not examined, this suggestion remains to be explored. Additionally, the increase in SERT and decrease in the 5-HT_4 receptor seen in males fed the Med-based diet may suggest a benefit of the diet in terms of visceral pain regulation, potentially blocked by the prenatal stressor. Tryptophan metabolism, SERT, and the 5-HT_4 receptor were not altered by the diet or the stressor in the colonic tissue of adult females, suggesting that sex hormones such as estrogen and progesterone, could potentially interact with these factors.

5.4 Hippocampal 5-HT receptor expression was influenced by diet in females

The hippocampus is a brain region implicated in MDD and sensitive to stress (Belleau et al., 2019). Considering that previous studies found that prenatal stress altered tryptophan metabolism in this region (Galley et al., 2021), this thesis examined if a Med-based diet could limit the alterations to tryptophan metabolism, SERT and 5-HT receptors in offspring subjected to a prenatal stressor.

Most studies to date looking at the effects of a prenatal stressor on the serotonergic system only included males (Akatsu et al., 2015; Guerrero et al., 2020), creating a large knowledge gap in the literature. In the present study, sex-specific effects of the dietary intervention on 5-HT rate-limiting enzyme and 5-HT receptors were reported in the hippocampus. Specifically, the 5-HT rate-limiting enzyme TPH2 was increased in males fed the Med-based diet compared to females, suggesting that the diet increased the potential to synthesize tryptophan into 5-HT to a larger extent in males. No effect of the stressor was noted on the expression of TPH2 in the hippocampus, which is consistent with findings from Galley et al (2021). Additionally, this thesis aimed to examine the

effects of a Med-based diet and a prenatal stressor on the serotonergic system in the hippocampus by evaluating the expression of SERT as well as the 5-HT_{1a}, 5-HT_{1b}, 5-$HT_{2c,}$ and 5-HT_4 receptors. This was not done in the report from Galley et al (2021). The Med-based diet increased hippocampal expression of the 5-HT_{1b} and 5-HT_{2c} receptors in females compared to the Purified control diet. Interestingly, the administration of 5-HT_{1b} and 5-HT_{2c} receptor agonists reduced the time immobile during the forced swim test, suggesting that increased activity of these receptors may contribute to a decrease in depressive-like behaviour (Cryan & Lucki, 2000; O'Neill, 2001). The increase in receptor expression may be representative of higher receptor activity. Unfortunately, the binding capacity of the 5-HT receptors was not assessed in this thesis and thus 5-HT receptor activity remains to be explored. Considering the increase of 5-HT_{1b} and 5-HT_{2c} receptors in the hippocampus of adult female offspring as a result of the Med-based diet in the current study, and previous reports of agonists of these receptors reducing depressive-like behaviour (Cryan & Lucki, 2000; O'Neill, 2001), this suggests that the increase in expression of these receptors may contribute to the normalization of depressive-like behaviour in prenatally stressed females in the present thesis. Importantly, whether the 5-HT receptors increased in female mice fed the Med-based diet were located on pre- versus post-synaptic neurons has not been determined. This is especially important as although most 5-HT_{1b} receptors are found on pre-synaptic neurons (Boschert et al., 1994; Boulenguez et al., 1996; Riad et al., 2000; Sari, 2004; Sari et al., 1997, 1999), their role in stress-related behaviour and mental health outcomes may vary according to their synaptic location (Tiger et al., 2018).

Many markers examined in the hippocampus had no significant changes. Notably, TDO2, one of the rate-limiting enzymes of the kynurenine pathway, SERT, the gene coding for the 5-HT transporter, the 5-HT_{1a} and 5-HT_4 receptors were all genes that were not altered in the

hippocampus. As SERT and the 5-HT_4 receptor were both altered by the Med-based diet in the colon of adult males, the results from the hippocampus suggest that the dietary intervention does not have the same effects along the gut-brain axis.

The data from the hippocampus indicates that the Med-based diet increased the 5-HT rate-limiting enzyme TPH2 in males, thus the potential to synthesize 5-HT in the hippocampus, while increasing the expression of the 5-HT_{1b} and 5-HT_{2c} receptors in females only. As genes that were altered in the colon of males, notably SERT and the 5-HT_4 receptor, were not altered in the hippocampus, this suggests that these changes did not persist along the gut-brain axis. Additionally, as the stressor did not increase TDO2 in the hippocampus of females or males, this suggests that, although it increased IDO1 in the colon, the stressor did not affect the kynurenine pathway in the hippocampus. However, the lack of effect seen in this enzyme may be due to the high variability. The Med-based diet increased the expression of 5-HT_{1b} and 5-HT_{2c} receptors in females while having no effect in males or on the 5-HT_{1a} and 5-HT_4 receptors. Although receptor binding capacity and 5-HT concentrations in the hippocampus remain to be analyzed, this data suggests that the Med-based diet increased 2 receptors that were linked to lower depressive-like behaviour when stimulated (Cryan & Lucki, 2000; O'Neill, 2001) in a sex-specific manner, potentially contributing to the decrease in depressive-like behaviour seen in females.

Taken together, this data demonstrates that the dietary intervention may modulate hippocampal tryptophan metabolism in males, while increasing specific 5-HT receptors in females. This suggests that the dietary intervention used in the present thesis affects the female hippocampus differently than the male hippocampus, highlighting an important sex difference.

5.5 Could postnatal handling have attenuated the effects on behavioural and serotonergic outcomes resulting from the prenatal stressor?

This research project involved handling pups daily and brief separation from the mother for the first 21 days of life. Specifically, dams in the current study were placed in a separate cage as their pups were weighed individually, whereas prior studies examining the effects of prenatal stress left the dams and offspring undisturbed (Galley et al., 2021; Gur et al., 2019; Mueller & Bale, 2006). Male and female offspring subjected to a prenatal stressor had a decrease in sociability in the three-chamber test however, neonatal handling for the first 21 days of life limited the effects of the stressor on the sociability index in males that were prenatally stressed (Vakili Shahrbabaki et al., 2022). Previous reports have also stated that offspring subjected to short sessions of handling during the first few weeks of life had a decreased response to stress in adulthood (Bondar et al., 2018; Levine, 1957, 1962; Levine et al., 1967), as well as increased 5-HT concentrations in three different brain regions of the offspring, including the hippocampus (Papaioannou et al., 2002). Together, these suggest that neonatal handling of the pups in this thesis may have attenuated the changes of the serotonergic system resulting from the prenatal stressor, particularly in the hippocampus, as no effects were seen in males, and no difference was seen in TDO2, SERT, 5-HT_{1a} and 5-HT_4 receptors.

Studies have found that a prenatal stressor reduced the care dams provide to their pups (Gatta et al., 2018) and neonatal handling attenuated these effects (Castelli et al., 2020). When the dam is returned to her home cage after being separated from her pups, she also increased the time spent breastfeeding (Pryce et al., 2001), which could result in an increased weight gain in those offspring. These findings suggest that neonatal handling and maternal separation could potentially explain the higher weight gain seen in prenatally stressed pups in early development. The potential interactions between maternal dietary patterns and maternal care remain an area of interest for future studies. Lastly, it has been reported that mothers provide more care such as breastfeeding to

male offspring than to their female offspring (Moore & Morelli, 1979). Considering these findings, the sex-specific effects reported in this present thesis could be mediated by alterations in maternal care resulting from neonatal handling.

It has previously been demonstrated that males subject to a prenatal stressor had decreased sociability (Gur et al., 2019) however, reports have indicated that handling the prenatally stressed pups during the first 21 days of life, which was done in this thesis, increased sociability (Vakili Shahrbabaki et al., 2022). Another study found that offspring that were prenatally stressed spent more time immobile during the forced swim test, a feature of depressive-like behaviour and neonatal handling decreased immobility time in prenatally stressed offspring performing the same behavioural test (Castelli et al., 2020), suggesting that neonatal handling may decrease depressive-like behaviour in offspring that were prenatally stressed. The findings to date demonstrate that neonatal handling modulates behavioural and 5-HT concentrations in the hippocampus, suggesting that handling of the pups may have acted as a potential mediator in the outcomes of this thesis by increasing sociability in adult females and decreasing depressive-like behaviour in prenatally stressed offspring.

5.7 Strengths and limitations and future directions

This thesis was the first study to evaluate the effects of a Med-based diet on depressive-like behaviour, tryptophan metabolism, the 5-HT transporter, and 5-HT receptors in the colon and hippocampus as a result of a prenatal stressor. An important strength of this thesis is the inclusion of both males and females, as many studies in the past have excluded females from prenatal stressor and serotonergic studies (Akatsu et al., 2015; Guerrero et al., 2020; Gur et al., 2019). Considering the important sex differences reported in this present study, researchers should focus on including females in their study and understanding the impact of their sex cycle on the brain.

Considering that MDD is twice as prevalent in females than males (GBD 2019 Mental Disorders Collaborator, 2022), studies aiming to understand the pathogenesis of the disorder and identify potential treatment options should include both sexes.

Another important strength of the present thesis is the development of the Med-based diet. This diet was created by adding ingredients to a modified version of the AIN 93G Purified diet (Barrington et al., 2018), which also served as the control diet in this research project. Given this information, effects seen in offspring fed the Med-based diet compared to those fed the Purified diet are resulting from the addition of ingredients found in the Mediterranean dietary pattern. Additionally, body weight and food intake were analyzed to confirm that both diets were palatable for the offspring and provided adequate nutritional value for growth. Considering MDD is very heterogenous (Nemeroff, 2020), a strength of the present thesis is the inclusion of three behavioural tests assessing three different features of depressive-like behaviour, as previous studies have used one to two tests (Chen et al., 2020; Gur et al., 2019; Soares-Cunha et al., 2018; Zhang et al., 2021). The inclusion of more behavioural tests allows for a more complete and thorough assessment of the effects of the prenatal stressor and the Med-based diet on behavioural outcomes. Lastly, the present thesis examined two distinct biological systems that are involved in tryptophan metabolism and 5-HT synthesis and regulation, namely the brain and intestinal systems. This allowed for a better understanding of the serotonergic system in adult offspring that were prenatally stressed.

A limitation of the present thesis is the small sample sizes in some of the experimental groups during the hippocampus analysis due to the removal of outliers. This resulted in a high variability for some markers, which may have contributed to non-significant effects in TDO2 and SERT expression for example. A larger sample size would help to mitigate the variability of the

results and uncover potential effects of the sex, diet, or stressor for these two genes. Additionally, tryptophan and 5-HT concentrations in the colon or the hippocampus have not been assessed, although these analyses are planned. This additional piece of information will give supplemental information that could potentially explain the sex-specific changes in the colon and hippocampus of adult males and females. As for the diet, the diet composition was analyzed using validated nutritional databases for the current thesis. A full diet composition analysis of the food pellets will be completed shortly and will provide complete nutritional information and confirm the theoretical analysis. Lastly, the female cycle was not noted prior to behavioural testing and tissue collection. This information could explain the presence or absence of sex differences seen in the present work.

Future work focusing on dietary patterns, stress-induced experiences, and mental health should assess the potential role of sex hormones, as the majority of the effects resulting from the dietary intervention and/or the prenatal stressor in the current study were sex-dependent. Understanding the potential biological processes behind these effects could provide key information in future prevention or treatment options for stress-related mental health disorders, including MDD. Along the same lines, animal and human studies should include females as this provides more knowledge and advances our understanding of the disorder, which predominantly affects women (GBD 2019 Mental Disorders Collaborator, 2022). In addition, as some gut bacteria have the potential to synthesize 5-HT (Reigstad et al., 2015), the impact of the Med-based diet and the prenatal stressor on the gut microbiota of the offspring used as part of the current thesis could be analyzed using intestinal and fecal samples already collected, as it is currently planned as part of a different study. This information will complement the colonic data obtained in the present thesis. Lastly, as this thesis highlighted a potential protective effect of maternal breast milk in dams fed the Med-based diet against the increase in weight gain induced by the prenatal stressor, the

breast milk intake and the breast milk composition could be analyzed using samples already collected.

5.8 Conclusions

The results obtained in this thesis demonstrate that the Med-based diet did normalize depressive-like behaviour and modulate tryptophan metabolism enzymes and 5-HT receptors in the colon and hippocampus in C57BL/6N mice, as well as the 5-HT transporter in the colon of offspring subjected to a prenatal stressor in a sex-specific manner. The Med-based diet also limited a higher body weight gain resulting from the prenatal stressor during early development, but this effect was no longer apparent in adult mice. The Med-based diet normalized the stress-induced decrease in grooming in males and females, and the increase in sociability elicited by the prenatal stressor in females. The Med-based diet also interacted with the prenatal stressor in a sex-specific way in the tail suspension test as it reduced immobility time only in stressed females. Sex effects independent of the diet and stressor procedures were also apparent in the behavioural tests as males spent overall less time grooming in the splash test and exhibited more active coping in the tail suspension test. In the colon, the Med-based diet increased the 5-HT rate-limiting enzyme and the 5-HT transporter and decreased the 5-HT_4 receptor in males, while having no effect in females or on the kynurenine metabolic pathway. In the hippocampus, the Med-based diet increased the 5-HT rate-limiting enzyme in males. Conversely, 5-HT_{1b} and 5-HT_{2c} receptors were increased by the Med-based diet in females, an effect not seen in males.

The results presented in this thesis indicate that the Med-based diet intervention in C57BL/6N mice normalized depressive-like behaviour predominantly in females, potentially increasing the capacity to synthesize 5-HT in the colon of males and increased 5-HT receptors in the hippocampus of adult females. The effects reported in this thesis were sex-dependent, possibly

due to an interaction between the diet and the stressor with sex hormones. A Mediterranean-style dietary pattern before conceiving and during pregnancy may thus have beneficial effects on the mental health of offspring exposed to early-life stressful experiences and therefore at a higher risk to develop MDD in adulthood. Additionally, normalized depressive-like behaviour in C57BL/6N offspring that were fed the Med-based diet may be associated with the modulation of tryptophan metabolism, the 5-HT transporter, and 5-HT receptors in the colon and hippocampus. The findings of this thesis suggest that the intake of a Mediterranean-based diet from conception onward could help promote better mental health, particularly in female offspring, potentially by modulating 5-HT receptors in the hippocampus.

Appendix 1: Diet Composition

Supplementary Table 1. Ingredient list and macronutrient distribution for the modified AIN-93G Purified Control and the Mediterranean-based diets.

	Modified AIN-93G Purified Control	Mediterranean-based
Ingredients (g)		
Casein	223	80
Fish protein isolate	0	18
Egg white	0	9
Beef, cooked	0	40
L-Cystine	3	3
Corn starch	467.4	0
Maltodextrin (for pelleting)	150	125
Wheat starch	0	198.5
Potato starch	0	0
Chickpeas, cooked, dried	0	36
Lentils, cooked, dried	0	36
Sucrose	0	0
Fructose	0	0
Cellulose, BW200	75	14
Inulin	0	5
Pectin	0	0
Beta-glucans	0	5
Soybean oil	70	0
Corn oil	0	0
Menhaden oil	0	9
Butter, Anhydrous	0	5
Flaxseed oil	0	6.5
Olive oil	0	105

Walnuts, dried, powdered	0	20
t-BHQ (antioxidant)	0.0049	0.005
Mineral Mix S10026	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate, 1 H_{2O}	16.5	16.5
Vitamin Mix V10001	10	10
Biotin (1%)	0	0.014
Choline Bitartrate	2	2
Cholesterol	0	0
Fruit and Veggie Blend	0	100
Resveratrol (50% trans)	0	0.045
Total	1045.405	872.064
Macronutrient + Fiber composition (g)		
Protein	197	156.4
Carbohydrate	552.9	402.1
Fat	72.7	157.8
Cholesterol	0	0.06
Total fiber	75	55
Insoluble fiber	75	37.8
Soluble fiber	0	18.4
Macronutrient + fiber composition (g%)		
Protein	18.28	17.9
Carbohydrate	52.9	46.1
Fat	7	18.1
Cholesterol	0	0.007
Total fiber	7.2	6.3
Insoluble fiber	7.2	4.3

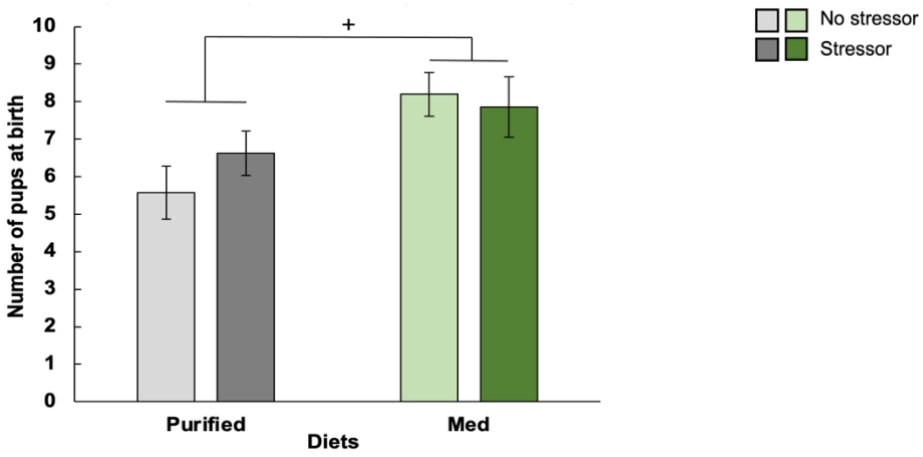
Soluble fiber	0	2.1
Macronutrient composition (kcal)		
Protein	788	626
Carbohydrate	2212	1608
Fat	654	1420
Total	3654	3654
Macronutrient composition (kcal%)		
Protein	22	17
Carbohydrate	61	44
Fat	18	39

Appendix 2: Primer Sequences

Supplementary Table 2. Primer sequences used in RT-qPCR analyses.

Gene	Gene sequence/Assay ID
Mus GAPDH	Forward: 5'- GGT CGG TGT GAA CGG ATT TG -3'
	Reverse: 5'- TGC CGT GAG TGG AGT CAT ACT G -3'
Mus B-Actin	Forward: 5'- GAA CCC TAA GGC CAA CCG TG -3'
	Reverse: 5'- GGT ACG ACC AGA GGC ATA CAG G -3'
Mus TPH 1	Forward: 5'- AACAAAGACCATTCCTCCGAAAG-3'
	Reverse: 5'- TGTAACAGGCTCACATGATTCTC-3'
Mus TPH 2	Forward: 5'- GCAAGACAGCGGTAGTGTTCT-3'
	Reverse: 5'- CAGTCCACGAAGATTTCGACTT-3'
Mus IDO 1	Forward: 5'- GCTTTGCTCTACCACATCCAC-3'
	Reverse: 5'- CAGGCGCTGTAACCTGTGT-3'
Mus TDO 2	Forward: 5'- ATGAGTGGGTGCCCGTTTG-3'
	Reverse: 5'- GGCTCTGTTTACACCAGTTTGAG-3'
Mus Slc6a4	Forward: 5'- TATCCAATGGGTACTCCGCAG-3'
	Reverse: 5'- CCGTTCCCCTTGGTGAATCT-3'
Mus Htr1a	Forward: 5'- GACAGGCGGCAACGATACT -3'
	Reverse: 5'- CCAAGGAGCCGATGAGATAGTT-3'
Mus Htr1b	Forward: 5'- CGCCGACGGCTACATTTAC-3'
	Reverse: 5'- TAGCTTCCGGGTCCGATACA-3'
Mus Htr2c	Forward: 5'- CTAATTGGCCTATTGGTTTGGCA-3'
	Reverse: 5'- CGGGAATTGAAACAAGCGTCC-3'
Mus Htr4	Forward: 5'- AGTTCCAACGAGGGTTTCAGG-3'
	Reverse: 5'- CAGCAGGTTGCCCAAGATG-3'

Appendix 3: Supplementary Data



Supplementary Figure 1. Number of pups at birth as a function of Diet (Purified versus Med-based [Med]), and Stressor (No stressor versus Stressor). Purified diet non-stressed mice (n = 8), Purified diet stressed mice (n = 9), Med-based diet non-stressed mice (n = 5) and Med-based diet stressed mice (n = 7). Data represent average litter size ± SEM. + $p < 0.05$ relative to dams fed the Purified diet.

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