SHORT-TERM ADOLESCENCE N-3 PUFA SUPPLEMENTATION AND ENVIRONMENTAL ENRICHMENT INDUCE SEX-SPECIFIC IMPACT ON EMOTIONALITY, STRESS COPING/REACTIVITY AND COGNITIVE PERFORMANCE.

DOCTORAL THESIS

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“The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say”

J.R.R Tolkien

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Abbreviations

A	Adult
AA	Arachidonic Acid
ALA	α-Linolenic acid
BMT	Barnes Maze Test
BDNF	Brain Neurotrophic Factor
CORT	Corticosterone
CSO	Control soybean oil
DG	Dentate gyrus
DHA	Docosahexaenoic acid
EE	Enriched Environment
EFA	Essential Fatty Acid
EPA	Eicosapentaenoic acid
EPM	Elevated Plus Maze
FO	Fish oil
FST	Forced Swim Test
G	Gavage Feeding
GR	Glucocorticoids Receptors
HPA	Hypothalamo-pituitary adrenal
HVF	Hydrogenated vegetable fat
J	Juvenile
LA	Linoleic acid
LT	Limited-Time Feeding
LTs	Leukotrienes
N-3 PUFA	Omega 3 Polyunsaturated acid
N-6 PUFA	Omega 6 Polyunsaturated acid
OFT	Open Field Test
PFC	Prefrontal cortex
PG	Prostaglandin
PLA	Phospholipase A2
PND	Post-natal days
PPAR	Proliferator-activated receptors
PVN	Paraventricular Nucleus
R	Restricted Feeding
RC	Regular Cage
RL	Reversal Learning
SAT	Social Approach Test
WME	Working memory errors

Contributions

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Abstract

Dietary N-3 PUFA plays a key role in brain maturation, development, stress response and cognitive abilities (Weiser et al., 2016; Devarshi et al., 2019). As adolescent's prefrontal cortex is maturating, the period becomes sensitive to external factors such as environment, nutrition, and stress (Petrovich et al., 2001; Calabro et al., 2020). In this thesis, we aim to expand our knowledge of the influence of external factors, such as dietary omega-3 supplementation and enriched environment, during this critical maturation period. By designing four distinct studies, we tested the hypothesis that visible sex-specific alterations would arise from adolescence targeted diet n-3 PUFA supplementation and enriched environment, which would act to modify physiological and stress responses, as well as socio-emotional and cognitive performance.

Our **first study** characterized the impact n-3 PUFA and n-6 PUFA regimen on corticosterone secretion and behavioural responses in adolescent male rodents. Additionally, it assessed the effects of delivery method (gavage versus restricted feeding) during this sensitive maturation period to ensure using a method with limited stress-mediated outcomes. This study highlighted gavage to induce reduced effects on corticosterone (CORT) secretion, regardless of the provided supplementation. On the last day of feeding, CORT secretion was diminished in fish oil (FO) fed rats exposed to restricted feeding, suggesting FO diet to promote physiological adjustments. Data also demonstrated that FO and soybean (CSO) rich diets were able to reduce anxiety-like behaviour compared to a high-fat diet intake (Hydrogenated Vegetal Fat - HVF), highlighting the role of n-3 PUFA dietary supplementation during adolescence on stress regulation.

Our **second study** assessed sex-specific impact of adolescence targeted dietary supplementation on brain Docosahexaenoic Acid (DHA), Arachidonic Acid (AA) and Linolenic

acid (LA) concentrations immediately following supplementation and during adulthood. Our findings demonstrated overall elevated DHA, AA and LA brain tissue concentrations in female compared to male rats, regardless of dietary supplementation. Benefit of supplementation were most apparent in adolescent males, where FO led to higher DHA concentrations compared to soybean oil supplementation, supporting a positive influence of FO dietary supplementation in males during intensive hormonal fluctuation and brain maturation. However, adolescent male rats showed reduced ability to extract nutrient essential fatty acids compared to female counterparts.

Our **third study** characterized sex-specific coping strategies, socioemotional responses, and glucocorticoid regulation following an n-3 PUFA rich diet and enriched environment (EE) during the adolescent period. While basal CORT secretions were not significantly altered by supplementation in males, a gradual increase in CORT was observed during supplementation, peaking at DAY21. Passive coping strategies was preferred in the FST in RC (Regular Cage)-housed females exposed to FO while RC-housed CSO-fed males opted for an active climbing coping strategy. Increase locomotion and anxiolytic behaviour were observed in CSO-supplemented males (exposed to EE), while CSO by itself promoted social recognition in males. In contrast, sociability was improved in FO EE exposed females, indicating possible synergic effects. Adulthood hippocampal GR-ir expression was reduced at the hippocampal CA3 region in FO/RC and CSO/EE rat groups, which could have influenced memory consolidation and stress resilience. Overall, results from this study provided insights on positive effects associated with short-term adolescent n-3 PUFA supplementation in females, while male appeared to most benefited from soybean diet supplementation.

Our **fourth and last study** assessed age- and sex-dependent influences of dietary supplementation on cognitive performance in the Barnes Maze Test. Our results showcase a gradual decrease in latencies to the escape box, as well as progressive decrease in working memory errors (WME) in adult compared to adolescent rats. Over the testing period, the FO females and CSO males showed improved performance through reduction of WMEs on specific days, which could subtend sex-related effects of dietary supplementations. However, while discrete effects of n-3 PUFA were more apparent in female rats, short-term supplementation appeared insufficient to promote consistent enhancement of visuospatial performance or cognitive flexibility that could be observed throughout the testing period.

In **conclusion,** our findings support the importance of studying single and combined factors to understand overall impact. We were able to consistently demonstrate beneficial effects on coping strategies, stress reactivity, sociability, and cognitive performance of adolescence-targeted fish oil supplementation, especially in female rodents.

General Introduction

Intrinsic and extrinsic conditions have been shown to shape an individual's trajectory in many ways. From exercise to food choices, living habits or pollutants, scientific groups have spent decades investigating the influence of exposure to various external factors on developmental trajectories, maintenance of homeostasis, and well-being of the mammalian body (Evan, 2003; Lenroot & Giedd, 2008; Gaber et al., 2017; Miguel et al., 2019). The main goal of studying these external factors is to better comprehend the impact of each of them on the development of a healthy individual, oftentimes studying isolated effects and neglecting co-exposure and synergism between these factors. Exploring the conditions in which extrinsic factors affect an individual experience has the potential to modify and/or reduce harmful and undesirable alterations. At present, different studies have supported a critical impact of environmental conditions on protein synthesis, brain development, and/or physiological regulation (Irvine et al., 2006; Berardi & Maffei, 2015; Miguel et al., 2019). These studies and several others have shown beneficial and deleterious effects of nutritional, biochemical, and social factors on human health (Owen et Corfe, 2017; Venn, 2020; Meng et al., 2020). Intuitively, one assumes that a combination of several factors will have a differential impact, likely more complex and difficult to establish. The following introduction will depict ways by which dietary supplementation, environmental enrichment, and sex, alone or combined, can influence biochemical, behavioural, and cognitive patterns in humans and rodents.

The last decades have seen emerged series of studies centered on understanding the biological, genetic, and behavioural influences of discrete nutrients. These findings have supported nutrition to not only impact physiological responses but to regulate neurobiological changes affecting emotional and cognitive responses in animals and humans (Jump, 2002;

Wurtman et al., 2009; Simopoulos, 2002; Gomez-Pinilla, 2008, Black, 2018; Moore et al., 2018; Firth et al., 2020). In recent years, increased awareness of such impact among the general population has promoted a renewed interest for research addressing the role of macro- and micro-nutrients and the possible impact of modulation of eating habits on optimal growth, development, and well-being (Fellows, 2009; Zoohori, 2020), especially in relation to the development and preservation of the brain and associated emotional and cognitive functions (Boitard et al., 2015; Reichelt & Rank, 2017; Kanoski & Davidson, 2011). While knowledge of the impact of unbalanced diet on obesity and cardiovascular disease has exponentially grown, the role of dietary nutrients in regulating emotional and cognitive changes as the brain develops needs to be further examined (Fellows, 2009; de Lorgeril, 2013; Padro & Dewey, 2014; Tapsell et al., 2019). Consequently, this thesis aims to examine the role of a specific nutrient consumption, polyunsaturated fatty acids, during the adolescent period on immediate and delayed neurochemical, behavioural and cognitive responses.

The radical changes in the food industry over the last century have prompted important shifts in dietary choices (Simopoulos, 2002). The rapid expansion of the food industry fostered an easier access to a variety of different nutrients, including an increased presence of processed foods and dietary supplements (Simopoulus, 2016). While increased food selection has health benefits, industrialization also involved unforeseen consequences. Indeed, the human diet drastically shifted from elevated lean meat, fish, fruits, and vegetables consumption to a grain-based diet characterized by elevated consumption of high fat red meat and low fiber intake (Gómez-Pinilla, 2008, Simopoulos, 2016). These changes led to adoption of a Western diet enriched in omega 6 fatty acids, saturated fat, and *trans*-fat content, and marked by a deficit in essential fatty acid such as n-3 polyunsaturated acid (n-3 PUFA), which by extent significantly altered the omega 6 to omega 3 dietary ratio (Simopoulos, 2002; Marion-Letellier, Savoye & Ghosh, 2015, Mani & Kurpad, 2016). Epidemiological data suggests that Western nations collectively share a dietary omega 6 to omega 3 ratio ranging between 15:1 to 20:1 compared to optimal 1:4 ratio (Simopoulos, 2002; Simopoulos, 2016). This ratio indicates a consumption of omega 6 critically above recommendations, which has shown effects on endogenous mechanisms regulating body homeostasis and affects health status in general. Notably, certain studies have shown an unbalanced and/or elevated omega 6 consumption to reduce nutrient absorption (Novak et al., 2008; Karr et al., 2011). The dietary shift observed in the last century has also been associated with increased risk of type-2 diabetes (Raheja et al., 1993, Micha et al., 2017), cancer cell proliferation (Ge et al., 2002, Bojková et al., 2020), and cardiovascular disease (Weber, 1989; Simopoulos et al., 1994a, Chareonrungrueangchai et al., 2020; Mazidi et al., 2021). Many studies, including earlier research from de Lorgeril and al. (1994), have demonstrated that within a two-year period, adoption of dietary omega 6 to omega 3 ratios ranging between 2.5:1 and 4:1 can reduce the risk of mortality from coronary heart disease, minimize inflammation and proliferation of certain cancer cells, and improve autoimmune disease (Simopoulos, 2002; Simopoulos, 2008; Duan et al. 2014; Ma, Xu & Lv, 2019; Khadge et al., 2020).

Whereas research continues to concentrate on determination of the effects of isolated factors, the mutual influence of external factors is acknowledged and consensual. Along with changes in dietary habits, adoption of a sedentary lifestyle has characterized Western societies, a phenomenon also noted among younger individuals (Martins et al., 2021). In a recent meta-analysis review, Martins et al. (2021) reported compiled evidence from 35 distinct studies to demonstrate the importance of habits and lifestyle. The authors indicated that around 23% of adults and 81% of adolescents are currently not achieving the minimal recommendation for weekly physical activity, despite knowledge of an increased risk for several major health challenges such as hypertension, cancer, stroke, and type-II diabetes associated with long-term, sedentary lifestyle (Blüher, 2019; Martins et al., 2021). These debilitating health conditions also commonly related to Western diet regimens support the importance of examining the communal effects of living styles and dietary conditions in regulating health conditions. To this effect, a growing body of research supports environmental factors including diet, exercise, and housing conditions to be associated with neural regulation and modification of behaviour (de Groot et al., 2012; Barnea-Goraly et al., 2005; Bechara & Kelly, 2013; van Praag et al., 2000; Bhadgya et al., 2017). The role of nutrients and housing conditions will be further developed in upcoming sections and their involvement in the alteration of neural and behavioural responses discussed.

Whereas the impact of adverse living conditions (stress, malnutrition, etc.) has been more closely examined, there is a gap of knowledge involving the role of dietary polyunsaturated fatty acid (omega 3 PUFAs) and environmental enrichment (EE) during critical maturation periods. My PhD thesis represents an attempt to foster new knowledge and aims to untangle the role of single versus combined exposure to external/environmental factors likely to induce key impact on physiological, emotional, and neurochemical regulation. The studies explored the effects of exposure to omega 3 dietary supplementation alone or combined to a cognitively stimulating environment. Importantly, my research uniquely features the study of sex differences as well as examine the impact of environmental changes at the critical adolescence period, a significant and sensitive developmental time. While other developmental phases are also crucial, proper development of the brain during adolescence relies heavily on fatty acids consumption, due to the rapid and time-sensitive growth of the prefrontal cortex and interconnected development with other brain structures (Ghashghaei et al., 2002; Naneix et al., 2012 Calabro et al., 2020).

The following sections will provide an overview of existing research addressing demonstrated effects of omega 3 dietary supplementation and deficiencies during different lifespan periods. Then, the impact of another major extrinsic influence, environmental enrichment will be described along with a discussion of sex-specific changes noted and the importance of including this intrinsic variable in studying these extrinsic factors. Considering that the subsequent thesis chapters also introduce these topics, we have tried to minimize redundancy.

1.1 Dietary Polyunsaturated acid (PUFA): Regulatory actions of body and brain functions

Polyunsaturated acids (PUFA) are part of macronutrients playing a critical role in proper maintenance of body homeostasis, which contributes to conferred health benefits from their ingestion in different functioning spheres of the mammalian's life. There are four types of fatty acid in the human diet: saturated fatty acids, monounsaturated fatty acids and the n-3 and n-6 polyunsaturated fatty acids (PUFA), commonly referred to as omega 3 and 6. The mammalian body does not naturally synthesize PUFAs (Jump, 2002). Consequently, bioavailability of these fatty acids in adequate concentrations rests on balanced nutrition (Jump, 2002). PUFAs are found in oily substances from plants and nuts, including soybean, corn and sunflower oil and fatty fish such as salmon, mackerel, herring, and trout (Simopoulos, 2002; Gómez-Pinilla, 2008). Following absorption of these macronutrients, PUFAs are transformed into several nutritive components (Jump, 2002). Linolenic acid (LA, C18:2n6) and arachidonic (AA, 20:4n-6) are issued from the omega 6 metabolic chain, whereas α-Linolenic acid (ALA, C:18; 3n-3), eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6 ω-3) are byproducts of omega 3 metabolism (Jump, 2002). In this metabolic chain of actions, the conversion of ALA in EPA and DHA and that of LA into AA occurs naturally in mammalian species, although the conversion process is relatively slow due to both omega molecules using the same desaturation enzymes (Jump, 2002; Shahidi & Ambigaipalan, 2018). An unbalanced diet increases the competing metabolism process between omega 3 and omega 6 leading to a reduced production of key metabolites from the omegas-3 (Saini et al., 2018 for a review). In other words, an increased presence of LA from an unbalanced diet interferes with the bioavailability and absorption of PUFAs, causing reduced EPA and DHA body concentrations. This strengthened the need for adding n-3 omega ingestion to our daily diet to maximize EPA and DHA actions to obtain an optimal ratio 4:1 (Jump, 2002; Shahidi & Ambigaipalan, 2018) (See Figure 1).

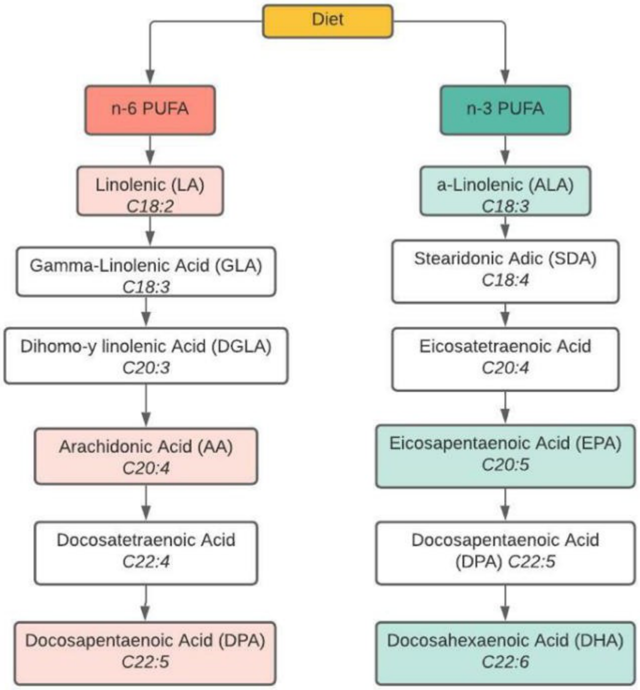

Figure 1. Metabolism of n-3 and n-6 polyunsaturated fatty acids (PUFAs).

A balanced ratio appears to be the most critical factor for increased benefits. n-3 and n-6 PUFAs have distinct functions in regulating body homeostasis. Omega 6 PUFAs (n-6 PUFAs) are necessary to produce eicosanoids, which are small lipids derived from arachidonic acid (AA), a metabolite of n6 PUFAs. A high concentration of AA leads to the activation of phospholipase A2 (PLA-2) and by extension influences the production of prostaglandin (PG), leukotrienes (LTs) and other proteins. PLA2 catalyzes the hydrolysis of the phospholipid membrane and leads to the release of AA from the membrane. Subsequently, cyclooxygenases (COXs) convert the free-AA to PG, LTs and thromboxane (Van den Bosh, 1980; Yoshida et al., 2007), which conversion has effects on different functional outcomes. For instance, the secretion of leukotrienes has been linked to inflammatory or/and allergic responses, which can create a rippling effect on body homeostasis (Norris, 2007). When the conversion rate is elevated, this translation mechanism can increase the risk of developing allergic reaction, asthma, cystic fibrosis, and other related diseases (Oddy et al., 2004; Norris, 2007). Furthermore, omega 6-derived eicosanoids can regulate gene expression by directly binding to peroxisome proliferator-activated receptors (PPAR) (Kliewer et al., 1997; Norris, 2007). These receptors are members of a superfamily of nuclear receptors with distinctive subunits (PPARa, g, and d) playing a role in energy homeostasis and regulation (Kliewer et al., 1997). Although most types of fatty acids can activate these receptors, omega 6's prominence in the Western diet is associated with an increased level of eicosanoids which exert a profound influence on PPAR-mediated gene expression. PPAR expression is also influenced by glucocorticoid and estrogen secretion, making it a key factor in stress regulation (Tyagi et al., 2011). The harmful effects associated with consumption of diet rich omega 6 and poor in omega 3 PUFAs are in part related to accumulation of elevated AA concentration in the body, causing an increase in eicosanoids

(Innes & Calder, 2018 for review). Among noted effects, elevated consumption of omega 6 impacts the immune system functions, hormonal balance, and the homeostasis of the mammalian body, increasing inflammatory responses, allergic reactions, and insulin resistance, and compromising recovery following cerebrovascular accidents (Simopoulos, 2009; Marion-Letellier et al., 2015).

Contrasting effects of elevated n-6 PUFAs, Sierra and al. (2006) indicated n-3 PUFA supplementation to diminish inflammatory responses, by reducing activation of the pro-inflammatory TH1 and TH2 cytokines, while increasing the expression of the immunomodulatory cytokine Interleukin-10 (IL-10) shown to prevent cell damage and reduce inflammation (Iyer & Cheng, 2012). A review article by Miles & Calder (2017) further supports omega 3's anti-inflammatory actions to be associated with a decline in allergic responses and in the risk of developing childhood allergic disease. Indeed, a study by Oddy et al. (2004) showed a balanced dietary consumption of omega 3 and 6 to protect against asthma in children. Additionally, Kuszewski et al. (2020) recently reported that regular 16-weeks supplementation of fish oil improved cerebrovascular function. Specifically, these authors showed that fish oil supplementation in males and female participants (median age 65.4-years) was associated with reduced artery stiffness, heart rate and serum triglycerides (Kuszewski et al., 2020). Regular supplementation also increased High Density Lipoprotein (HDL) cholesterol, known to promote cardiovascular health (Kuszweski et al., 2020). Lastly, a review by Innes and Calder (2020) revealed protective effects of DHA and EPA supplementation related to anti-inflammatory effects, reduced hypertension and blood lipid concentrations, effects associated with a reduced risk of developing cardiovascular disease, including coronary heart disease and myocardial infraction (Innes and Calder, 2020).

In the brain, studies have supported omega 3 supplementations to promote membrane fluidity, synapse formation, and improve cognitive and attentional capacities (Jump, 2002; Gómez-Pinilla, 2008, Wurtman et al., 2009; Sidhu et al., 2011; Woo et al., 2014, Gow & Hibbeln, 2014; Ayee et al., 2020). Among the active metabolites resulting from omega 3 and omega 6 consumption, docosahexaenoic acid (DHA) plays a critical role in regulating the development of the human brain via its ability to penetrate the cellular/neuronal phospholipid membranes (Souied et al., 2013). The observation that DHA content accounts for approximately 10% of the human brain's wet weight has contributed to maintain a vivid interest in understanding its function (Wurtman et al., 2009; Sidhu et al., 2011; Gharami et al., 2015, von Schacky, 2021 for a review). In this context, the modification of the membrane composition and architecture of the cells favours a wide range of essential brain functions to which DHA could contribute, including vesicle formation, protein transport and synaptic function (Kitajka et al., 2001; Sidhu et al., 2016). In rodents, DHA deficiencies have been associated with decreased synapsin expression in hippocampal neurons, a factor known to affect synapse formation and remodeling, and have a role in regulating neurotransmitter release and brain plasticity (Cao et al., 2009). The same group described diminished glutamate receptor expression in DHA deficient rats, which affect distinct functions of the most widespread excitatory neurotransmitter of the brain glutamate (Cao et al., 2009). At the hippocampus, glutamate transmission is essential for long-term potentiation and memory consolidation, and decreased receptor expression at that site can have deleterious impact on cognitive processing (Zhou & Danbolt, 2014; Gonçalves-Ribeiro et al. 2019). Importantly, although beneficial effects of dietary omega 3 supplementation on memory, cognition and emotional response have been demonstrated in multiple age groups, the impact has been most prominent when intake occurred during the early developmental periods, in terms of prevention of future health problems as well as cognitive development (Karr et al., 2011; Lauritzen et al., 2016; Thesing et al., 2018; Comitini et al., 2020).

1.2 Physiological, Molecular, Behavioural, and Cognitive Influences of n-3 PUFA diet.

As elucidated in the previous section, intake of essential fatty acids has shown a crucial role in maintaining homeostatic balance as well insuring cell viability and development of optimally functioning brain networks. To this effect, deficiency in omega 3 consumption has various detrimental effects, many of which have been demonstrated in early stages of the ontogenic development, although supplementation in aging has also been linked to emotional and cognitive benefits (Daniels et al., 2004; Devarshi et al., 2019). Omega 3 metabolites accumulate in the brain's membranes early during the third trimester of gestation, a period associated with neurogenesis (Sidhu et al., 2011; Malik et al., 2013; Lo Van et al., 2016). Therefore, a balanced diet from gestating mothers is crucial (Basak et al., 2020 for a review). Sidhu and colleagues (2011) showed DHA supplementation during gestation and lactating periods to promote neurite outgrowth and synaptogenesis in infants. Importantly, they also reported increases of pre- and post- synaptic proteins involved in long-term potentiation in the hippocampus, a decisive factor related to learning and memory consolidation, essential to long-term cognitive development (Sidhu et al., 2011). Consistent with delayed effects of PUFA supplementation, Daniels and colleagues (2004) reported that children of mothers consuming fish four times a week during the gestation period have higher developmental scores compared to the control group, such differences being maintained until 18 months of age. Infant cognitive development similarly appeared influenced by the DHA supplementation. Multiple studies reported higher IQ scores and better school performance in supplemented as compared to non-supplemented children (Helland et al., 2003; Kidd, 2007; Richardson et al., 2012; Gharami et al., 2015; Bernard et al., 2017). Likewise, Uauy & Dangour (2006) showed omega 3 supplementation during breastfeeding to promote cognitive and physical abilities in children. In rodents, studies showed gestational omega 3 supplementation through milk feeding to improve balance and coordination in adolescent rodents (Dunstan et al., 2007; Coluccia et al., 2009; Brenna, 2011), further supporting remote benefits from perinatal omega 3 exposure.

To date, current knowledge on the impact of supplementation during the adolescent period is based on a limited number of studies. Researchers have reported omega 3 supplementation during the adolescent period to have positive impact on cognitive abilities while reducing physical and mental health problems. For instance, studies in humans have supported a relationship of omega 3 consumption with increased vocabulary, academic performance (c.f., grades obtained) and higher IQ test scores in adolescent cohorts, with similar cognitive influences being noted in developing infants (Åberg et al., 2008; Kim et al, 2010; De Groot et al., 2012; Nieto-Ruiz et al., 2022). Interestingly, Soni and colleagues (2015) demonstrated that while mice fed a high fat diet during the adolescent period showed increased fat accumulation in the liver and reduced activation of specific coding genes related to cytoplasmic lipid droplets, while supplementation of DHA and EPA to such diet successfully reversed these negative effects. Fatty liver accumulation being associated with various diseases, including type- 2 diabetes and cardiovascular problems, omega 3 consumption during this developmental phase appears to play a significant role in promoting health as well as preventing negative impact of an unbalanced diet (Soni et al.,2015). Importantly, studies have also established a relationship between early omega 3 consumption and improvement of neurodevelopmental disorders, including attention deficit and hyperactivity disorder (ADHD) (Woo et al., 2014; Parletta et al., 2016; LaChance et al., 2016, Pei-Cheng, 2021). It is important to note that such impact has mainly been observed in studies assessing n-3 deficiencies. Among important findings, deficiencies in n-3 PUFA consumption during childhood and/or adolescence have been associated with higher prevalence of ADHD and reduced dopamine levels in the prefrontal cortex, a brain region regulating attention processes (Woo et al., 2014, Gow & Hibbeln, 2014; Pei-Cheng, 2021). Studies have also supported beneficial effect of PUFA on mood and anxiety disorders (Gow & Hibbeln, 2014; Hallahan et al., 2016; DiNicolantonio and O'Keefe, 2020). Thus, deficiency in dietary omega 3 consumption has been associated with elevated glucocorticoid levels and depressive symptoms (Anisman, 2002; Ginty & Conklin, 2015; Rice and al., 2015). Concordant with this, Nemets and his colleague (2006) have shown omega 3 supplementation to alleviate depressive symptoms in young adults, while Ginty & Conklin (2015) showed that within 21 days of omega 3 supplementation, 67% of individuals part of the experimental group (omega 3 supplemented) no longer met DSM's depression criteria. Considering the suggested benefits of omega 3 consumption on anxiety and depression, my PhD studies have been developed with the aim to address effects of omega 3 supplementation in mediating physiological responses to stress, a critical component in developing depressive states. To do so, our research characterized the impact of n-3 PUFA supplementation in rodents on peripheral and brain responses regulating stress reactivity, namely corticosterone secretion and hippocampal glucocorticoid receptor expression.

The influence of omega 3 intake in the perinatal and infancy period on cognitive responses have been extensively examined (Lauritzen et al., 2016; Weiser et al., 2016 for a review). Nonetheless, it became apparent a decade ago that, in comparison to perinatal states, the adolescence period had received minimal attention (Karr, 2011; Fuhrmann et al., 2015, Gerhard et al., 2021). The adolescence period, defined by sexual maturation and is characterized by the opening of the vaginal opening in females and balanopreputial separation in males (McCormick and Green, 2007; Sengupta, 2013), reached between postnatal days (PND) 32 and 34 in females, while in males it is reached later between PND45 and 48 (Sengupta, 2013). However, sexual maturation can considerably vary in males (Sengupta, 2013), with balanopreputial separation having been noted from PND32 to PND46 (see review by McCormick and Green, 2007).

The lack of studies covering this period is intriguing considering that it is characterized by critical hormonal and brain changes. Indeed, adolescence represents a developmental milestone that is marked by extensive maturation of brain tissue, particularly the prefrontal cortex and associated mesolimbic system and hippocampal region (Akirav et al., 1999; Petrovich et al., 2001; Ghashghaei et al., 2002; Naneix et al., 2012, Calabro et al., 2020). Barnea-Goraly et al. (2005) established that the increased cognitive abilities from childhood to adolescence was correlated with a significant growth of myelinated non-cortical white matter in the prefrontal cortex. Related to ongoing plasticity of this structural network, the frontal cortex and associated structures can most benefit from positive environmental, such as an enriched environment or a n-3 PUFA dietary supplement, while also showing increased vulnerability to stress exposure (Bathia et al., 2011; McEwen & Morrison, 2013; Wu & Mitra, 2021). For instance, Bhatia et al. (2011) demonstrated that long-term mother and pup's dietary supplementation can modify prefrontal brain development. Sprague-Dawley male rats exposed to an omega 3 deficient diet fo 15 weeks showed reduced p-Tropomyosin receptor kinase B (TrkB) receptor expression in the frontal cortex, hypothalamus, and hippocampus (Bhatia et al., 2011). Additionally, the researchers found diminished levels of brain-derived neurotrophic factor (BDNF) in the hypothalamus and hippocampus, which has been linked to depressive-like behaviours (Bhatia et al., 2011). Considering these effects, it is interesting to note that Wu and Mitra (2021) recently showed exposure to an enriched environment to confer protection against reduced plasticity in the prefrontal cortex of 7-week-old Wistar male rats exposed to stress. Such observations strengthened the importance of both adolescence dietary and environmental living conditions in the optimal development of the prefrontal cortex and the interconnected circuitry of the amygdala, hippocampus, and mesolimbic/nigrostriatal systems. These factors have the ability to minimize the disruption of brain function in case of failed optimal environmental/living conditions (Akirav et al., 1999; Petrovich et al., 2001; Ghashghaei et al., 2002; Naneix et al., 2012 Calabro et al., 2020).

1.3 Intrinsic factor: Sex differences related to PUFAs

In the last decades, efforts have been multiplied to fulfill the lack of knowledge on the influence of sex on behavioural and brain responses, created by centuries of almost exclusive studies of males' biobehavioural responses (Beery and Zucker, 2011; Will et al., 2017). AS studies started including both sexes, it rapidly became evident that male and female responses in various domains are distinct (Simone et al., 2021; Bangasser & Cuarenta, 2021). While n-3 PUFA has been shown to impact different neurobiological and behavioural processes in male rodents, including cognitive performance and reduce hyperactivation of the HPA axis, these effects can greatly be influenced by sex hormones (Lovick & Zangrossi, 2021; Trova et al., 2021). Indeed, research support females to experience benefits from omega 3 supplements compared to males (Lassek and Gaulin, 2011). Directly related to n-3 PUFAs' metabolic chain of actions, Extier et al. (2010) demonstrated mRNA expression of Δ5 and Δ6 desaturase to be significantly elevated in female rodents, and Δ6 desaturase expression to be increased following estrogen administration. Increased detection of these enzymes, known to play a key role in the conversion of n-3 PUFA, may promote post-ingestive effects in females. In this context, Childs et al. (2012) have indicated a positive correlation between progesterone and Δ6 desaturase level, proposed to contribute to the relationship between hormonal levels and efficiency of n-3 PUFA conversion.

Sex-dependent variations have also been noted when assessing n-3 PUFA's main active metabolite, docosahexaenoic acid (DHA). For instance, female rodents have been shown to have higher levels of DHA in their bloodstream, both in plasma and erythrocytes, compared to males (McNamara et al, 2009; Child et al, 2012). Similar patterns have been demonstrated in the human population, women showing up to 12% increased fatty acid content in total plasma compared to men (Lohner et al., 2013). Lohner et al. (2013) also supported females' DHA concentrations acquired from fish oil supplemented diet to be increased, women showing a 24% mean increase in plasma DHA levels compared to men. These authors also mentioned heightened DHA levels in female livers. Furthermore, Boot et al. (1997) demonstrated that during puberty, female tends increased their fat percentage (from 14.8 to 25.5%), providing additional storage capacities for DHA fatty acids. Harris et al. (2013) added an interesting age-related element, their findings supporting DHA levels to be higher in women compared to men, from teen to 30 years of age. They suggest this observation to be related to heightened level of erythrocytes in women during that period of life (Harris et al., 2013). These findings bring perspective on some reasons that could underlay sexually dimorphic n-3 PUFA effects during the developmental stage of adolescence.

As previously mentioned, gonadal hormones show an ability to modify the efficiency of n-3 PUFA conversion into its distinctive metabolites. Of interest, Giltay et al (2004) reported that DHA conversion is higher in females who use contraceptive pills and individuals going through estrogen replacement therapy. In an older experiment, Lee et al (1988) had been among the first to show heightened conversation rate associated with estrogen treatment in post-menopausal women. In turn, oestradiol was shown to facilitate the conversion of omega 3 to DHA, increasing its abundance in liver, brain tissues and plasma (Alessandri et al., 2007; Childs et al, 2008). Childs et al (2012) have also noted heightened progesterone levels in women to have similar effects on n-3 PUFA conversion, although other studies, including Giltay et al. (2013), have found no correlation. Since estrogen and progesterone share certain signaling pathways, it is plausible that both could facilitate metabolic conversion, although this remains to be confirmed (Vicent et al., 2006; Sibbons et al, 2014). In contrast, testosterone has shown opposite effects, acting to reduce the conversion rate of dietary macronutrient into DHA (Schuchardt et al, 2010; Giltay et al., 2004). Studies have shown testosterone treatment in females undergoing transitioning process to significantly reduce DHA concentrations obtained from dietary n-3 PUFA ingestion (Giltay et al, 2004, Childs et al. 2008). Marra & Alaniz (1989) showed testosterone treatment to increase Δ9 desaturase and inhibit Δ5 and Δ6 desaturase levels associated with n-3 PUFA ingestion, favouring n3-PUFA conversion into AA rather than DHA (see Gonzalez-Soto and Mutch, 2021 for review). These observations suggest that male and females could be differentially affected by omega 3 deficits or react differently to equivalent consumption. From these studies, one can presume the importance of omega 3 consumption during the sexual maturation period of adolescence. At present, limited research has been conducted assessing sex-specific mechanism and behaviour effects of adolescence dietary PUFA supplementation. Acquisition of knowledge on these aspects is necessary to better understand supplementation effects associated with distinctive lifespan periods, which have a potential to influence dietary recommendations of omega 3 consumption.

1.4 Extrinsic factor and adolescent development: Living in an Enriched Environment

Aside from diet, other factors, including daily activity levels, have remarkably evolved over the last century. A shift in lifestyle, associated with technological advancement and industrialization, have led to a decline in physical demands related to work. Over time, more sedentary lifestyles have been associated with negative consequences on mental and physical health statuses (Deslandes et al., 2009, Loewen et al., 2019). Loewen et al., (2019) demonstrated that a considerable proportion of Canadian adolescent with a mental health diagnosis do not follow physician's recommendation regarding sleep, physical activity, sedentary lifestyle and/or dietary requirements. Importantly, within the first year of adolescents' adherence to such recommendations, up to 47% reduction of metal health-related visits have been observed (Loewen et al., 2019). More generally, research clearly support that among many benefits, exercise shows significant effects in reducing the risk of heart disease, hypotension, and osteoporosis in humans (Joubert et al., 2018; Rêgo, Cabral, Costa, & Fontes, 2019). It appears evident that among several factors known to influence behaviour and stress reactivity, environmental conditions represent a key element. Rosenzweig & Bennett (1996) defined the environment as a "combination of inanimate and social stimulation". In rodents, environmental enrichment (EE) paradigms have been used to determine the various roles of living/environmental conditions (Schub & Eisenstein, 2003; Hutchinson, Avery, Vanderwoude, 2005; Smith & Corrow, 2005; Girbovan and Plamondon, 2013). Although environmental conditions have not been standardized in rodents' EE studies, the premises remain similar between experiments. In most cases, studies have used a mixture of elements such as: an increased number of animals per cage (social housing), additional space using larger size cages, and the presence of continually changed items such as toys, nesting material and tubes (Kempermann, 2019). Materials added in the environment can help develop certain skills, while articles such as paper and tissue, facilitate the development of nesting behaviour in rodents. Thus, larger living spaces offer additional hiding places, providing comfort and safety for rodents, while group housing enhanced social interactions. By extent, an enriched environment tends to promote motor, sensory, and cognitive skills (Kempermann, 2019).

In animals, research has shown the addition of running wheel and treadmill activities in rodents' routine to stimulate rodents both physically and cognitively (Bechara & Kelly, 2013). Notably, the sole presence of a running wheel (even if locked) can stimulate cell proliferation and improve cell survival (Bednarczyk et al., 2011; van Praag et al., 2000; Olson et al., 2006). Bednarczyk et al., (2011) also demonstrated exercise to impact neuronal differentiation, improving the development of neuronal networks during ontogeny. Additional to the heightened exercise level in the enriched environment, rodents are exposed to toys, nesting material and promote social interaction (Kempermann, 2019). These elements have been shown to alter several mechanisms, involving cognitive abilities, stress reactivity and even the immune response (Rattazi et al.,2016; Hu et al., 2013; Veena et al., 2009, Kondo et al., 2016). For instance, EE exposure has been shown to modify gene expression, alter protein productions, improve neurogenesis in the hippocampus and regulate neurotrophic factors such as BDNF (Hu et al., 2013; Kempermann, 2019). Interestingly, several of these changes can be seen after minimal exposure. Gabriel et al (2020) reports that even a 4-day exposure to EE could increase cortical tissue development. While short term exposure was shown to be beneficial on some levels, living in an EE for a longer period promotes many more changes. Thus, a 10-day EE exposure was shown to promote newborn cell differentiation and attenuate depressive-like symptoms in rodents exposed to chronic stress exposure (Veena et al., 2009). Similarly, Gonzalez-Pardo et al. (2019) have shown a longer 39-day EE exposure to enhance the maturation of the medial prefrontal cortex and ventral hippocampus of adolescent rodents. Behaviourally, EE created by the presence of toys and social stimulation has shown beneficial effects on spatial memory performance in the Morris Water Maze in adolescent and adult rodents (Kondo et al., 2016). Bhagya and colleagues (2017) also reported reduced anxiety-like behaviour and improved working memory associated with a short 10-day EE exposure in rodents. Notably, studies have reported EE to reduce stress reactivity and promote resilience following stress exposure (Rosenzweig et al. 1996; Marcon et al., 2018). In addition to these important variables, EE can modify important health regulators, including the immune system responses. Rattazi et al. (2016) provided the first evidence that a short exposure to EE, as brief as 2 weeks, can modify mice immune profile of T cells. More precisely, they found an increased production, among other things, of interleukins (IL-10 and IL-17) and a reduction of Interferon (IFN-y) (Rattazi et al., 2016), resulting in a positive effect of the environment on the differentiation of T cells and the immune response. These observations indicate exposure to environmental enrichment to foster discrete regulatory actions affecting brain development, socioemotional and cognitive processes, stress reactivity and immune system functions.

One particularity of EE is group housing, fostering increased exploration and social encounters between cage mates (Kempermann, 2019). During the adolescence period, studies show that increased interactions between rodents can increase playful behaviour, an essential characteristic for adolescent rodents (Pellis and Pellis, 2009; reported in review by Burke et al., 2017). Importantly, same species interactions have been shown to influence the maturation of prefrontal (PFC) networks, including neuronal pathways from the PFC to the reward system (Pellis and Pellis, 2009; Calabro et al., 2020). Consistent with this, playful interactions during adolescence have been shown to increase dopamine release and improve the development of the circuitry (Felix-Ortiz et al., 2016; Shams et al., 2018; Wang et al., 2021). Further, Neal et al (2018) demonstrated that exposure to a socially rich environment promoted social grooming, increased oxytocin secretion, and improved performance in cognitive tasks. Finally, combined exposure to cognitively stimulating toys and exercise was shown to increase neurogenesis in the dentate gyrus of adult rats (van Praag et al., 2000; Olson et al., 2006, Garthe et al., 2016; Kempermann, 2019), which if associated with increased structural activity could improve memory consolidation.

While beneficial effects are generally noted, Tanner et al (2019) have reported responses to voluntary exercise and EE to be sex dependent. They reported voluntary exercise to improve fear extinction in males following an uncontrollable tail shock stress, while such cognitive effect was not visible in females (Tanner et al., 2019). On the other hand, 6-weeks voluntary exercise benefited stress resistance in both sexes and reduced shock-elicited freezing behaviour (Tanner et al., 2019). A study by Whitaker et al. (2016) reported EE with a running wheel to promote prosocial behaviour in female rodents compared to males. Interestingly, male mice exposed to EE including nesting material and running wheel access spent reduced time in the open arms in the elevated plus maze and less time interacting with strangers in social test, supporting a lower risk-taking behaviour, while males from a different mice strain showed a contrasting and opposite behavioural response profile. Of interest, Guidotti et al. (2016) further demonstrated that independently bred females tend to favor voluntary exercise, even in the absence of a running wheel, compared to in-bred females. Moreover, several studies demonstrate that female Wistar rats tend to show increased locomotion in mazes such as the open field compared to male counterparts (Blizard et al., 1975; Belviranli et al. 2012). These differential responses have in part been attributed to gonadal hormone levels. In this context, Rosenfeld (2016) reported elevated estrogen levels to promote physical activities through increased overall brain activity. Different studies have similarly supported estrogen level to regulate physical performance in women by decreasing fatigue, favoring optimal muscle functioning and endurance increasing bone mass, muscle health and mitochondrial function (Ikeda, Horie-Inoue & Inoue, 2019). On the other hand, studies examining testosterone effects on voluntary exercise can be conflicting. While Rosenfield (2016) found elevated testosterone levels to reduce motivation for exercise, Saad et al. (2012) reported opposite effects (Saad, Aversa, Isidori & Gooren, 2012). In sum, multiple factors, including sex and genotypes, are likely to modulate the effects of EE exposure and exercise, pressing for additional research.

Although many studies support the beneficial impact of an enriched environment (Jankowsky et al., 2005; Costa et al., 2007; Hu et al., 2010; Ortiz-Perez et al. 2016), conflicting sex-dependent effects of EE have been reported, particularly concerning physiological effects and anxiety-like behaviours (Girbovan & Plamondon, 2013; Kempermann, 2019). For instance, Martin & Brown (2010) showed that female rodents CORT secretion was heightened following enriched environment housing, suggesting the environment created additional stress for the female rodents. Alongside, Welberg & Plotsky (2005) observed that female exposed to control environment had reduced ACTH level following an acute stressor. Such changes were undetected in male rodents, regardless of the environment, reinforcing the potential dichotomic influence of sex following an EE prolonged exposure (Welberg et al., 2005; Martin et al., 2010). On the other hand, Peña et al. (2009) reported contrasting effects, with the sex and not the housing condition affecting females and males CORT and ACTH secretion. Adding to these observations, Harati et al. (2013) reported EE-exposed females to show reduced stress reactivity compared to regularly housing females. Consistent with this, Kulesskaya et al. (2011) found post-weaning female mice exposed to nesting material (as provided in EE housing) to display reduced anxiety-like behaviour. Such variability in the results commends additional research on enriched environment exposure and stress reactivity in female rodents (Bakos et al., 2009; see review by Girbovan and Plamondon al., 2013).

Concerning the impact of enriched environmental exposure on cognitive performance, distinct beneficial effects have been noted in female rodents (Girbovan et al., 2013). For instance, Kulesskaya et al. (2011) found a 8-week EE exposure in female rodents to increase Morris Water maze spatial memory performances compared isolated housed counterparts. Similarly, Yang et al. (2015) found 4 months of EE housing in middle aged female rats to not only improve spatial learning in the Morris Water Maze, but also enhanced white matter volume and induced a surge of myelinated fibers through the whole brain. More recently, Sadegzadeh et al. (2020) reported 40-days EE housing in adolescent female rodents to significantly elevate prefrontal cortex BDNF levels, providing insights on EE’s physiological regulation of executive and working memory functions (Sadegzadeh et al., 2020). Enhanced social interaction provided through EE exposure also appears to play a critical role in enhanced cognitive performance, Doulames et al. (2014) showing social isolation to be associated with declined Y maze performance in rodents (Doulames, Lee & Shea, 2014). Overall, the combination of a cognitively stimulating and socially prone environment during peak sociability period (PND30-35), proposed through EE housing, seems to enhance cognitive performance in both female and male rodents, with a few discrepancies in studies (Girbovan et al., 2013). For example, Beck & Luine, (2002) found that EE housing condition failed to foster improvement in object recognition in females Sprague-Dawley rats.

1.5 Thesis objective

Considering promising yet limited findings on changes observed upon exposure to environmental and dietary changes during the adolescent period, one main goal of this thesis aimed to extend knowledge on the influence of external factors during this critical maturation periods (Calabro et al., 2020). An important element in assessing such impact was the examination of sex-specificity of the responses, the inclusion of females in research having been omitted for too long (Will et al., 2017). Specifically, the studies in this doctoral thesis evaluated the effects of n- 3 PUFA supplementation provided during the adolescent period, in combination or not with EE, on immediate and delayed physiological, behavioural, and cognitive responses in male and female rodents.

The first thesis study characterized the impact of different dietary supplementations on stress-mediated CORT secretion and behavioural responses in adolescent male rodents. Three dietary supplements were compared 1) Soybean Oil (CSO), a non-hydrogenated oil presenting a 7.4/1 n-6/n-3 ratio, 2) Fish Oil from menhaden (FO), a highly rich omega 3 supplement (Sigma Aldrich; contains 20.0 to 31.0% omega 3 fatty acids) or 3) Hydrogenated Vegetable Fat (HVF) (High fat content: 18% of saturated fat and 0% of trans fat in 12g of HVF). The study also aimed to examine differences that could be related to using supplementation by gavage or through limited time feeding in adolescent rats, as minimal stress associated with supplementation was a primary goal. Rodents' corticosterone secretion and behavioural responses in the open field and elevated plus maze test were used to determine discrete outcomes of the supplementation methods. We predicted that rodent exposed to gavage and FO would show reduced CORT secretion, elevated locomotion in the OFT and EPM, as well as increased time spent in the OFT centre zone and the EPM open arm areas. On the other hand, high-fat diet (HVF) shall promote anxiogenic behaviour, especially when combined with the restricted feeding method.

Following validation of gavage as our method of choice for supplementation, the second study aimed to determine sex-specific impact of fish oil versus soybean oil supplementation during the adolescent period on fatty acids concentration profile in brain tissue, which was measured immediately following the adolescent 20-day supplementation period and at a delayed interval as rats reached early adulthood. These two studies provided valuable information supporting sex specific differences associated with n-3 PUFA supplementation on brain fatty acid concentrations. Previous studies supporting testosterone interference in PUFA dietary nutrient conversion into DHA, we predicted that female rats would have greater DHA brain concentrations than males.

After clarifying the general impact of the n-3 PUFA supplementation and the method of delivery in adolescent rodents, the third study characterized the impact of adolescent fish-oil supplementation, alone or combined to EE, on social and emotional responses, stress coping, as well as corticosterone and glucocorticoid receptor expression in male and female rats. One particular interest was to assess how environmental conditions would impact coping to acute stress exposure, anxiety-like responses, sociability, and social recognition and fear conditioned responses in adolescents. Biochemically, we measured effects of the various conditions on adulthood glucocorticoid receptor expression in the hippocampus. We predicted that a synergic effect of combined condition of EE and FO, would decrease anxiety-like behaviour, reduce corticosterone, and facilitate social behaviours, especially in male rodents. While we strongly suspect that female rats would benefit from FO supplementation, based on mixed findings in the literature regarding stress reactivity in females exposed to EE led to believe that observations in females exposed to both FO and EE might be ambiguous. Nonetheless, we believed that combined FO and EE could create a beneficial impact of sociability in females compared regular housing.

Finally, the fourth thesis study assessed immediate and delayed sex-specific effects of adolescent fish oil supplementation on cognitive performance and flexibility. The study was designed to evaluate the immediate -and longer-term impact of adolescence FO supplementation in male and female rats. Based on the positive impact of FO on cognitive abilities, we predicted that FO adult rats, both females and males, would show reduced working memory errors, shorter latencies and distance travelled to reach the Barnes maze hidden box as testing progressed compared to rat fed a soybean supplemented diet.

Study 1

Delivery method matters: Omega 3 supplementation by restricted feeding period and oral gavage has distinct impact on corticosterone secretion and anxious behaviour in adolescent rats.

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Abstract

Objectives: Oral gavage and time-restricted feeding are common delivery methods for dietary supplementation to rodents. However, the stress associated with selected feeding regimens could represent a confounding variable. In rodents, the adolescence period is particularly vulnerable to stressful events, in part related to ongoing maturation of the brain. In this context, omega 3 dietary supplementation has shown beneficial effects on neuronal growth, cognitive performance, and stress regulation, while high-fat diet (HVF) has been associated with enhanced stress and anxiety. Therefore, this study has two aims: (1) evaluate the influence of 21-daysupplementation with soybean oil (control group; CSO), fish oil (FO) or hydrogenated vegetable fat (HVF) fatty acids (FA) during the adolescence period on corticosterone secretion and anxiety-like behaviour and,
(2) compare the impact of dietary supplementation using oral gavage or time-limited feeding on these measures. **Methods:** Oral gavage or restricted feeding were used to daily feed adolescent rats (PND28–47; n=49). On supplementation days 1, 7, 14 and 21, droplets of blood were collected for corticosterone (CORT) assessments. The Open Field (OFT) and the Elevated-Plus Maze (EPM) tests served to assess anxiety-like behaviour on PND50. **Results**: Our findings indicate increased CORT secretion in restricted-(R) compared to gavage-fed animals on DAY7 and DAY14, suggesting heightened HPA-axis reactivity. Notably, CORT secretion diminished in FO-R-rats (DAY21), suggesting improved coping/adjustment. Consistent with CORT assessments, findings in the OFT and EPM supported attenuated anxiety in gavage versus restricted groups. FO and CSO supplementation reduced anxiety compared to HVF intake. **Conclusions:** Our findings uncover a significant impact of feeding methods on anxiety-like behaviour and physiological stress response in rodents, supporting oral gavage as a less stressful option during the adolescent developmental stage. Supplement-specific effects on CORT secretion further indicated an influence of fish oil in regulating the stress response.

Keywords: Omega 3, High fat diet, fish oil, Gavage, Stress response, Anxiety-like behaviour, Corticosterone

Introduction

A balanced diet plays an important role in cognitive, memory and social development (Karr, Alexander & Winingham, 2011). The Western Diet (WD) is characterized by high omega 6, saturated and trans-fat content, as well as increased sugar, salt, and total energy to the detriment of essential nutrients such as fibers and omega 3 (Marion-Letellier, Savoye & Ghosh, 2016). While the optimal omega 3: omega 6 ratio shall approach ~ 4:1, WD ratios can reach 1:20 (Mario-Letellier, Savoye & Ghosh, 2016). Advantages of increased omega 3 consumption are numerous, including enhanced cell membrane fluidity, synapses formation and improved cognitive and attention abilities (Simopoulos, 2002; Gómez-Pinilla, 2008), while elevated omega 6 consumption is associated with reduced immune system function, increased allergic and inflammatory responses, altered hormonal balance and homeostasis, insulin resistance and compromised recovery following cerebrovascular disease (Marion-Letellier et al., 2016).

During the adolescence period, which ranges from postnatal days 28 to 47 in rodents, brain maturation is ongoing with major changes in the prefrontal cortex and interconnected mesolimbic system, which evolves into decisive functional networks (Jump, 2002; Wurtman, Cansex, Sakamoto & Ulus, 2009). During this period, the brain is highly vulnerable to environmental changes, and acute or prolonged stress exposure (Akirav & Richter-Levin, 1999; Ghashghaei & Barbas, 2002) has been associated with heightened corticosterone secretion and persistent dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis responses in adulthood (Petrovich, Canteras & Swason, 2001). Considering this situation, experimental procedures selected to study this period should aim to minimize associated stress.

Gavage or time-limited feeding are common methods to deliver drug or dietary supplementation to animals. However, assessment of the stressful nature of these procedures in young animals is pending, despite differences in stress responses (individual or age-related) being well documented (Goldman, Winget, Hollingshead & Leving, 1973). During the pre-pubertal and adolescence periods (Maslova, Bulygina & Markel, 2002), fluctuations in gonadal hormones are associated with greater imbalances of the physiological responses. For instance, adrenocorticotropic hormone (ACTH) and corticosterone (CORT) elevations in 30-day old rodents following a brief stress extended 45 to 60 min longer than those of adults' rodents (Golman et al., 1973). Studies also suggested abrupt changes in HPA axis reactivity at the puberty period, especially affecting CORT secretion in 30 to 40 days old rats (Turner et al., 2012; see review by Kane and Ismail, 2017). Oral gavage enabling direct delivery into the stomach of the animal via a feeding tube or syringe is widely used to deliver supplements to various age groups providing excellent control of supplement intake and drug dosage (Jahng et al., 2010). However, Brown (2000) showed oil supplementation by gavage to elevate CORT measurements collected 4 h following the procedure. Notably, such an effect was related to volume administered and fed substrates; injection volumes exceeding 10 mL/kg, and high-density lipids, having a stronger effect. The need for animal restraint can also generate stress, although habituation usually occurs over days. In this context, time-limited feeding characterized by minimal handling, no restraint and ad libitum feeding, represents an alternative method. This procedure can be valuable in feeding studies, as steady, consistent dietary intake patterns can stabilize diurnal hormones. Thus, in context where nutriment intake systematically occurs at a time of the day, endocrine secretion shift promotes pre-prandial hormonal secretion and facilitates digestion (Romeo, 2013). However, the 22 h feeding restriction period normally characterizing this regimen represents a stressful event, sometimes included in chronic mild stress protocols (Foilb, Lui & Romeo, 2011). Moreover, the rat's separation from their cage mate to control intake and avoid competition for food, represents a form of isolation (Turner et al., 2012).

Therefore, objectives of this study are two-fold: 1) to evaluate the impact of gavage versus time-restricted feeding on CORT levels and anxiety-like behaviours in the adolescent male rats, and 2) to compare effects related to administration of different fatty acid supplements, namely hydrogenated vegetable fat, fish oil and soybean oil. We predict that restricted-limited feeding will be associated with increased CORT secretion and anxiety compared to the oral gavage. This is based on rapid handling and small oil volume used in oral gavage, which shall facilitate habituation to the technique and minimally affect daily ab libitum feeding.

2. Methods

2.1 Subjects

Twenty-three days old Male Wistar rats (n=49), weighing between 80-100g, were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Rats were housed 2-3/cage with free access to standard rat chow (Teklad Global 18% Protein Rodent Diet, Envigo) and water. Upon arrival, a 5-day period facilitated the animals' vivarium acclimation. During this period, rats were handled for 2-3 min daily. Rats were housed in Plexiglas cages with beta chip bedding and maintained on a 12 h L:12 h D photoperiod (lights on at 07:00) with room temperature ~21-23°C and 60% relative humidity. A black polycarbonate tube (3.5-inch diameter) placed in each cage provided a secluded environment where rodents can rest and/or hide. The experiment was initiated at adolescence on postnatal day 28 (PND28) as described by Schneider (2013). All procedures were in accordance with the Canadian Council of Animal Care and approved by the Animal Care Committee. Experimentation complied with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

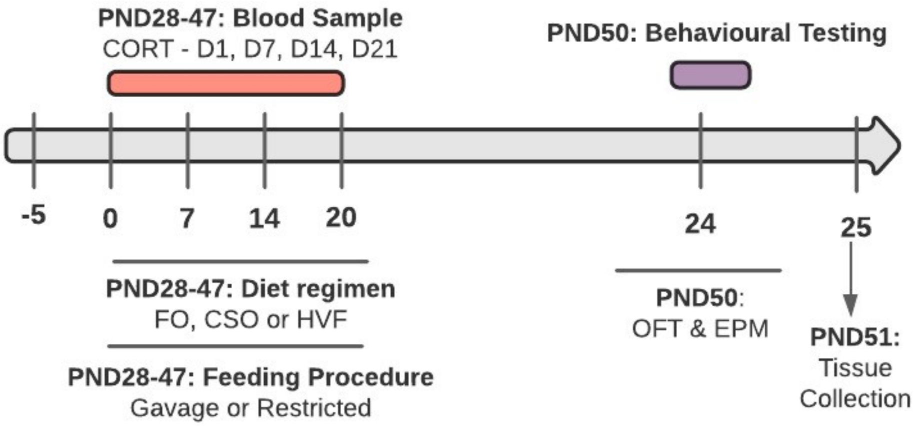

Figure 1. Timeline of the experiment. Animals arrived at the facility and were given 5 days of acclimation prior to the start of the experiment. On DAY0, rats were separated in the following groups: G-CSO, G-FO, G-HVF, R-CSO, R-FO or R-HVF. From PND28 to PND47, oil supplementation was provided using oral gavage (G) or restricted feeding (R). On experimental day DAY24, rats (PND 50) underwent OFT followed by the EPM testing to measure anxiety-like behaviour. The experiment ended on DAY28.

FO: Menhaden fish oil; CSO: Control Soybean oil; G: Gavage; R: Restricted; CORT: Corticosterone; OFT: Open Field Test; EPM: Elevated Plus Maze.

2.2 Feeding procedures/Experimental timeline

Upon arrival, rats were given 5 days of acclimation prior to the start of the experiment. At PND28, rats were divided into two groups according to oral gavage or time-restricted feeding. Dietary supplementation (via oral gavage or restricted diet) started at the PND28 and continued daily until PND47, which consists in a 21-day supplementation. Rats were fed daily between 7:30 and 9:30 am. Three oil supplements were compared 1) Soybean oil (CSO), a non-hydrogenated omega 6 rich supplement a 7.4/1 n-6/n-3 ratio, 2) Fish oil from menhaden (FO), an omega 3 rich supplement (Sigma Aldrich; 20.0 -31.0% omega 3 fatty acids content; Eicosapentaenoic Acid (EPA - 10-15%)/ Docosahexaenoic Acid (DHA 8-15%) or 3) hydrogenated vegetable fat (HVF) a high fat supplement containing 18% saturated and 0% trans-fat. On experimental day 24 (PND 50), daily handled ad libitum fed rats from all groups underwent behavioural testing (OFT and

EPM) to measure anxiety-like behaviour. Rodents' brains were collected on DAY28 (See Fig. 1 for the timeline of the study).

2.2.1 Oral Gavage (-G)

Animals (n=25; 8 CSO; 9 FO; 8 HVF) were daily fed a soybean oil (CSO), fish oil (FO) or hydrogenated vegetable fat (HVF) supplementation (Sigma-Aldrich Canada) (0.3ml/100g body weight) for a 20-day period (Brown et al., 2000). The complete procedure, including handling, lasted 5-10 min/rat.

2.2.2 Restricted Feeding (-R)

Rats were fed every morning for a 2 h period (between 7:30am and 9:30am) in individual cages (n=24; 8 CSO and 8 FO, 8 HVF) (Sunderram et al., 2014). The CSO, FO or HVF supplements were mixed with the regular rodent chow immediately prior delivery (15% supplement content in regular rat chow - Teklad Global 18% Protein Rodent Diet) as per Plamondon and Roberge (2008). To ensure a consistent supplementation intake, adjustments were made by measuring food intake throughout the rodent's development to ensure maintaining an equal supplementation ratio according to growth (consumption was between 15-25g per day) and taking individual chow consumption differences into account. Following individual feeding sessions, rats reunited in their home cages. Food intake was monitored to ensure adequate nutrition and weight gain.

2.3 Behavioural Testing

The Open Field test (OFT) and Elevated-Plus Maze (EPM) served to assess anxiety-like behaviour and locomotion the day following completion of the 21-day dietary supplementation. Prior to testing on PND50, rats were moved to an acclimation room for at least 30 min (7h00 AM). Rats underwent the OFT in the morning (between 7h30 to 12h00) and the EPM in the afternoon (12h00 to 16h00). Rats were first exposed to the OFT. The testing area was lit with 400 lux overhead lights. The researcher was separated from the testing area by a white curtain, minimizing external visual cues to the animals. Testing sessions were recorded using a ceiling mounted camera (Panasonic® Analog Camera, Model: WV-CP284) and behavioural responses coded using ODlog^{TM} (trademark of Macropod Software). Both apparatuses were thoroughly cleaned with a 70% Ethanol solution between each rat.

2.3.1 Open Field Test (OFT)

The test consists in a 75 cm x 75cm open arena with 15 cm height walls. Thirty-six equally sized squares divide the floor (20 in the periphery and 16 in the centre). The rat is initially placed in the centre of the arena and left to freely move for 10 min. Global activity is recorded using a video camera. The number of entries in the centre and peripheral zones, the latency to enter the centre and immobility (the rat is still, but not freezing) were determined using computer-assisted data logging (ODlog software). Locomotion was determined by counting the number of squares crossed in the centre and periphery. A rat was considered in the centre or periphery when its head and two front paws were in this area.

2.3.2 Elevated-Plus Maze (EPM)

The apparatus consists of a plus-shaped Plexiglas structure with two open and two closed arms, each measuring 50cm x 10cm. The closed arms have 40 cm high walls while edges of the open arms consist in a 5mm Plexiglas lip. The rat is initially placed in the EPM centre, head facing an open arm and allowed to freely explore the maze for 5 min. A video camera recorded activity, and the latency to open arm entry, the number of open and closed arm entries, resting periods (as defined previously), centre zone crossings and time spent in the open and closed arm were determined using computer assisted ODlog data logging software. A rat was considered in a particular area when its head and two front paws were in this area. Risk assessment was operationally defined as the rat having its snout protruding in the centre zone while having its body (including the two front paws) in a closed arm.

2.4 Blood corticosterone collection (tail venipuncture)

Blood samples were collected (between 7:00-:00AM) at predetermined intervals on days 0 (before supplementation was initiated), 7, 14 and 21 of the feeding paradigms. Following a small incision on the lateral tail vein, two blood droplets were collected in less than 3 min on Whatman BFC180 bloodstain cards (Sigma-Aldrich, Canada) as previously described (Guan et al., 2014) Cards were let to dry overnight at room temperature prior to storage in -80°C.

2.5 Blood corticosterone immunoassay

CORT levels were quantified in duplicates using an enzyme-linked immunosorbent assay (ELISA) kit (Corticosterone EIA kit, AD1-901-097, ENZO Life Sciences). Standards and samples were prepared as recommended by the manufacturer. Whatman cards were allowed to thaw for 30 min at room temperature (RT) after removal from -80°C storage. As previously reported Azogu & Plamondon (2017), a 3.0mm diameter circle was punched from the cards' blood samples using a Gem Hole Punch (McGill Inc., Marengo, IL) (n = 4/group/collection intervals). Punches were placed in glass tubes containing 280 µl of assay buffer covered with parafilm and shaken for 24 h at room temperature on a Belly Dancer® (Structure Probe Inc., West Chester, PA). Following incubation, 214.5 µl of each sample was mixed with 5.5 µl of steroid displacement reagent (SDR) to minimize corticosterone binding to proteins. Samples were then diluted 1:5 with the assay buffer. CORT concentrations were determined using a PowerWave™ XS2 Microplate Spectrophotometer (BioTek, Winooski, VT), and calculated using a four-parameter equation derived from the standard curve values. Intra- and inter-assay variability was determined. The analytic range of the assay was 32–20,000 pg/ml

2.6 Statistical Analyses

Data are expressed as mean + S.E.M. A two-way ANOVA and Repeated Measure General Linear Model served to analyze the behavioural and CORT data, respectively. Prior to analyses, homogeneity of variance, skewness and kurtosis were determined and corrected when necessary. A boxplot and Z scores (above 2.58) served to identify outliers and analyses were adjusted for unequal groups. Main and simple effects were measured using IBM SPSS 21 software. Between group differences were determined for seven behaviours in the OFT and six in the EPM. Differences were considered statistically significant when p-value is lower than 0.05. Simple effects were confirmed using Bonferroni corrected post-hoc comparisons.

3. Results

3.1 Open Field Test (OFT)

3.1.1 Time spent in centre and periphery

Analysis of the time spent in the centre zone revealed a main effect of the feeding method [F (2, 49) =10.216, *p*=.003], supplement [F (1, 49) =5.8, *p*=.006] and an interaction between feeding*supplement [F (2, 49) =7.01, *p*=.002]. Simple effect indicates that reduced centre time in G-FO compared to G-CSO (*p*=.001) while comparison with G-HVF did not reach significance (p=0.061). Notably, R rats showed reduced open zone exploration compared to G rats (*p*=.04). (See Figure 2a). For the time spent in the periphery, a main effect was observed for the feeding method [F (1,49) =16.57, *p*<.001], supplement [F (2, 49) =9.778, *p*<.001] and a procedure*supplement interaction [F (2,49)=6.840, *p*<0.01]. Simple effect tests revealed differences between the G-FO and G-CSO and G-HVF rats (*p*≤.001). Consistent with time spent in open zones, G-CSO and HVF rats differed from the R-CSO and R-FO (*p*<.001; *p*<.01, respectively) (See Figure 2b).

3.1.2 Immobility in the centre and periphery

In the centre zone, analysis revealed a main effect of feeding method [F (1, 49) =58.118, *p*<.001], supplement [F (2, 49) =25.041, *p*<.001] and a method*supplement interaction [F (2, 49) =22.721, *p*<.001]. Simple effect test indicates that the G-CSO fed rats spent more time resting/grooming/washing compared to all other conditions (*p*<.001). In the periphery, a main effect of supplement [F (2, 49) =3.35, *p*<.05] was found, largely related to reduced resting time by R-HVF rats compared to R-FO and R-CSO rats (*p*<.05). As described in the method section, the rodents in an immobile state are not freezing, which is normally associated to a stressful reaction (See Figure 2c and d).

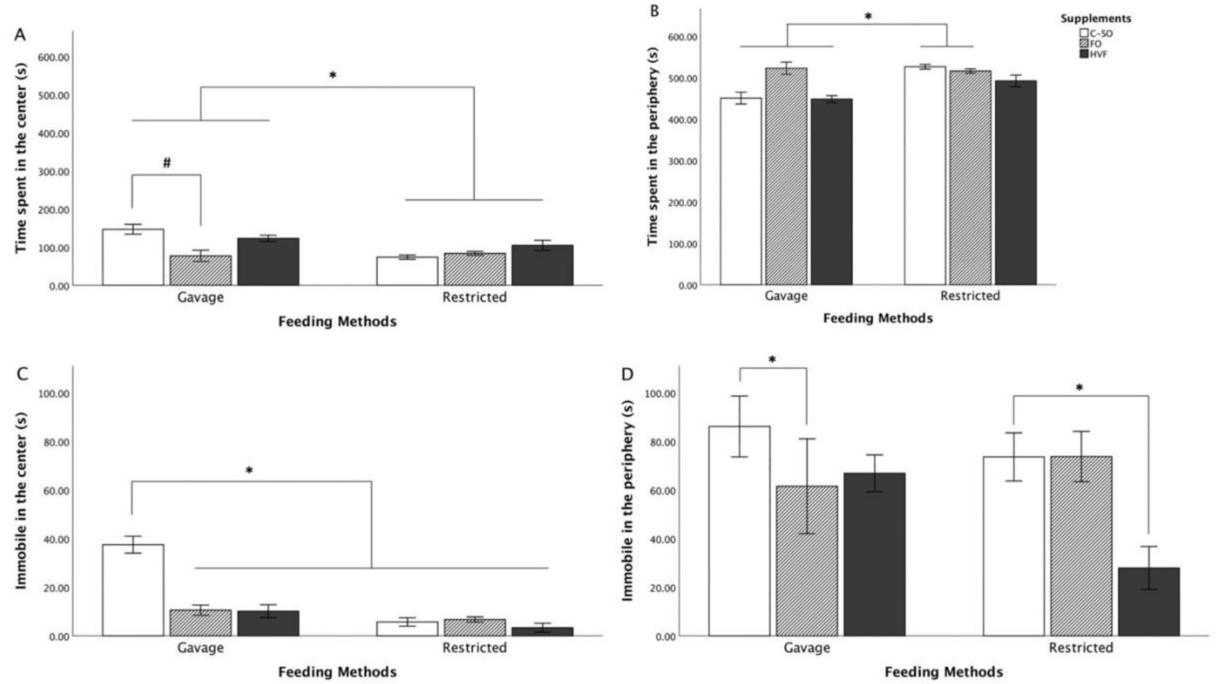

Figure 2. Effect of feeding procedures and dietary supplements in the Open Field Test (OFT) for the time spent in the centre (2a) and in the periphery (2b). Dietary supplementation and delivery method influenced immobility in the centre (2c) and peripheral (2d) zones. *Indicates statistical significance (*p*<0.05). Values represent means ± S.E.M.

CSO: Soybean; FO: Fish Oil; HVF: Hydrogenated Vegetable Fat

3.1.3 Frequencies of arm entries, locomotion, and latency to centre zone entry

For the frequencies of entries in the centre zone, analysis revealed a main effect of the feeding method [F (2, 49) =8.354, *p*<.01]. G-fed rats entered the centre zone more frequently than R-fed rodents, supporting reduced stress. Consistently, a main effect of the feeding method [F (2, 49) =9.364, *p*<.01] was found for frequencies of entries in the periphery due to G-fed rats entering this zone more frequently than R-fed rats. The number of squares walked in the centre and periphery served to determined locomotion. For centre zone locomotion, a main effect of method [F (2, 49) =7.260, *p*<.05] and supplementation [F (2, 49) =5.391, *p*<.01] was found. Simple effect tests showed increased exploration of the centre zone in G- compared to R rats (*p*=.010). The FO and HVF fed groups also differed, the former showing increased centre zone exploration (*p*<.01).

In the peripheral zone, only a main effect of method [F (2, 49) =9.732, *p*<.01] was found. Simple effect tests indicated that R-rats spent more time walking in the peripheral zone compared to G-fed rats. Finally, no group differences were observed for the latency to enter the centre zone.

3.2 Elevated-Plus Maze (EPM)

3.2.1 Time spent in open and closed arms of the EPM

For the time spent in the open-arms, analysis revealed main effects for the feeding method [F (1, 49) =5.483, p<.01] and supplement [F (2, 49) =13.322, p<.001]. Simple effect was analyzed, and we found that the HVF group spend statistically more time in the open arm compared to the FO (p<.001) and the CSO (p<.001) conditions (See Figure 3a)

For the time spent in the closed arms, a main effect of the supplement [F (2, 49) =13.755, *p*<.001] and a supplement*method interaction [F (2, 49) =4.722, *p*<.05] was observed. Consistent with observation in the open arms, G-HVF-fed rats spent less time in the closed-arm compared to

G-CSO and G-FO rats (*p*<.001). The G-HVF also spent reduced time in the closed-arm compared to the R-FO (*p*<.05) and R-CSO (*p*<.001) conditions. (See Figure 3b)

3.2.2 Frequencies of open/closed arm entries and arm crossings.

Analysis of the frequencies of open-arm entries revealed main effects of method [F (1, 49) =13.834, *p*<.01] and supplement [F (2, 49) =6.477, *p*<.001]. G-rats showed increased open arms entries compared to R-rats (*p*<.001), G-HVF rats entering the open arms the most compared to other supplementations (See Figure 3c). For frequencies of closed-arm entries and arm crossings, analyses revealed main effects of the feeding method [F (1, 49) =10,237, *p*<.01; F (1, 49) =4.67 *p*<.05, respectively], mainly attributable to G-HVF rats showing elevated closed-arm entries, and G-CSO displayed reduced arm crossings compared to R-counterparts. (See Figure 3d and 3e)

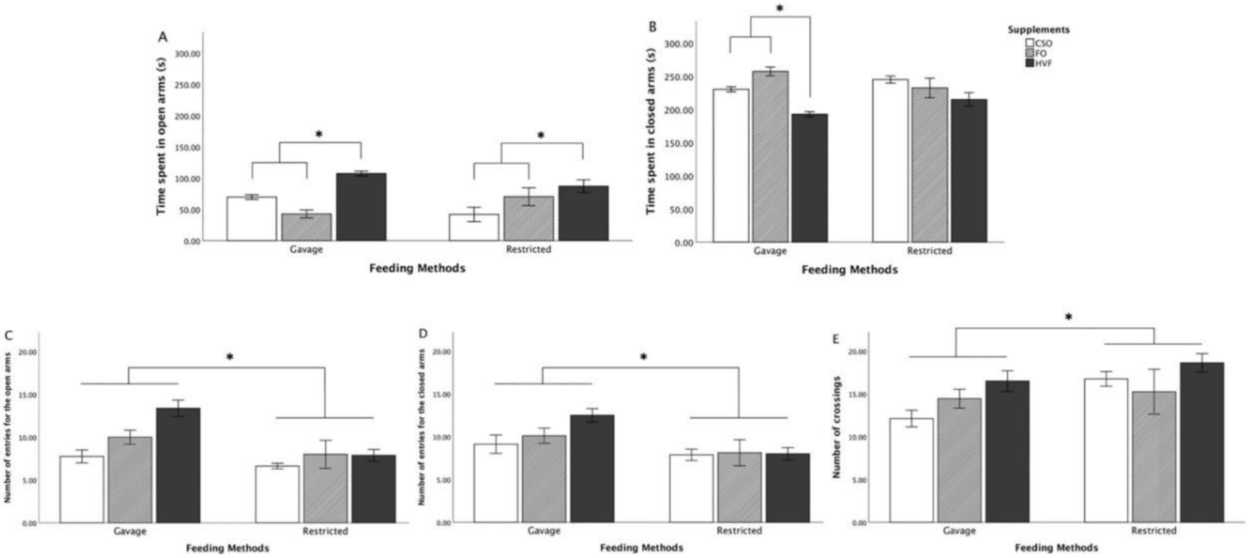

Figure 3. Effect of dietary supplement and feeding methods (EPM) on time spend the open-(3a) and closed (3b) arms, for the number of open and closed arm entries (3c & 3d), and arm crossings (3e). *Indicates statistical significance (p<0.05). Values represent means ± S.E.M.

CSO: Soybean; FO: Fish Oil; HVF: Hydrogenated Vegetable Fat

3.2.3 Risk Assessment time and frequencies

All groups spent comparable time performing risk assessments although risk assessment frequencies showed feeding method [F (2, 49) =30.457, p<.01] and supplement [F (2, 49)=6.647, *p*<.01] effects, related to G-rats making increased risk assessments compared to R-rats (p<0.01). (See Figure 4a and b)

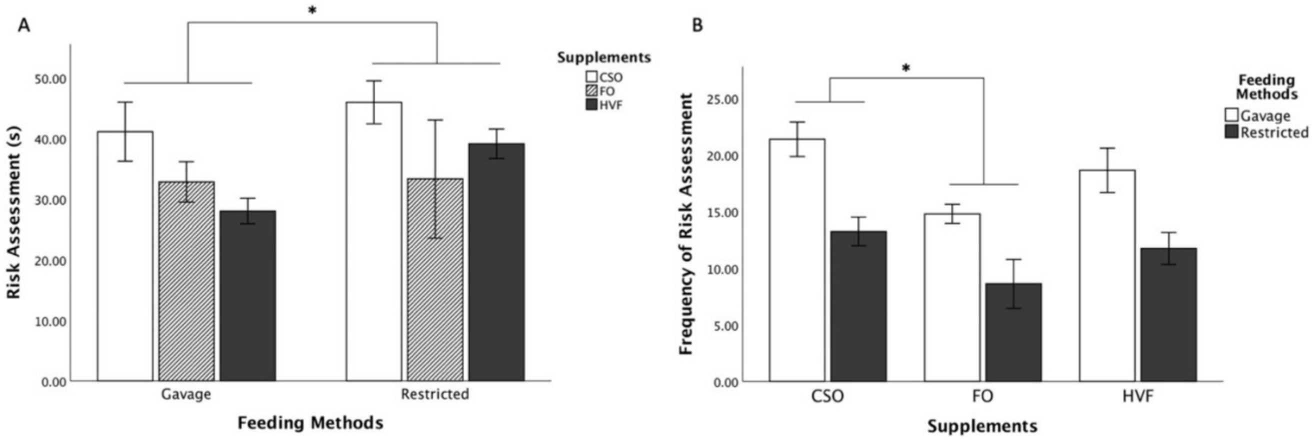

Figure 4. Effect of feeding procedures and supplement in the Elevated-Plus Maze (EPM) for Risk Assessment behaviour (4a) and the frequency of risk assessment (4b). *Indicates statistical significance (p<0.05). Values represent means ± S.E.M.

CSO: Soybean; FO: Fish Oil; HVF: Hydrogenated Vegetable Fat

3.3 Corticosterone secretion

Two-way ANOVAs revealed main effects of the feeding method [F (2,25) = 30.014, *p*<.01] and supplements [F (2,25) = 4.015, *p*<.01] and on CORT levels assessed at 4-time intervals during the 20-day feeding period. Baseline CORT levels did not differ between the groups. In support of behavioural observations, R-rats showed increased stress levels compared to G-rats when comparing DAY 1 (baseline values) to all other sampled days (*p*<.001) (see Fig. 5a). DAY7 revealed differences related to feeding method [F (1, 25) = 34.088, *p*<.001] and supplementation [F (2,25) = 6.152, *p*<.001], again highlighting reduced CORT secretion in G-fed rats. Notably, a trend toward reduced CORT secretion was observed in R-CSO rats compared to R-FO rats (p=0.051) at this time interval (See Figure 5a). At DAY14, CORT levels related to the two feeding methods remained statistically different (*p*<.001). Regardless of the supplement provided, R-fed groups showed increased CORT secretion (See Figure 5a). On the last collection (DAY21) (n= 8/group), analysis showed main effects of feeding method [F (1, 49) =63.426 p<.001, η^2=0.406], Supplements [F (2, 49) =3.659, *p*<.05, η^2=0.145] a method*supplements interaction [F (2, 49) =3.689, *p*<.05, η^2=0.146]. Simple effect tests show this effect to be due to elevated CORT secretion in R-CSO and HVF compared to R-FO supplemented rats (*p*<.05 and p=0.05). R-FO rats no longer differed from G-fed rats (*p*<.01 for all comparisons), suggesting facilitated adaptation of R-rats fed FO (See Figure 5b).

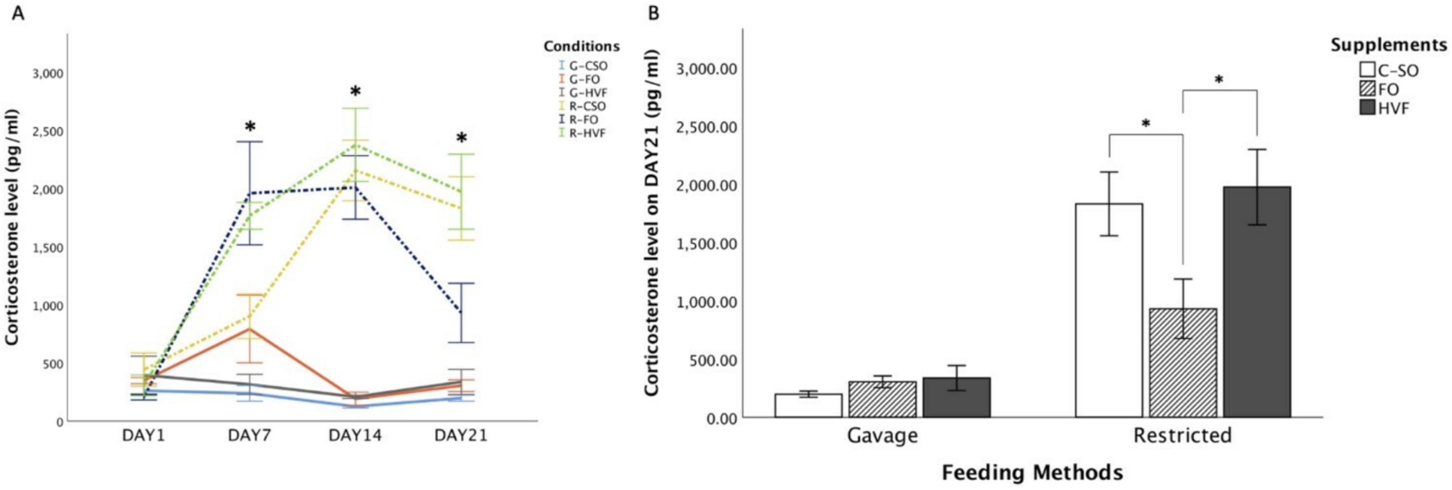

Figure 5. Corticosterone levels (pg/ml) assessed on Experimental Days 1, 7, 14 (n=4 rodent per group) (Fig 5a) and at DAY21 (CSO & HVF n=8/group; FO, n=9) (Fig 5b). Data suggest an important impact of the selected feeding method on CORT secretion. *Indicates statistical significance (p<0.05). Values represent means ± S.E.M.

CSO: Soybean; FO: Fish Oil; HVF: Hydrogenated Vegetable Fat; G: Gavage; R: Restricted

3.4 Weight monitoring

A Repeated Measure ANOVA revealed differences in weight gain over days related to feeding methods [F (1, 49) = 9.123, *p*<.01, η^2=0.175], the R-rats showing reduced weight gain. Furthermore, main effect of supplements and method*supplements interaction were found [F (2, 49) =3.613, *p*<.05, η^2=0.144 and F (2, 49) =3.265, *p*<.05, η^2=0.132]. Surprisingly, at the end of the 21-day feeding period reduced weight gain was observed in gavage rats fed HVF compared to CSO and FO groups (*p*<.05 and *p*<.05, respectively). Body weight assessments 7 days following the supplementation period support a lasting impact of ingested supplement [F (2, 49) =6.08, *p*<.01, η^2=0.221], feeding method [F (2, 49) =45.02, p<0.001, η^2=0.511] and a supplement*method interaction [F (2, 49) =3.21, *p*<.05, η^2=0.130]. Most notable is the observation that HVF fed rats, G and R, failed to gain similar weight to other rodents during this period (See Figure 6).

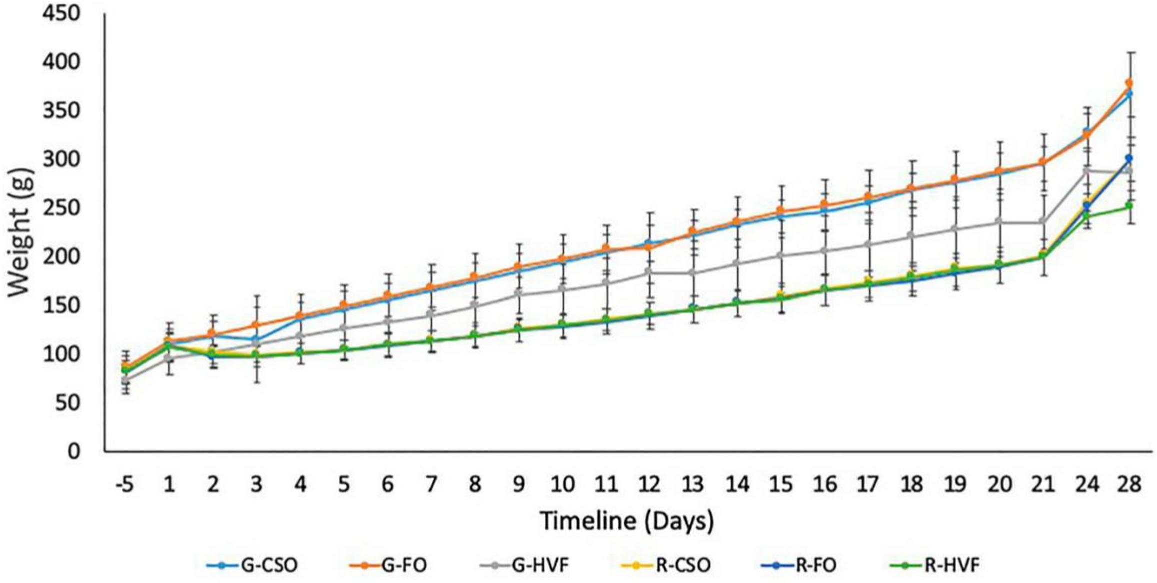

Figure 6. Weight monitoring (g) from the arrival day (5 days prior to start of the experiment) until euthanasia DAY 28. Values represent means ± S.E.M.

CSO: Soybean; FO: Fish Oil; HVF: Hydrogenated Vegetable Fat; G: Gavage; R: Restricted

4. Discussion

To our knowledge, this study is the first to characterize effects of dietary supplementation dispensed during the adolescent period on CORT response at different time intervals during this process and ensuing impact on anxiety-like behaviour in 50-day old male rats. Despite extensive research on nutraceuticals over the last decade, the influence of omega 3 supplementation in adolescence remains understudied (Karr et al.,2011; Akirav et al., 1999, Ghashghaei et al., 2002).

Importantly, studies have used different administration regimens to best control intake and prevent oxidation of oil supplements, and there is a lack of research assessing the impact of different feeding regimens on bio/behavioural responses, especially in young rats. Our data shows that oral gavage and time-restricted feeding have a distinct impact 1) on basal CORT secretion measured at multiple time intervals during exposure to the feeding protocols and 2) on anxiety-like behaviours in the Open Field and the Elevated-Plus Maze tests.

The effects of adolescence exposure to dietary supplements on emotional responses is of particular interest considering an important gap in knowledge, and maturing brain connections between the prefrontal cortex and the limbic system, including the amygdalar complex, which characterizes this developmental stage (Karr et al., 2011). Our findings indicate that time restricted feeding induced a significant rise in CORT secretion, gradually achieving a 4-5-fold increase compared to secretion values associated with oral gavage, independently of the ingested oil supplement. While CORT secretion remained largely stable over time in G-fed rats, CORT values in R-fed groups significantly increased over time, peaking on Day 14. Comparing intraperitoneal and oral gavage administration of glucose in adult rodents, Pilon et al. (2018) similarly showed rapid restoration of basal CORT levels in gavage animals, while IP administration led to sustained CORT elevations over a 2h period following glucose administration levels.

4.1 Time-limited feeding, corticosterone secretion and nutrient effects

Past studies have supported increased CORT secretion in fasted rodents (Correia Bacarin et al., 2013; Inou et al., 2004), which could explain distinctive impact of the two feeding paradigms in this experiment. Furthermore, Jensen et al. (2019) showed endocrine fluctuations to be more pronounced if the fasting period was performed during the “night” (dark cycle of the rodent) (Plamondon & Roberge, 2008). In this study, fasting occurred in large part during the night cycle, possibly inducing larger CORT morning secretions. Of note, rodents exposed to limited feeding time tend to over-consume food in a rapid fashion, an intake pattern similar to that observed in binge eating. A recent study supported this behavioural response to promote increased CORT secretion in rodents (Milot et al., 2012). Importantly, restricted feeding has been associated with changes in food anticipatory activities and phase shift in endocrine rhythms (Gharami et al., 2015), affecting CORT measured at precise time intervals in restricted fed rats. In this context, including CORT measures at other times of the day would be informative. Our observations also support a possible effect of separation at the adolescent and adolescent periods, which has yet to be understood, although the lasting impact of maternal separation (3-6 h) in the neonatal period is well acknowledged (Gharami et al., 2015). This is an interesting avenue for future studies to explore.

In this context, when tested for anxiety as young adults (PND 52), differences related to feeding methods emerged over those associated with the dietary supplements. These were characterized by increased novel field exploration, frequencies of entries in centre and peripheral zones, and locomotion in the anxiogenic centre zone in G-fed compared to R-fed animals. These observations support long term benefits of using gavage over limited feeding at this developmental stage on emotionality. Studies have shown that mate separation and food restriction, which are part of time-limited feeding, participate in increased anxiety-like behaviour and elevated corticosterone secretion measured in adult rats (Foilb et al., 2011; Turner et al., 2012; Honma et al., 2009). Our findings thus indicate that R-fed adolescent rats respond in a similar fashion to adult rats.

Interestingly, we observed synergistic effects between dietary supplements and feeding methods, the impact of dietary supplementation being better distinguished using the less stressful gavage procedure. Using this feeding regimen, CSO supplementation led to increased OFT centre zone exploration compared to HVF and FO. This observation is consistent with cognitive benefits related to a 4-week CSO supplemented diet in rats, as well as soybean oil regulatory effects on serotonin level, a critical mood regulator (Helland et al., 2003). Fish oil also increased walking in the OFT centre zone compared to HVF, notwithstanding the feeding conditions, indicating attenuated novelty induced anxiety. To these effects, reduced n-3 PUFA and DHA plasma levels have been linked to dysregulation of three biological stress systems (i.e., the immune-inflammatory system, the HPA-axis and the autonomic nervous system) in 2724 participants from the Netherlands Study of Depression and Anxiety (Soni et al., 2015) and omega 3 PUFA supplementation via fish oil reported to reduce depressive-like symptoms through eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derivatives (McGhee et al., 2009). Proposed mechanisms underlying PUFAs effects on mood regulation have included increased secretion of brain derived-neurotrophic factor (BDNF) accompanied by reduced corticotrophin-releasing hormone (CRH) release (Shojaie, Ghanbari & Shojaie, 2017). In this study, FO showed comparative beneficial effects to attenuate CORT secretion in R-fed rats exposed to a more stressful ingestive condition, supporting advantages of such diet in promoting improved coping responses. FO supplementation in the R-feeding condition was associated with delay-dependent attenuated CORT secretion, which reached statistical significance compared to the CSO and HVF on Day 21. Knowing that CRH reduces BDNF secretion while omega 3 consumption can mitigate this effect, we propose that interplay between these systems could participate in reduced CORT levels at remote intervals (Jense et al., 2019).

4.2 Hydrogenated vegetable fat consumption and anxiety-like behaviour in the Elevated Plus Maze

In the EPM, a similar pattern emerged between the G- and R- feeding conditions. Anxious behaviours were more frequently observed in R-rats, which spent increased time in the anxiogenic zones. Although feeding methods yield no difference in the time spent performing risk assessment, G-rats showed increased frequencies of risk assessment compared to the R-fed rats. Noteworthy, some observations in the EPM contrast OFT behavioural findings. For instance, HVF supplemented rats (G- and R) showed a trend towards spending increased time in the open arms of the EPM, suggesting reduced anxiety-like behaviour. This is an interesting finding, which is difficult to reconcile with these studies showing HVF supplementation to enhance stress responses. Thus, mice fed high-fat diets showed increased anxiety-like behaviour in the OFT, elevated zero maze (EZM) and forced swim test (FST) (Hussain et al., 2018). Of note, Steele et al (2019) reported that rodents fed HVF-enriched diet for 8-week lack the ability to delay lever press in a delay-discounting task, suggesting increased impulsivity. Thus, it is possible that impulsivity accounts for HVF-fed rats rapidly entering the open arms, without assessing the risk.

5. Concluding remarks

The experimental design was closely examined prior to testing. However, although both supplementation methods are commonly used in nutritional and pharmaceutical research, inequalities in ensuring exact and consistent supplementation may remain. As described in the methods section, administrating a supplement via gavage is a precise means, the measured amount being directly placed in the stomach. Using restricted feeding, despite taking the necessary measures to ensure proper supplementation, it is possible that small amount of oil is wasted during feeding (Cameron-Smith et al., 2015). If the oil supplementation is not freshly mixed daily, it can create oxidation and represent an issue possibility introducing undue bias. Our findings clearly demonstrate that the procedure used for administrating a supplement has a significant effect on the adolescent rat stimulation of CORT secretion and later affect anxiety levels. In adolescent rats, gavage had minimal impact on CORT secretion and is the best suited procedure to assess intrinsic effects of dietary supplementation in regulating behavioural and biochemical responses. To our surprise, dietary supplements showed mitigated effects in regulating behavioural responses. In this context, Pase et al. (2013) showed that the type of fatty acids (soybean, fish oil or hydrogenated vegetable fat) provided via oral gavage supplementation during development and growth over two generations is able to modify the brain oxidative status and response to acute stressor, which was particularly adversely affected by trans-fat. Further investigation of the impact of nutritional composition at this critical developmental period is commendable.

Study 2

Fish oil supplementation during adolescence induced sexually dimorphic changes in DHA, LA and AA brain concentration measured post supplementation and during adulthood.

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Abstract

Food diversity and increased intake of un-synthesized molecules such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) promote cell membrane formation and brain maturation. Omega-3 supplemented diets (DHA and EPA enriched) have been associated with improved cognitive flexibility and performance, and attentional control. DHA and EPA storage in brain structures such as the hippocampus and prefrontal cortex has been linked to improved memory function and associated to brain plasticity during sensitive developmental period, such as adolescence. This study assessed the impact of dietary supplementation during adolescence on brain DHA levels measured in males and female rats immediately following supplementation and during adulthood. **Methods**: 48 post-weaning Wistar rats (n=24 males; n=24 females) were randomly assigned to one of the 4 groups. Control animals received soybean oil (C-SO and the experimental groups received menhaden fish oil supplement (FO) daily (n=12/sex/oil supplement). Rats were daily supplemented via oral gavage (0.3ml/100g body weight) throughout the early and mid-adolescence period (from PD28-47). Whole brain extraction was performed upon termination of the gavage procedure (PND48) or during adulthood (PND90). Fatty acid methyl esthers, sterol esthers, phospholipids and triacyclycerols were extracted by gas liquid chromatography using the FAMEs procedure**. Results**: Our findings indicate increased DHA levels in females compared to male rats in adolescence and adulthood. Importantly however, the benefit of supplementation appears sex-specific, elevated adulthood DHA concentrations from dietary sources being only achieved in males as they reach adulthood. **Conclusion/Implications**: Dietary supplementation during the adolescence period influence brain composition at long intervals, with fatty acid supplementation being influential to fatty acid composition of the adult male brains.

Keywords: Omega 3, Docosahexaenoic acid, Essential fatty acid, Brain maturation, Sex-dependent

Introduction

Two types of essential fatty acid (EFA) are present in the human diet: the omega 3 and 6 polyunsaturated fatty acids (PUFA), also referred as n-3 and n-6 PUFAs (Gómez-Pinilla, 2008; Jump, 2002; Simopoulus, 2002; Wurtman et al., 2009). At present, the omega 6 and omega 3 diet ratio in North America ranges between 15:1 to 20:1 when the optimal ratio is around 4:1 (Simopoulus, 2002; Gómez-Pinilla, 2008; Karr et al, 2011). Such abundant omega 6 ingestion has an effect to reduce nutrient absorption, which can disrupt the nervous system physiology (Metzler-Zebeli et al., 2013). Elevated n-6/n-3 dietary ratios have been associated with increased risk of type-2 diabetes (Lazic et al., 2014; Ma et al., 2021), cancer cell proliferation (Ge et al., 2002; Dermadi et al., 2017), and cardiovascular disease (Simopoulos, 2008; Gomez-Delgado et al., 2021). Studies demonstrate that reaching a 4:1 dietary ratio appears sufficient to reduce many health risks, including cardiovascular problem. Findings from de Lorgeril and al. (2013) support a substantial reduction (approaching 70%) in the risk of mortality from coronary heart disease in people adopting a 4:1 consumption ratio over a two-year period. Other studies showed ratios between 2.5:1 and 4:1 to be associated with reduced cancer cell proliferation and improved inflammatory and autoimmune disease (Simopoulos, 2002; Zárate et al., 2017; Maillard et al., 2017).

Not being synthesized by the mammalian body, essential PUFAs are uniquely available via nutrition (Simopoulos, 2002; Gómez-Pinilla, 2008), and dietary intake or supplementation is critical to maintain optimal brain and body functions. PUFAs influence cell membrane composition and architecture and a wide range of brain functions, including vesicle formation and protein transport (Kitajka et al., 2002; Echeverría, Valenzuela, Catalina Hernandez-Rodas, & Valenzuela, 2017). Rapidly crossing the blood brain barrier, EPA and DHA act to promote neuronal growth, synapse formation and long-term potentiation, enhancing memory consolidation cognitive performance/flexibility, and attentional processes (Gharami, Das, & Das, 2015; Sidhu, Huang, & Kim, 2011; Wurtman et al., 2009). Consistently, DHA deficiencies have been associated with reduced synapsin and glutamate receptor expression in the hippocampus (Cao et al., 2009).

The two essential PUFAs are linolenic acid (LA, C18:2n-6) and α-linolenic acid (ALA; 18:3n-3). LA is metabolized to arachidonic acid (AA; 20:4n-6), adrenic acid (22:4n-6) and docosapentaenoic acid (DPA; 22:5n-6), while ALA is transformed to eicosapentaenoic acid (EPA; 20:5n-3), docasapentaenoic acid (DPA;22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Jump, 2002). This conversion process can be relatively slow mostly due to the poor conversion through the delta-6 desaturase-catalysed step (Shahidi & Ambigaipalan, 2018) (See Figure 1). An unbalanced n6/n3 ratio common to the Western diet leads to reduced production of key metabolites from n-3 PUFA ingestion and reduced systemic EPA and DHA concentrations, with increases LA metabolites (Jump, 2002; Shahidi & Ambigaipalan, 2018).

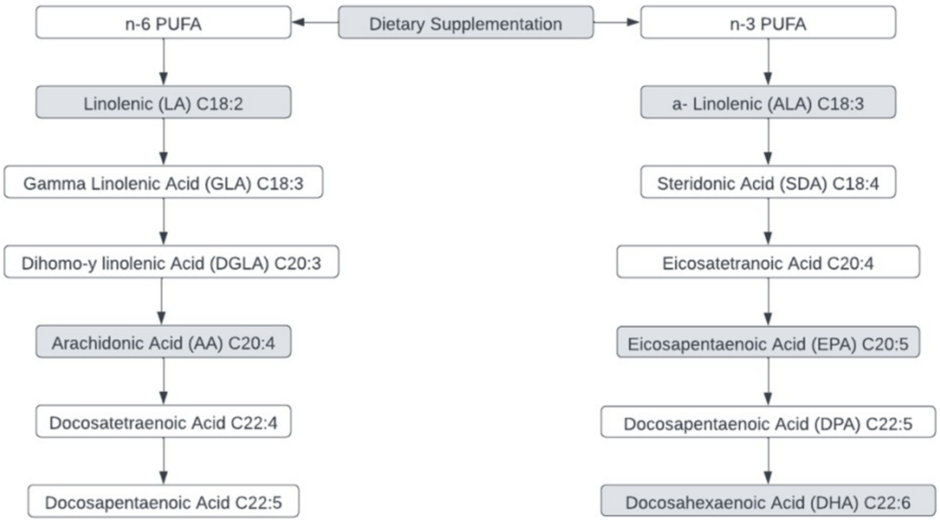

Figure 1. Elongation and desaturation of omega 3 and omega 6 chain of action

Interestingly, studies suggest females to most benefit from omega 3 PUFA supplementation compared to males. This is in part attributed to testosterone reducing the conversion rate of 18-carbon essential PUFA rich diets into DHA, while estrogen facilitates such conversion (Marra et Alaniz, 1989; Schuchardt, Huss, Stauss-Grabo, & Hahn, 2009; Childs, 2008) and promotes the shunting of free fatty acids toward oxidation and away from triglyceride storage (D'Eon et al., 2005; Huang & Horrobin, 1987). Estrogen is also known to exert beneficial effects on cognition, although relationship to hormonal effects on PUFA metabolism have not been determined (Huang & Horrobin, 1987; Marra & Alaniz, 1989; Schuchardt et al., 2009). In contrast, exposure to elevated testosterone levels during development has been proposed to influence attentional processes and sociability in children (Tammam et al., 2016).

Recent studies have indicated DHA consumption to exert restricted impact on brain DHA content in adult mice compared to ALA supplemented diet, despite isotope ratio mass spectrometry experiments supporting the half-life of brain DHA to be much longer than that assessed in metabolic tissues like the liver and adipose tissue (Lacombe et al., 2020). Furthermore, the displacement of brain DHA by DHA derived from dietary ALA is much slower compared to that of dietary DHA (Lacombe et al, 2020). However, whether dietary supplementation with DHA during adolescence has a lasting impact on brain fatty acid composition in adulthood, despite cessation of dietary DHA consumption, is not known.

The current study compares sex-specific effects of a 20-day dietary supplementation in male and female rats with dietary fish oil (source of EPA+DHA) or soybean oil (source of ALA) during adolescence on brain fatty acid composition immediately following the supplementation period or at a delayed interval during adulthood.

2. Methods

2.1 Subjects

Wistar rats (n=48; 24 females and 24 males) arrived at the animal facility at PD23 from Charles River Laboratories (Rochefort Quebec, Canada). They acclimated to the vivarium for a 5-day period prior initiation of the experiment. Male and female rats were randomly assigned to one of two supplementation groups, receiving an elevated source of omega 3 from menhaden fish oil (FO; Sigma-Aldrich Canada) or a control supplement consisting of soybean oil (C-SO; Sigma-Aldrich Canada). Dietary supplementation ranged from PND28 to PND47, covering the early to mid-adolescence period. Rats were group housed 2-3 per cage and had ad libitum access to rat chow (Teklad Global 18% Protein Diet® manufactured by Envigo®) and water throughout the experiment. They were kept on a 12h light/dark cycle (light on at 7AM). Half of the male and female rats from the FO and CSO groups were killed immediately following the 20 days supplementation (PND 48; n=6 per condition), while the other half groups were kept in the animal facility on a regular diet, until they reach adulthood (PND 90, n=6 per condition) (See Figure 2 for the study timeline). All procedures were carried out in accordance with the Canadian Council of Animal care (CCAC) and approved by the University of Ottawa Animal Care Committee. Experimentation complied with the ARRIVE guidelines and was in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

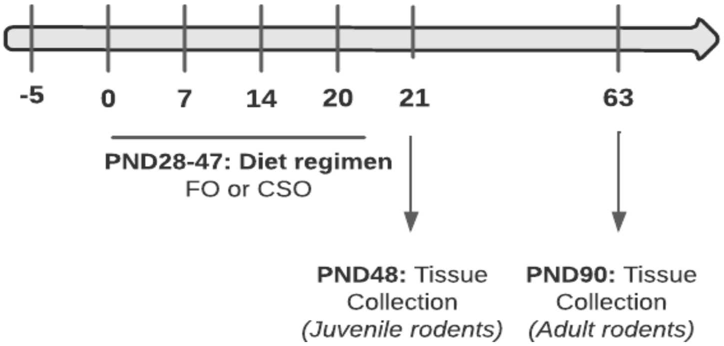

Figure 2. Experimental Timeline. Dietary supplements began on PND28, following a 5-day acclimation period to the animal facility, and ended on PND47 (20-day supplementation period). Adolescent's brain was collected on PND48, while the adult cohort remained housed in the vivarium until PND 90.

FO: Menhaden fish oil; CSO: Control Soybean oil.

2.2. Feeding and Weight Monitoring

Menhaden fish oil (FO; Sigma-Aldrich Canada) and soybean oil (CSO; Sigma-Aldrich Canada) respectively provided an elevated omega 3 dietary supplement or a Soybean oil (CSO), a non-hydrogenated omega 6 rich supplement a 7.4/1 n-6/n-3 ratio. Daily supplements were given via oral gavage at a concentration of 0.3ml of supplement per 100g of weight (Raymond, Morin & Plamondon, 2020). The oil supplement was freshly prepared daily and administered between 7 and 9 AM. To facilitate the procedure and limits gavage induced stress, the tip of the gavage syringes was lightly dip in condensed milk (Eagle Brand® Low Fat Sweetened Condensed Milk). Weight was monitored each day during oral gavage, and once a week until euthanasia to ensure proper weight gain and development (See Table 1,2 and 3 for nutrient composition).

Table 1. Nutrient composition of fish oil (Sigma-Aldrich)

Nutrient composition of Menhaden Fish Oil (FO)	Percentage of nutrients (%)
14:0 Myristic acid	6-9
16:0 Palmitic acid	15-20
16:1 Palmitoleic acid	9-14
18:0 Stearic acid	3-4

18:1 Oleic acid	5-12
18:2 Linoleic acid	< 3
18:3 Linolenic acid	< 3
18:4 Octadecatetraenoic acid	2-4
20:4 Arachidonic acid	< 3
20:5 Eicosapentaenoic acid	10-15
20:6 Docosahexaenoic acid	8-15
Unidentified fatty acids	20

Table 2. Nutrient composition of soybean oil (Sigma-Aldrich)

Nutrient composition of Soybean Oil (CSO)	Percentage of nutrients (%)
16:0 Palmitic acid	10
18:0 Stearic acid	4
18:1 Oleic acid	18
18:2 Linoleic acid (LA)	55
18:3 a-Linolenic acid	13

Table 3. Nutrient composition of Teklad Global Rodent Diet

Nutrient composition of Teklad Global Rodent Diet	Percentage of nutrients (%)
Crude Protein	18.6
Fat	6.2
Metabolizable Energy	3.1 kcal/g
Isoflavone Concentration	150 – 250 mg/kg
18:0 Stearic acid	0.2
18:1 Oleic acid	1.2
18:2 Linoleic acid (LA)	3.1
18:3 a-Linolenic acid	0.3
Total Saturated	0.9
Total Monounsaturated	1.3
Total Polyunsaturated	3.4

2.3 Sample collection

On PND48 and PND90, rats were euthanized with isoflurane (3% in oxygen) and quickly decapitated. Brains were rapidly extracted and snap frozen with liquid nitrogen for 10-15 seconds. Whole brains were kept on dry ice and later stored at -80C until analysis.

2.4 Fatty Acid Methyl Ester (FAME) Gas-Liquid Chromatography Analyses

Samples were weighted and 9x mass of phosphate buffer solution was added (PBS, i.e., 9mls PBS per 1g sample) prior to the samples being processed with a Potter-Elvehjem homogenizer. Following this step, 800μL of homogenate was added to a solution of 2mL of MeOH and 1mL of $CHCl_3$ containing 50μg of 1,2-diheptadecanoyl- sn-glycerol-3-phosphorylcholine (Matreya LLC, State College, PA, USA) (DHDPC) as internal standard. After 15 minutes, 2ml of $CHCl_3$ were added, the chloroform layer was collected and dried under a stream of N_2, samples were then hydrolysed with 0.5 M KOH in methanol (100°C for 15 min), and FAMEs were prepared by adding 14% BF_3 in methanol (100°C for 15 min). FAMES were extracted into hexane and the methylated samples were analysed by Gas Chromatography (GC) with Flame-Ionization Detection and compared with authentic fatty acid methyl ester standards for retention time and quantification as previously described (Robichaud et al., 2016; Lefort et al., 2016).

2.5 Statistical Analyses

Two-way ANOVA was performed using SPSS Statistics 27.0 software. All assumptions were met prior to the analysis. Homogeneity of variance was confirmed by using Levine's Test, while the Mauchly's test for sphericity. In case of violation of sphericity, Huynh-Feldt correction was used for these datasets. Three-way analyses of variance (ANOVA) were performed with between factors of sex (male vs female), supplementation (control soybean oil vs fish oil), and age (adolescent vs adult). Significance was set at $\alpha<0.05$ and Bonferroni correction was applied in pairwise comparisons. Data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Results for DHA concentrations [ug per Sample (mg)] detected using FAMEs

Statistical Analyses showed main effects of age [F (1, 42) = 54.041, $p<.001$, $\eta^2=0.607$], sex [F (1,48) = 54.041, $p<.001$, $\eta^2=0.628$] and supplement [F (1, 42) =10.418, $p=.003$, $\eta^2=0.229$]. Significant interaction between sex*age [F (1, 42) =70.284, $p<.001$, $\eta^2=0.668$].

Bonferroni corrected pairwise comparisons revealed adult rats to have heightened brain DHA content compared to juveniles. FO-fed rats also showed increased DHA levels compared CSO-fed counterparts *(p*=.003). Similar pattern was observed in female rats, which had increased DHA levels compared to male rats (*p*<.001). The juvenile rats' brains (CSO and FO fed rats) showed lower DHA concentrations compared to levels measured in the adult brains (*p*<.001). Interestingly, increased DHA levels observed in females compared to males' brains during the adolescent period was no longer present once rats reached adulthood (*p*<.001), regardless of the supplemented diet (CSO or FO) (*p*<.001). The interaction between sex, diet and age is attributable to changes in male rats. Both CSO and FO fed juvenile males showed reduced DHA brain concentrations, with CSO showing the lowest DHA level of all conditions (*p*<.001). When analyzing results from male rats, fish oil supplementation showed a crucial role in regulating DHA levels in brain tissue (See Figure 3). It must be noted that a few brain samples with extremely low DHA values (below 0.2 ug FAME/mg sample), lower than the levels reported in the cortex by Sugasini et al. (2017) were excluded from analyses, leaving a n=4/group for male adults fed with FO.

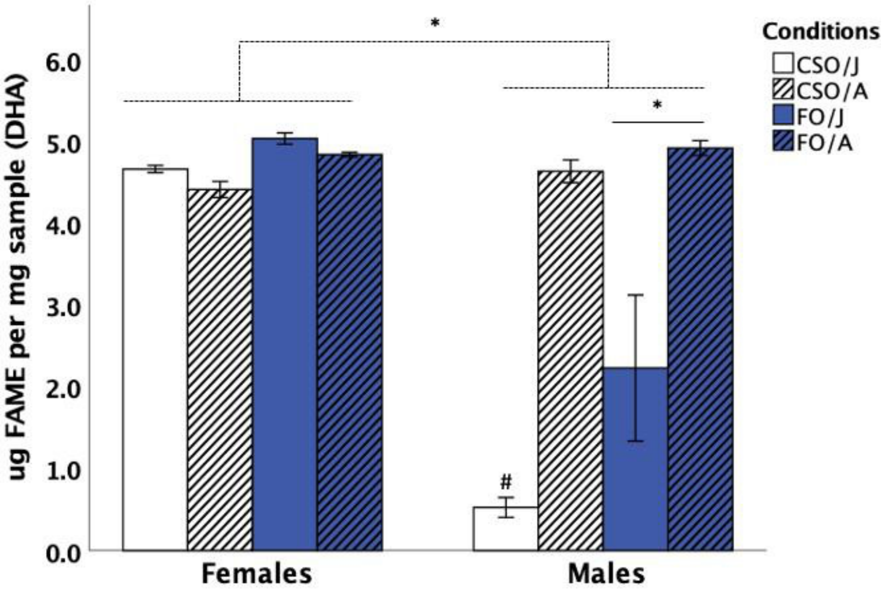

Figure 3. FAMEs (ug) per Sample (mg) of Docosahexaenoic Acid (DHA; 22:6n−3) in the whole brain. Analyses revealed a main effect of sex, age, supplements (p<.001), as well as an interaction between age and sex (p<.001). Elevated DHA was found in FO- compared to CSO-supplemented rats (p=.003), females compared to males (p<.001) and adults contrasting to juveniles (p<.001). FO-supplemented juvenile males had lower DHA concentration compared to adults (p<.001). CSO juvenile males showed significantly lower DHA levels than all other groups (p<.001, #). Data are presented as mean ± S.E.M. * and # Indicates statistically significant difference between groups at p < .05.

FO: Menhaden fish oil; CSO: Control soybean oil; J: Juveniles; A: Adults; DHA: Docosahexaenoic Acid

3.2 Results for LA concentrations [ug per Sample (mg)] detected using FAMEs

Analyses of LA concentration ratios indicated a main effect of age [F (1, 40) =26.640, *p*<.001] and sex [F (1, 40) =19.951, *p*<.001]. Analyses also revealed increased LA levels immediately following supplementation (juvenile endpoint) compared to concentrations detected in adult rats *(p*<.001). Additionally, females showed significantly higher LA levels compared to males (*p*<.001). No significant effects of the supplements or interactions were observed (see Figure 4).

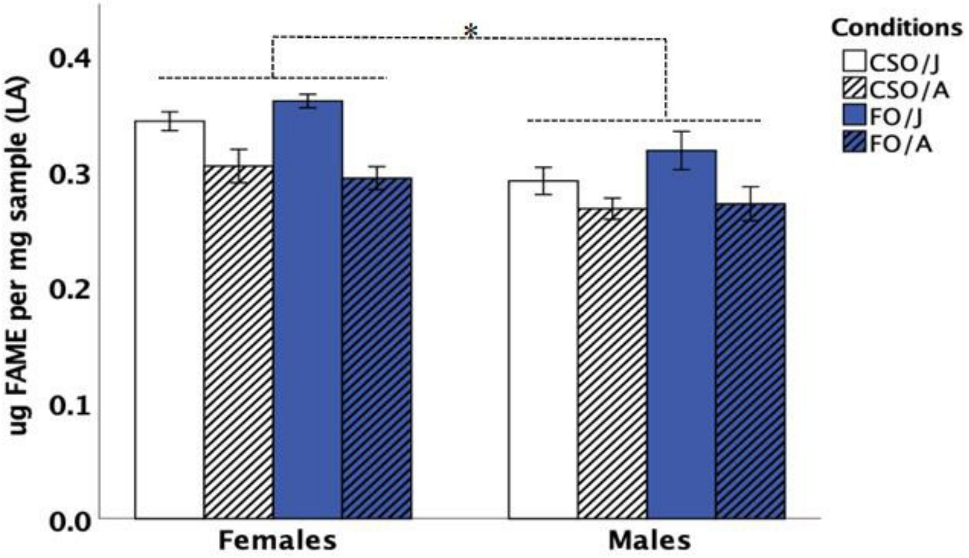

Figure 4. FAME (ug) per Sample (mg) for Linolenic acid (LA, C18:2n6). For the LA ratios, a main effect for sexes and age (p<.001; *) was detected. Data are presented as mean ± S.E.M. * Indicates statistically significant difference between groups at p < .05.

FO: Menhaden fish oil; CSO: Control soybean oil; J: Juveniles; A: Adults; LA: Linoleic Acid

3.3 Results for AA concentrations [ug per Sample (mg)] detected using FAMEs

Analyses of AA concentration ratios indicated a main effect of sex [F (1, 40) =48.603, *p*<.001], attributable to higher AA levels in females compare to males (*p*<.001). No significant main effects of age, supplements or interactions were observed (see Figure 5).

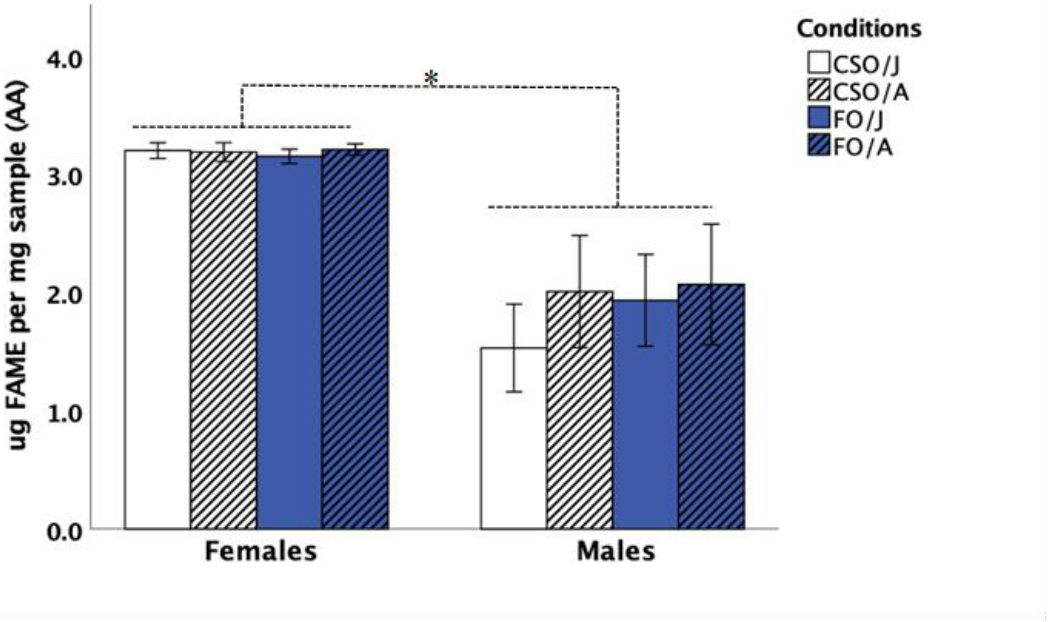

Figure 5. FAME (ug) per Sample (mg) Arachidonic Acid (AA; 20:4n-6). For the AA ratio, a main effect for sexes (*p*<.001; *) was detected. Data are presented as mean ± S.E.M. * Indicates a statistically significant difference between the male and female groups at $p < .05$.

FO: Menhaden fish oil; CSO: Control soybean oil; J: Juveniles; A: Adults; AA: Arachidonic acid

3.4 Results for EPA concentrations [ug per Sample (mg)] detected using FAMEs

No statistical analysis could be performed on EPA levels, which were too low to be systematically detected.

4. Discussion

Sex differences in essential fatty acids metabolism have been observed following long-term ingestion of different diets in humans and animals (Huang & Horrobin, 1987; Lohner, Fekete, Marosvölgyi, Decsi, 2013; Lin et al., 2016). However, little is known about the effect of omega-3 or omega-6 rich dietary supplements during the adolescent period on the immediate and delayed changes in brain essential fatty acid levels (EFA), including DHA, ALA and LA, in male and female rats (McNamara & Carlson, 2006). Sexual dimorphic changes in brain's essential fatty acid concentrations occurring during this critical maturation period is likely to have immediate and/or delayed effects on multiple brain-mediated responses.

4.1 Sex-specific effects of fish oil supplementation

Our findings revealed sex-specific differences in EFAs brain content during the adolescent period, which support differential abilities for adolescent male and female rats to benefit from adolescent n-3 PUFA supplementation. Thus, although adolescent females readily showed ability to metabolise nutrients from the n-3 PUFA supplementation, being able to extract DHA from other dietary sources, males failed to show the same benefits from n-3 supplements. Indeed, while both the CSO- and FO- supplements led to elevated DHA brain concentrations in adolescent females, males fed the same supplementations appeared deficient in their ability to metabolically extract EFAs from dietary sources. For instance, although males benefited from FO supplementation as adolescents, this benefit was twice reduced compared to that observed in FO-fed adolescent females. Remotely however, adult male and female rats showed comparable metabolic abilities, FO-supplemented rats showing slight elevations of brain DHA concentrations compared to CSO-supplemented counterparts.

Previous studies have supported sex-specific differences in DHA content following omega-3 supplementation (Alessandri et al., 2008; Extier et al., 2010; Decsi & Kennedy, 2011; Childs et al., 2012). Thus, Decsi and Kennedy (2011) showed that females tend to have greater liver and plasma DHA levels following the consumption of omega-3 compared to males. Their findings indicated female rodents exposed to FO supplementation to show elevated DHA levels both in brain and liver tissues compared to male counterparts (Decsi & Kennedy, 2011). Many explanations have been proposed to explain sex-specific changes in brain DHA content in rodents fed omega-3 enriched diets. Notably, evidence suggests gonadal hormones to play a determinant role in the conversion of dietary omega-3 into DHA (Alessandri et al., 2007; Childs et al., 2008). In this context, testosterone has been found to mitigate the conversion rate of

PUFAs, leading to reduced DHA absorption as testosterone levels increased (Schuchardt et al, 2010; Giltay et al., 2004). In contrast, estrogen appears to facilitate the conversion of an omega-3 rich diet into increased DHA brain concentrations (Extier et al., 2010, Giltay et al., 2004). Similarly, estradiol has been shown to facilitate the conversion of dietary omega-3 from ALA into DHA, increasing DHA abundance in liver and brain tissues (Alessandri et al., 2008; Decsi & Kennedy, 2011). Consistent with these findings, Herrera et al. (2018) reported reduced fatty acids metabolism in ovariectomized versus intact females to inhibit post-synaptic density protein expression (i.e., PSD-95) in cerebral cortex tissues (Herrera et al., 2018). Together, these observations strengthen a regulatory role of gonadal hormones on the conversation rate of dietary omega-3 into EFAs.

In this research, rats were CSO- or FO- supplemented from PND 28 to 47, and brain tissues collected at PND48 and PND 90. Pubertal maturation has been shown generally completed by PND36 in females and PND44 in males (Vetter-O’Hagen & Spear, 2012), majority of males showing balano-preputial skinfold separation and sperm presence at PND 40, where testosterone achieved detectable levels (Vetter-O’Hagen & Spear, 2012; Sengupta, 2013). In females, estradiol and progesterone are already detected around PND 28, although significant increase is noted from PND40 to 75 (Vetter-O’Hagen & Spear, 2012). While the timeframe of puberty can slightly vary between individuals, one can presume dietary supplementation in this study to have fell during a period where estradiol versus testosterone may have differentially interfere and/or contribute to sex-specific impact of supplementation measured at adolescence. Furthermore, since estradiol secretion increases during the early puberty period, and elevated estradiol levels favor conversation of omega-3 into DHA, this secretion likely contributes to distinctive DHA patterns noted in males and females immediately following the 20-days supplementation period

(Vetter-O”Hagen & Spears, 2012; Childs et al, 2008). Interestingly, other groups have observed sexually dimorphic effects of dietary conditions. For instance, Rodriguez-Navas et al. (2016) showed ingestion of a high fat diet for a 16-week period to significantly increase the percentage of saturated fatty acids while reducing brain n-6 PUFA levels in male but not female mice. Notably, this study also supported ingestion of a regular diet to elevate DHA and AA levels in the brains of female compared to male mice (Rodriguez-Navas et al., 2016). In conclusion, our findings appear concordant with that of other researcher also supporting sex-related effects in DHA concentrations following consumption of a rich n-3 PUFA diet. More studies are required to closely examine the physiological determinants associated with the sex-specific changes.

4.2 Sex-specific effects of soybean supplementation

Akin to FO, CSO supplementation in females was associated with substantial DHA levels in the adolescent and adult brains. Although this finding was not expected, soybean oil contains elevated levels of Linoleic Acid (18:2) (LA), representing over half of the total oil composition (see Table 2). LA being a precursor of AA, dietary supplementation with CSO was found to be unlikely to promote DHA conversation (Jump, 2002). Nonetheless, two factors may have influenced CSO conversation into DHA. First, while the main derivative of soybean oil is linoleic acid (LA), the small percentage of alpha-linolenic acid (ALA) provided through SO intake may have been converted into DHA in sexually maturing females under the influence of gonadal hormones. Past studies assessing sex differences have supported elevated estradiol combined to low testosterone concentrations, to favor DHA conversion rates in females (Alessandri et al., 2008; Decsi & Kennedy, 2011). Second, the presence of isoflavones in the rodent’s diet could influence essential fatty acid conversation pathways in several ways. Phytoestrogens such as isoflavones have a similar structure to that of 17B-estradiol and have been shown to interact with estrogen receptors (Zaheer & Humayoun Akhtar, 2017). Acting similarly as estrogen, they may have facilitated the PUFA metabolic pathway (Mezei et al., 2006), contributing to increased DHA levels in adolescent females. In this context, related to their estrogen-like nature, phytoestrogens, as well as their metabolites, increase the encoding of certain proteins essential to EPA and DHA biosynthesis (Fickler, Staats, Rimbach et Schulz, 2019). Recently, Gou et al., (2020) reported significantly enhanced plasma and muscles EPA and DHA levels in chickens fed a combination of soy, isoflavones and linseed oil, a mixture characterised by a high ALA content. This supports that above any supplementation, some phytoestrogens present in the rat chow have an ability to be differentially metabolized and consequently affect brain fatty acid total composition in a sex-specific manner (Gou et al., 2020). However, it must be noted that DHA levels remained slightly lower in females supplemented with CSO compared to FO. In sum, it appears plausible that DHA elevations in CSO-supplemented adolescent female rats be attributable to sex-specific hormonal signatures leading to differential EFAs conversation rates.

4.3 Dietary supplementation led to increased immediate and delayed brain AA and LA levels in female compared to male rats.

Notwithstanding post supplementation intervals, females showed increased LA and ALA brain levels following FO and CSO supplementation compared to males. Using a comprehensive mass spectrometry fatty acid analysis, which includes a profile for 52 different fatty acid isomers, Rodriguez-Navas et al. (2016) have reported discriminant fatty acid storage in the brain of female and male mice fed a conventional rat chow, with males having an 30% increase in brain saturated fatty acids and depletion of n-6 PUFAs compared to females. These sexually dimorphic fatty acid brain profiles were even more pronounced following consumption of the high fat Western diet (Rodriguez-Navas et al., 2016). These authors further reported that females exposed to n-6 PUFAs enriched diet showed higher levels of AA and adrenic acid compared to males, indicating sex-specificity in the desaturation process leading to essential fatty acid storage in females. They speculated these differences to be related to increased lipogenesis of saturated FAs, a slower degradation or turnover of FAs, and/or an impaired mechanism of uptake of FAs from the periphery through the BBB in the brain of male animals. (Rodriguez-Navas et al, 2016). These findings are concordant with our observations, supporting increased level of both AA and LA in females, regardless of diet or age. Additional research is required to better understand the physiological mechanisms responsible for sexual dimorphism underlying the differing FA concentrations in the brain.

4.4 Adolescent FO supplementation has a minimal impact on adulthood DHA brain levels

One of the most insightful findings of this research stems from the minimal effects induced by elevated omega-3 consumption measured in males immediately following supplementation compared to levels generated from dietary intake as adults. While DHA levels are significantly higher in FO- compared to CSO-fed male rats immediately post supplementation, a 20-day adolescent FO supplementation had modest impact on brain DHA elevations observed in adulthood. Noteworthy, when tissues were assessed on PND 90 (42 days post-supplementation), FO-supplemented adult males had reached equivalent DHA concentrations as females assessed in both periods. These observations suggest that compared to females, males appear to show greater benefit from prepubescent FO supplementation immediately post regimen. Our data also indicate that through maturation, male rats develop the ability to extract EFAs more efficiently from dietary elements, both the adolescent CSO and FO supplemented male rats showing increased DHA brain concentrations as adults.

4.5 Limitations

Our study compared the effects of FO and CSO supplementations on DHA, LA, and ALA brain concentrations in female and male rats. Our experimental design is however lacking groups of male and female rats not exposed to oil supplementation, which would have enabled determining the impact of ingestion of the regular diet on EFAs concentrations and better ascertain effects of the supplementation regimens. Nonetheless, many studies have privileged a study design using an oil-based control groups, to best control energy intake. Such design also offers a control for stress possibly related to the gavage procedure, by providing all rats with a similar environmental experience (Allaire et al. 2017; Child et al. 2014; Leng, Winter & Aukema 2017; Raymond, Morin & Plamondon, 2020). Although this study assumed pubertal maturation to be completed by PND 36 in female and PND 44 in male rats (Vetter-O'Hagen and Spear, 2012), environmental conditions and stress levels could have slightly shifted puberty onset and influence our results. Lastly, our analysis required to modify sample size in certain group due to undetectable DHA level in the brain. Samples were therefore lower in certain groups for the DHA analysis and led to an interpretation based on a lower sample.

5. Concluding remarks

This experiment demonstrated that females' rats supplemented with both fish and soybean oil during the adolescent period show elevated DHA, ALA and LA brain levels, immediately post supplementation (PND48) and as adults (PND90). Notably, our findings support sexual dimorphism, male rats showing delay-dependent increase of essential fatty acid content from FO supplementation, DHA content achieving maximal expression in adult brain. This research strengthens the importance of sex differences previously noted following omega-3 consumption, showing an association with discriminant brain essential fatty acid levels. Importantly this study points to critical difference in the immediate effects of adolescent FO supplementation, which shows maximal impact on brain fatty acid profiles in female rats, with 50% reduced DHA brain concentration in the male brain.

6. Acknowledgement

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Study 3

Adolescent omega 3 and environmental enrichment exposure exerts sex-specific effects on adulthood coping, socioemotional responses, and CA3 glucocorticoid receptor expression

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Abstract

Introduction: Omega 3 supplementation and environmental enrichment (EE) have shown beneficial effects on cognition and stress regulation in adult rats. However, sex-specificity, impact at adolescence, and synergistic effects remain to be clarified. This study assessed sex-specific effects of omega 3 and enriched environment during the adolescent period, critical for brain maturation, on adulthood coping, socioemotional responses, and glucocorticoid regulation. **Methods:** 64 Wistar rats [n=32/sex; postnatal day (PND) 23] were assigned to daily supplementation of control soybean oil (CSO) or omega 3 menhaden fish oil (FO; 0.3 mL/100 g) from PND28 to 47 and exposed to EE or regular cage (RC) housing (PND28-58). Weekly-collected blood droplets assessed corticosterone (CORT) during supplementation. On PND90-91, adult rats were exposed to repeated forced swim sessions to measure coping responses. Twenty-four hours later, anxiety, sociability/social preference, and conditioned fear learning were assessed. Immunohistochemistry determined hippocampus CA1 and CA3 glucocorticoid receptor (GR) expression in samples collected on PND95. **Results**: CORT secretion gradually increased in females over the supplementation period reaching a peak at DAY21, contrasting minimal changes in males. In the FST, coping strategies significantly differed between males and females, where FO/RC significantly increased floating behaviour in females, while males tended to preferentially select tail support for immobility, and CSO/RC males displayed increased climbing. In the Open Field, CSO/EE males spent increased time in the centre zone, supporting anxiolytic behaviour. FO/EE promoted sociability in females, while CSO favoured social recognition in males. Notably, CSO/RC males showed increased fear responses in the Y maze passive avoidance test, which was prevented by EE housing. Adulthood hippocampal GR-ir was marked by reduced CA3 GR-ir in FO/RC and CSO/EE rat groups. **Conclusion:** Together, our findings support adolescent dietary supplementation and environmental enrichment to distinctly modulate adulthood stress coping, activity level, sociability, and fear responses in male and female rats.

Keywords: Fish Oil Supplementation; Adolescents; Sex-Dependent Effects; Enriched Environment; Coping Responses; Sociability.

Introduction

Among essential dietary fatty acids, fish-derived omega 3 polyunsaturated fatty acids (n3-PUFAs) are necessary for optimal mammalian nervous system development (Gómez-Pinilla, 2008; Jump, 2002; Simopoulos, 2002a; Wurtman et al., 2009a), and play a critical role in cell membrane, synapse formation, and membrane fluidity during brain maturation (Gómez-Pinilla, 2008a; Jump, 2002b; Kitajka et al., 2002; Simopoulos, 2002a; Wurtman et al., 2009). Over the last century, a shift towards grain-based diets has promoted diets rich in omega 6 polyunsaturated fatty acids (n-6 PUFA), saturated and trans-fat content, at the expense of n-3 PUFA intake (Gómez-Pinilla, 2008; Simopoulos, 2002). Insufficient n -3 dietary intake has resulted in omega 6/omega 3 ratios approaching 20:1 against a recommended 4:1 (Marion-Letellier et al., 2016; Simopoulos, 2002), which combined to a sedentary lifestyle threatens health status, predisposing individuals to develop cardiovascular disease, cognitive problems, and mental health disorders (De Lorgeril, 2013; Gómez-Pinilla, 2008; Jump, 2002; Kitajka et al., 2002; Simopoulos, 2002; Wurtman et al., 2009). Diets rich in n-6 and/or deficient in n-3 PUFAs are associated with increased hypothalamic-pituitary-adrenal (HPA) axis activation, glucocorticoid secretion (Rosenberger et al., 2004; Yoshida et al., 2007), and depression/anxiety-related symptoms (Green et al., 2013; Gow & Hibbeln, 2014; Hallahan et al., 2016). In contrast, n-3 supplementation has been associated with reduced depression symptoms in young adults (Nemets et al., 2006), with Ginty & Conklin (2015) reporting 67% of individuals with depression to no longer meet diagnostic criteria following a short-term 21-day n-3 supplementation. Notably, females have shown increased benefits from n-3 supplementation, in part related to estradiol and progesterone facilitation of n-3 conversion into docosahexaenoic acid (DHA), which promotes brain development (Extier et al., 2010; Child et al., 2012). In turn, testosterone is known to reduce conversion rates in males (Schuchardt et al., 2009). Notably, increased dietary n-3 consumption in children aged 6 to 16 years revealed effects on cognitive performance to be twice as important in girls compared to boys (Lassek & Gaulin, 2011). While some of the effects of fish oil during the adolescence period can be proposed, studies have concentrated on assessing the impact of n3-PUFAs supplementation exposure during perinatal development. Considering sexual maturation related to the adolescence period, it appears important to gather additional information on fish oil-related regulation of behavioural responses and stress reactivity during this crucial developmental period.

Akin to diet, living conditions greatly impact brain and behavioural responses. Several studies have shown environmental enrichment (EE), defined by provision of heightened social and environmental stimulation through renewed external stimuli, increased physical activities, social interactions, and living space (Harland et Dalrymple-Alford, 2020; Slater et Cao, 2015), to exert positive effects on behaviour. EE exposure thus enhances social development in rodents by increasing exploration and play (Kempermann, 2019), and attenuating anxiety-like and depressive-like behaviours (Bechara & Kelly, 2013; van Praag et al., 2000; Olson et al., 2006; Bednarczyk et al., 2011; Veena et al., 2009). Veena et al. (2009) further supported a minimal 10-day EE exposure period to suffice in attenuating depressive-like symptoms related to chronic stress exposure in adult rodents, while Belz et al. (2003) reported reduced basal adrenocorticotropic hormone (ACTH) and corticosterone (CORT) secretion, both being stress response markers, in enriched male and female rats. EE also promotes secretion of several neurotrophic factors [i.e., brain-derived (BDNF), nerve (NGF), insulin-like growth factors (IGF) and neurotrophin-3 (NT-3)] (Hu et al., 2013) shown to impact mood regulation (Hill et al., 2013;

Jeanneteau et al. 2011), and is associated with hippocampal neurogenesis (van Praag et al., 2000; Olson et al., 2006). EE exposure also improve spatial memory performance (Kondo et al., 2016).

The adolescent period, marked by increased plasticity within the frontal cortex and interconnected mesolimbic circuitries, represents a critical, time-sensitive window to evaluate effects of diet and environment (Akirav & Richter-Levin, 1999; Ghashghaei & Barbas, 2002; Petrovich et al., 2001). It is also during adolescence and early adulthood where higher vulnerability to anxiety disorders and depression onset is noted (Ginty & Conklin, 2015b; Rice et al., 2015). Indeed, adolescent exposure to stressors induces sustained elevations in glucocorticoid secretion which, among other effects, alters various intracellular processes and white matter organisation in the rodent hippocampus (see Lupien and al., 2009 for a review; Chetty et al., 2014). Stress reactivity during this specific developmental interval is also highlighted by important sex-specific differences (McLoughlin, 2002). Upon reaching adolescence, girls become twice as likely as boys to develop depression (Rutter et al., 2006). Gonadal hormones seem to be mediating sex-dependant stress reactivity during this developmental period (Albert et al, 2015). In fact, Albert et al. (2015) demonstrated that females with high estradiol levels during certain phases of their menstrual cycle were experiencing less distress, a phenomenon associated with attenuated brain activation and negative mood responses.

The shared ability of n-3 PUFA-supplemented diet and EE to regulate common endocrine and plasticity makers warrants an evaluation of synergistic actions susceptible to affect adolescent brain maturation and delayed biobehavioural responses. Furthermore, most published research has focussed on perinatal or adulthood exposure, leaving social and fear-related behaviours during the adolescence period, especially in females, undetermined. Specifically, this study determined sex-specific effects of single and combined adolescent exposure to dietary omega 3 supplementation and enriched environment on (1) immediate CORT secretion and delayed regulation of glucocorticoid receptors in the CA1 and CA3 hippocampus layers, (2) coping behaviour in the forced swim test (FST) and (3) socioemotional responses as assessed in the open field test, elevated-plus maze, social approach test, and Y-maze passive avoidance test 24 h following repeated FST exposure. It is predicted that females and males fed with FO would demonstrate reduced anxiety and heightened sociability. While mitigated effects of EE have been reported in females, we expect a synergic, beneficial impact of EE and FO supplementation in male rodents.

2. Methods

2.1 Animals

Female (F) and male (M) Wistar rats (n = 32/sex) were obtained from Charles Rivers Laboratories (Rochefort, Québec, Canada) and arrived at the vivarium on postnatal day (PND) 23. Upon arrival, rats were pair-housed with same sex cage mate and handled daily for 2-3 min for habituation prior to start of experiment. All rats had ad libitum access to regular rat chow and water and were kept on a 12 h light/dark cycle (lights on at 7 a.m.) in a temperature-controlled environment (21-23 °C) with 40-60 % relative humidity. All procedures were carried out in accordance with the Canadian Council on Animal Care (CCAC) and approved by the University of Ottawa Animal Care Committee. Experimentations complied with ARRIVE guidelines and National Institutes of Health Guide for the Care and Use of Laboratory Animals (See Figure 1 for study timeline.

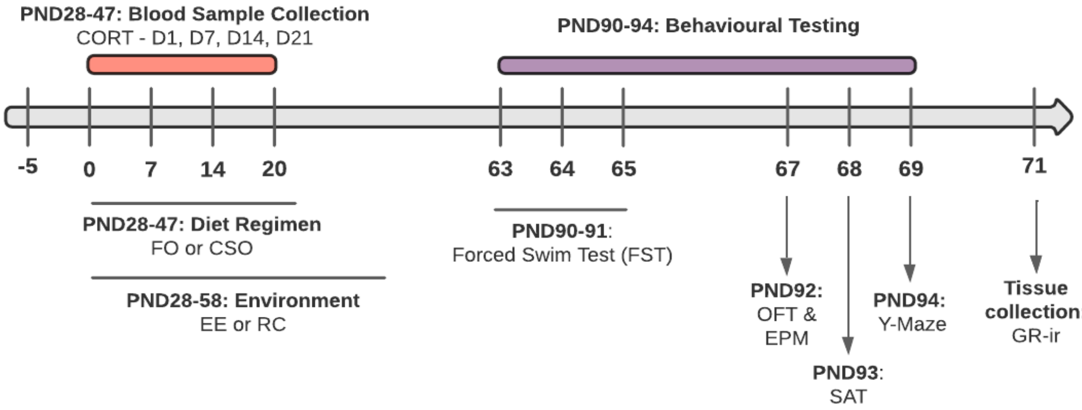

Figure 1. Experimental timeline. Wistar rats (n=64), females (F) and males (M), arrived at the facility at PND23. FO or CSO dietary supplementation was provided daily by oral gavage from PND28 to PND47. Rats were exposed to EE or RC for a 31-day period from PND28 to PND59. Four (4) experimental conditions were examined: CSO/RC, CSO/EE, FO/RC, and FO/EE. Behavioural Testing started with the OFT and EPM [PND92] followed by the SAT [PND93], and the Y Maze test [PND94].

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage; CORT: Corticosterone; FST: forced swim test; OFT: Open Field Test; EPM: Elevated-Plus Maze; SAT: Social Approach Test; GR-ir: Glucocorticoid's receptors.

2.2 Dietary Supplementation and Environmental Conditions

On PND28, male and female rats were randomly assigned to one of the four experimental conditions based on dietary supplementation and housing. Dietary supplements consisted of omega 3-rich menhaden fish oil (FO; 0.3 mL/100 g body weight; F8020-1L; Sigma-Aldrich Canada) or control soybean oil (CSO; S7381-1L; Sigma-Aldrich Canada), supplemented daily from PND28 to 47 using oral gavage, a delivery method shown less stressful in adolescent rats than time-restricted feeding (Raymond et al., 2020). Both FO- and CSO- supplemented groups were further divided into two housing conditions: Environmental Enrichment (EE) or regular cage (RC) for a 31-day period. EE rats were group housed [4 to 5 rats] in a large cage (LWH: 44 cm x 23 cm x 20 cm), with access to acrylic tubes, paper for nesting, toys, a running wheel, and chains (for similar protocols, see Ismail et al., 2021; Mileva et al., 2017a and b). The environment was slightly modified every 2-3 days by changing available toys/elements to maintain rats' interest. Following adolescent EE exposure, rats were transferred to smaller cages (LWH: 21 cm x 42 cm x 20 cm) equipped with an acrylic tube and paper for building nests without being separated from their cage mates. RC housing was maintained, with rats' pair-housed with one acrylic tube. To minimise confounds related to increased exercise in EE-housed rats, RC rats were placed in an empty water maze pool (DH: 86 cm x 15 cm) for 15-min sessions, three times a week. All other conditions related to housing (water and food intake) were identical between groups. In total, the four experimental conditions tested in male and female rats are identified as follows: FO/EE, FO/RC, CSO/EE, and CSO/RC.

2.3 Blood Sampling

From PND28, a two-drop blood sample was collected every 7 days between 7 and 9 a.m. over a 4-week period using the tail vein nick procedure as previously described (Milot et al., 2012). Blood collection took under 3 min to prevent variations in CORT secretion related to sampling stress. The two blood drops were collected on Whatman paper (Sigma-Aldrich Canada) and samples were left to dry for 24 h before being stored at -80 ºC until CORT secretion analysis.

2.4 Female's Estrus Cycle Assessment

Daily monitoring of females' estrus cycle was initiated one week prior to behavioural testing through collection of vaginal samples. Briefly, a sterile pipette containing a small volume of distilled water (0.2-0.25 mL) was placed in the vaginal canal opening (< 1 cm) and the liquid gently expelled. This procedure was repeated 4-5 times enabling the collection of sufficient number of cells per sample. Fluid was transferred to a glass slide and left to dry at room temperature. Vaginal cytology was determined immediately following collection using a light microscope and the McLean et al. (2012) and Goldman et al. (2007) stage assessment guidelines.

2.5 Adulthood Behavioural Testing

Behavioural testing (PND92 to 94) was initiated 24 h following repeated FST. Akin to the FST, rats were moved to a room adjacent to the vivarium from 7 a.m. on test days to acclimate for 30 min before testing. Temperature and humidity conditions were equivalent to those of the vivarium with □ 400 lux illuminations. A white curtain surrounded the behavioural testing area, preventing rats' visual access to the researcher and to remote visual cues. Testing apparatuses were cleaned with 70% ethanol between each session. Sessions were recorded using a ceiling-mounted camera (WV-CP284; Panasonic®, Canada) and coded using ODlog^{TM} (Macropod Software, Australia). Behavioural testing sequence is depicted in the experimental timeline (see Figure 1).

2.5.1 Repeated Forced Swim Test (FST) Exposure

As adults (PND90-91), rats were exposed to a repeated FST stress. On the first day, rats were placed in a transparent plastic cylinder (DH: 28 cm x 54 cm) filled with lukewarm (20-22 °C) water for a 15-min period (as described by Lemos et al., 2012). Black cardboards were placed between each cylinder to prevent rats from seeing each other during testing, and a white curtain separated the rat testing area from the researcher's recording station. Four to six rodents underwent testing at the same time, ensuring that female and male rats were tested in different sessions. Twenty-four hours later, rats were exposed to the same cylinder for four, six-min swim sessions with 15 to 20 min intervals between the tests. The swim sessions acted as a repeated stressor, as per Lemos et al. (2012). Swim cylinders were emptied and cleaned with 70 % ethanol between trials. After removal from the cylinder, rats were dried with a towel and placed back in clean cages on a heat pad until the next swim session. Testing was recorded using an analogue camera (WV-CP284, Panasonic®, Canada) and coded using ODlog^{TM} (Macropod Software). Behavioural analyses were limited to the first session of the second day as per the original FST protocol (Porsolt et al., 1977). Time spent climbing, swimming, and immobile was analysed. In this context (i.e., testing rats not previously exposed to other documented stress conditions), the FST has been used to infer adaptive stress-coping strategies linked to energy conservation (Kloet & Molendijk, 2016; Commons et al., 2017).

2.5.2 Open Field Test (OFT)

The OFT consisted of an open square area surrounded by walls (LWH: 75 cm x 75 cm x 30 cm) made of grey opaque plexiglass and with flooring being a removable grey mat divided in 36 equally sized squares: 20 limiting the peripheral region and 16, the centre zone. Rats were initially placed in one corner and allowed to move freely for 10 min. Locomotor activity and anxiety-like behaviours were assessed via latency to enter the anxiogenic centre zone (s) as well as time spent (s) and frequency of entries in the centre and periphery (Campos et al., 2013).

2.5.3 Elevated Plus Maze (EPM)

Three to four hours following the OFT, rats were tested in the EPM for 5 min to assess anxiety-like behaviours (Campos et al., 2013). The cross-shaped apparatus was made of 2 open and 2 closed arms, each measuring 50 cm x 10 cm, attached to a central, square platform. Closed-arms were surrounded by 40 cm-high protective black walls while open arms only had a 5 mm plexiglass border exposing the rat to an open area. Rats were initially placed in the centre zone of the maze, facing an open arm. Using video camera recordings and ODlog, time spent (s) and frequency of entries in open and closed arms were determined. Risk assessment behaviours were scored when a rat dipped its head (tip of nose up to shoulder) over the open arm, with its full body remaining in the closed arm or centre zone. Duration (s) and frequency of risk assessments were recorded.

2.5.4 Social Approach Test (SAT)

On PND93, the three-chamber social approach (SA)/social preference test (SP) test was used to assess rodents' social affiliation/motivation and social memory/preference for social novelty (Moy et al., 2004). The apparatus consisted of a modified open field arena (LWH: 75 cm x 75 cm x 30 cm; standing on a table 90 cm above the floor) with removable clear plexiglass partitions dividing the arena into three chambers of equal size. An overhead camera recorded each behavioural session. White curtains separated the experimenter's recording area. As per Kaidanovich-Beilin et al. (2011), each rat was initially placed in the middle chamber and left to habituate to the apparatus for a 5-min period, allowing exploration of the two outer chambers, each containing identical empty wire containment cups. A few minutes following habituation, the rat was placed in the middle chamber of the SAT, with a stranger rat (S1) now placed inside one of the wire cups. During the first 10-min session (assessing sociability), the rat was free to engage in direct or indirect interactions with S1 in one side chamber or spend time exploring the empty cup (EC) in the second side chamber or the middle area. For the second 10-min session (assessing social preference), the rat was returned to the middle chamber and could now freely engage in direct and indirect interactions with a novel conspecific (S2; stranger) placed under the previously empty wire cup or revisit S1. Session 2 was completed immediately following habituation and session 1. Side chamber localization of stranger rats was counterbalanced between animals. Measures included the time spent (s) in direct contact (i.e., with the rodent's muzzle directly touching or/and sniffing) with the conspecific or the EC in session 1, and with S1 (now-familiar rat) or stranger S2 in session 2 and the time spent performing indirect behaviours in the chambers (i.e., walking, grooming, or being immobilized) while no observable direct contact with conspecifics or EC is performed (Kaidanovich-Beilin et al., 2011). Exploration ratio in the SAT was calculated as $[T_{S1} / (T_{S1} + T_{EC})]$, thus direct or indirect interactions with S1 were deemed elevated relative to EC when exploration ratio > 0.5, while an exploration ratio < 0.5 indicated reduced interactions with S1 relative to the EC. In session (2), exploration ratio was defined as $[T_{S2} / (T_{S2} + T_{S1})]$, a > 0.5 ratio indicating increased preference for S2 relative to S1 (i.e., social memory and predilection for novelty).

2.5.5 Y-Maze Passive Avoidance Test (Y-Maze)

The Y-Maze was used to assess fear-conditioned memory (Azogu et al., 2019) and consisted of a Y-shaped, 3-arm plexiglass maze (35.5 cm x 15 cm x 30 cm). One arm was used as a starting point (start arm) while the two others were possible aversive arms. Different visual cues were placed at the end of the two arms to facilitate spatial memory consolidation. Each animal was initially placed facing a wall in the start arm and left to roam freely until it entered one of the two others. Once the rat entered an arm, the exit was blocked with an opaque plexiglass sliding door and the rat received 4 puffs of air jets (dispensed every 15 s over 1 min), rendering this arm “aversive”. The rat was then removed from the arm and left to rest in its home cage for 30 min, after which it was placed back in the start arm. The initially unselected arm was now blocked by a grey plexiglass door, making the aversive arm the only available choice. If the rat failed to re-enter the aversive arm within 5 min, it was removed from the test and placed back in its home cage. Latency to enter the aversive arm (s) was logged. Time spent making risk assessments (i.e., rat’s head – nose to shoulder – peeking into the aversive arm; s) was noted. Wire grates placed on top of the three arms prevented escape during testing.

2.6 Post-mortem Brain Tissue Collection

On PND95, rats were lightly anesthetised using 3 % isoflurane dissolved in oxygen prior to rapid decapitation. Fresh brain tissue was extracted, quickly frozen on dry ice and stored at

- 80 °C prior to cryostat slicing (Leica CM1900, Leica Microsystems, Germany) to collect 14 µm-thick coronal brain sections mounted on glass slides (Fisherbrand Superfrost® Plus Microscope Slides, USA). Slides were stored at - 80 °C until immunohistochemistry was performed. Sections were collected using coordinates from the Paxinos & Watson (2006) atlas for identification of CA1 and CA3 hippocampus subfields (- 2.80 to - 4.16 mm from Bregma).

2.7 Blood Corticosterone Assessment Using Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA; Corticosterone EIA Kit; ADI-900-097, Enzo Life Sciences, USA) was used for blood CORT level determination. Briefly, 3.0 mm diameter circles were micro-punched from the Whatman collection paper using a Gem Hole puncher (McGill Inc). Samples were placed in glass tubes filled with 200 µL of Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St-Louis, MO) containing 0.1 % gelatin (Avantor Performance Materials, Phillipsburg, NJ). Tubes were shaken at 90 rpm for one hour at 24 ºC and refrigerated for 48 h at 4 ºC. Using volumes of the buffered solution containing the dissolved blood samples, an ELISA procedure was conducted to determine CORT levels in duplicates as previously described (Morin et al., 2021). Following sample distribution into the wells of the ELISA plates, the primary and secondary antibodies were added, and shaken at 500 rpm for 2 h at room temperature (RT) on a plate shaker. Then, plates were rinsed three times and samples were incubated in pNpp at RT for 1 h before a stop solution was added and plate read using a Powerwave XS2 Microplate Spectrophotometer (BioTek, USA). Assay detection range was 32–20,000 pg/mL and intra- and inter-assay consistency was confirmed.

2.8 Immunohistochemical Detection of Glucocorticoid Receptors (GR-ir)

Cryostat-sectioned brain tissue was pre-soaked in a paraformaldehyde fixing solution (4 % PFA, 0.2 % picric acid) for 15 min at RT and subsequently rinsed with 0.01 M phosphate-buffered saline (PBS; pH = 7.4; 3 x 5 min) before incubation in blocking solution (5 % donkey serum; 0.2 % Triton-X-100; PBS) for 30 min at RT. Tissues were rinsed prior to overnight incubation at 4 °C with the polyclonal rabbit anti-GR primary antibody (1:500; ab3578; Abcam, Canada) mixed in blocking solution. The following day, brain sections were washed in PBS (3 x 5 min) and incubated with Alexa 594-conjugated donkey anti-rabbit secondary antibody (1:1000; A-21207; Invitrogen Canada Inc.) at RT in the dark for 1 h. Sections were rinsed with PBS (3 x 5 min), immersed in Hoechst adenine-thymine-binding dye (1:20,000; Hoechst 33342, Invitrogen Canada Inc.) for 5 min at RT. Following a last series of rinses, an anti-fade medium containing 0.1 % p-phenylenediamine in phosphate buffered glycerol was applied, and slides were cover-slipped and sealed with nail polish. All slides were kept at - 80 °C until analysis. Special controls were run to test for antibody specificity, i.e., incubation of tissue slices in PBS-Triton-Serum without the primary antibody prior to incubation in the secondary antibody.

2.9 Quantification of Immunoreactivity

Photomicrographs of fluorescence immunolabelling were obtained using an Olympus BX51 microscope (Center Valley, PA, USA) and the ProgRes Capture Pro 2.7.6 software under a 20-x objective lens (eyepiece 10x; numerical aperture 0.75). GR-ir was manually scored by two blinded examiners and was defined as clearly visible, circular Alexa 594 fluorescence superimposed on Hoechst dye. Four anatomically matched pictures of both hemispheres were assessed to produce an average immunoreactive score for the two brain regions of interest. Final sample sizes for females and males were as follows: FO/EE (F = 8; M = 7), FO/RC (F = 8; M = 3), CSO/EE (F = 7; M = 5), CSO/RC (F = 4; M = 7).

2.10 Statistical Analyses

All statistical analyses were performed using IBM© SPSS Statistics 27 (IBM, USA). Datasets were first screened for outliers and extreme datapoints by use of stem-and-leaf plots. If identified, such datapoints were replaced with their respective group's now most extreme datapoint plus or minus one for high and low datapoints, respectively. Normality was then verified with skewness and kurtosis, while homogeneity of variance was confirmed using Levene's test. Statistical corrections (square root, log10 or inverse) were performed when appropriate. Sphericity was determined using Mauchly's test, and Greenhouse-Geisser correction applied when sphericity assumption was violated. Three-way analyses of variance (ANOVA) with between-factors sex (female vs male), supplementation (fish oil vs control soybean), and environment (enriched vs regular) served to analyse behavioural and immunohistochemical data. CORT data was analysed using a three-way repeated ANOVA, the between factors being sex, supplementation, and environment with collection times as the within factor (1-, 7-, 14-, and 21-days post-supplementation). Significance was set at $\alpha < 0.05$ and Bonferroni correction was applied in pairwise comparisons. Data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1 Behavioural Responses in the FST

3.1.1 Time Spent Climbing

Three-way ANOVA revealed a main effect of sex [F (1,56) = 25.913, $p < .001$, $\eta^2_p = .316$] and interactions between sex*environment [F (1,56) = 18.403, $p < .001$, $\eta^2_p = .247$], sex*supplementation [F (1,56) = 21.301, $p < .001$, $\eta^2_p = .276$], supplementation*environment [F (1,56) = 8.705, $p = .005$, $\eta^2_p = .826$], and sex*supplementation*environment [F (1,56) = 4.981, $p = .030$, $\eta^2_p = .592$]. The main effect of sex was related to reduced climbing in females compared to males (*p* < .001). Post-hoc comparisons further showed climbing to be elevated in RC housed CSO fed rats compared to the other groups (*p* < .001). In addition, CSO/RC males spent the highest time climbing (three times that of CSO/RC females), a response prevented by EE (*p* < .001). In females, FO significantly increased climbing compared to CSO supplementation (*p* < .001), the effect being observed in both RC (*p* = .004) and EE (*p* = .005) conditions. Our findings also supported CSO/EE rats to show reduced climbing compared to FO-fed counterparts (*p*=.022) (See Figure 2a).

3.1.2 Time Spent Swimming

Three-way ANOVA revealed main effects of sex [*F* (1,56) = 7.723, *p* = .007, η^2_p= .121] and supplementation [*F* (1,56) = 8.262, *p* = .006, η^2_p= .129] and interaction between sex*supplementation [*F* (1,56) = 4.6, *p* = .036, η^2_p= .076] on time spent swimming. Pairwise comparisons indicated males to show significantly reduced swim time (*p*=.007). Additional post-hoc analyses revealed CSO supplementation in males to be associated with reduced swim time compared to female counterparts (*p* < .001). CSO-fed male rats exposed to RC showed reduced swim time compared to all other male and female groups (*p* < .01 for all comparisons). This effect is attributable to CSO/RC male rats favouring climbing over swimming. EE exposure acted to normalise swimming behaviour in CSO fed male rats (See Figure 2b).

3.1.3 Time Spent Immobile: Floating and Tail Support

In this study, rats were unlikely to develop depressive-like symptoms, being not exposed to chronic or repeated stress exposure. In that regard, the FST was used to assess coping and energy conservation, and tail support enabled as an alternate strategy to passive floating, helping to further characterize sex-specific coping strategies [as per described by Abelaira et al. (2013)]. Although no overall changes in time spent immobile (using floating or tail support) were observed between the groups, distinctive coping strategies were found influenced by sex, diet, and housing conditions.

For floating, three-way ANOVA revealed main effects of sex [F (1,56) = 35.877, $p < .001$, $\eta^2_p = .390$], supplementation [F (1,56) = 7.234, $p = .009$, $\eta^2_p = .114$], and environment [F (1,56) = 9.906, $p = .003$, $\eta^2_p = .150$] as well as significant interactions between sex*supplementation [F (1,56) = 7.230, $p = .009$, $\eta^2_p = .114$], sex*environment [F (1,56) = 8.685, $p = .005$, $\eta^2_p = .134$], supplementation*environment [F (1,56) = 17.347, $p < .001$, $\eta^2_p = .237$], and sex*supplementation*environment [F (1,56) = 18.369, $p < .001$, $\eta^2_p = .247$]. Pairwise comparisons revealed that females used floating to maintain immobility for significantly more time compared to males ($p < .001$). Floating was maximal in FO/RC females [significantly elevated compared to all other groups ($p < .001$)] and was significantly reduced by EE housing ($p < .001$). Together, female groups showed increased time floating compared to male rats ($p < .001$) (See Figure 2c).

For tail support, a main effect of sex [F (1,56) = 4.661, $p = .038$, $\eta^2_p = .075$] was observed. Post-hoc analyses revealed that females spent significantly less time using tail support to maintain immobility compared to males ($p = .035$). Analyses also showed an interaction between sex, supplementation, and environment, attributable to FO-fed males (exposed to RC or EE) spending more time using tail support compared to FO females similarly housed ($p = .001$). In contrast, CSO/RC females largely used tail support compared to all other female groups ($p < .01$, for each group comparisons). EE housing significantly reduced immobility using tail support in CSO females ($p < .01$) (See Figure 2d).

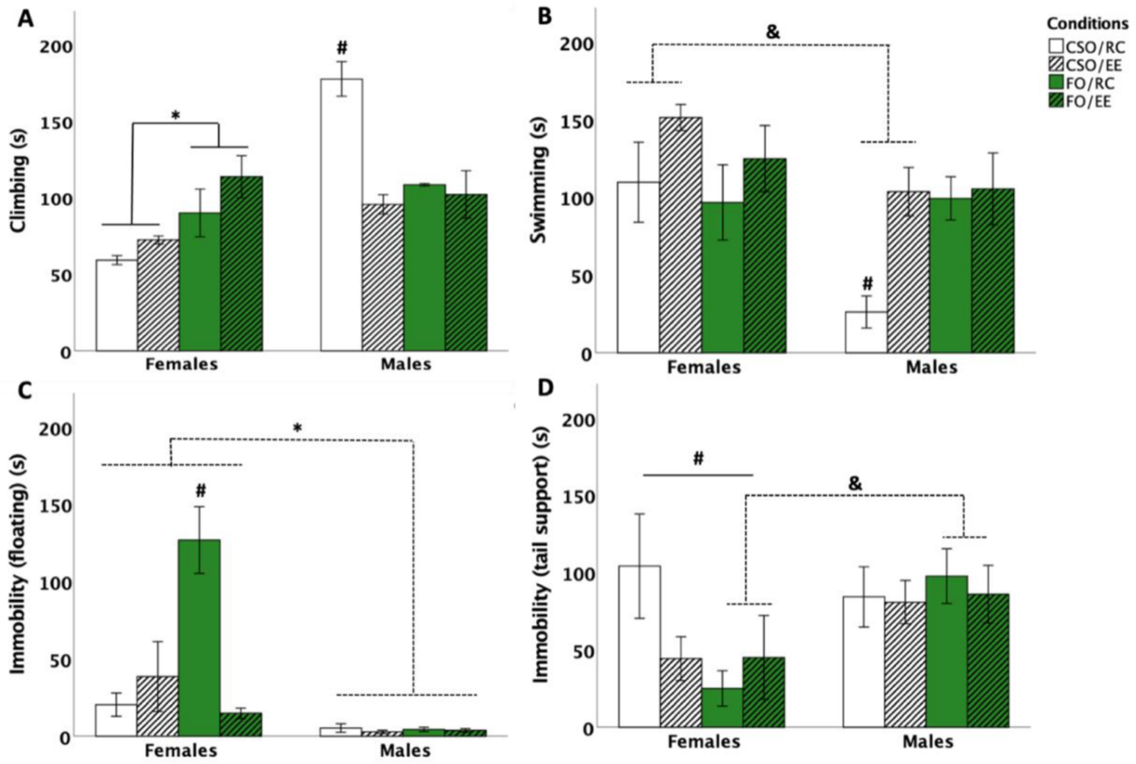

Figure 2. Effect of supplementation, sex, and environment in the Forced Swim Test (FST) on Time spent climbing (Fig 2a), Swimming (Fig 2b), Immobility by floating (Fig 2c), and Immobility by tail support (Fig 2d). Statistical increase of climbing was observed for CSO/RC males compared to other groups (*p* < .001; #). Females exposed to FO were climbing more than CSO, regardless of environment (*p* < .001; *) (2a). Swimming was enhanced in female rodents fed with CSO compared to their male counterpart in both RC and EE (*p* < .001; *). CSO/RC males swam less compared to all other groups (*p* < .01, #) (2b). Immobility was increased in FO/RC females compared to all other groups ((*p* < .001; #) (2c). FO-fed male rats showed increased use of tail support immobility ((*p* = .001; *), while females generally spend less time using this strategy (*p* < .001; #) (2d). Data are presented as mean ± S.E.M. * and # Indicates statistically significant difference between groups at *p* < .05. & Indicates significant impact of the condition (supplementation only) without an influence of sex or housing at *p* < .05.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.2 Open Field Test (OFT)

3.2.1. Time in the centre zone

Analyses were performed on log10-transformed data to correct for variance heterogeneity. Three-way ANOVA revealed main effects of sex [*F* (1,56) = 4.486, *p* = .023,

η^2_p= .089], supplement [*F* (1,56) = 6.988, *p* = .011, η^2_p= .111], and interactions between sex*supplement [*F* (1,56) = 4.220, *p* = .045, η^2_p= .070], supplement*environment [*F* (1,56) = 7.303, *p* = .009, η^2_p= .115] and sex*supplement*environment [*F* (1,56) = 6.851, *p* = .011, η^2_p= .109]. Post-hoc tests indicated reduced time spent in the centre zone in female compared to male rats (*p*=.023). CSO- spent increased time in the centre zone compared to FO fed rats (*p*=.011). The interaction between sex and supplementation was related to CSO-fed females spending reduced time in the centre zone compared to CSO-fed males (*p* =.003). EE potentiated centre exploration in CSO but not FO-fed rats ($p < 0.01$). Lastly, the sex*supplementation*environment interaction is related to CSO/EE males spending increased time in the centre zone compared to CSO/EE females ($p < 0.01$; See Figure 3a).

3.2.2. Time in the peripheral zone

Analyses revealed significant interactions between environment*supplementation [*F* (1,56) = 5.319, *p* = .025, η^2_p = .087] and environment*supplementation*sex [*F* (1,56) = 4.042, *p* = .049, η^2_p= .067]. Post-hoc analyses indicated these differences to be attributable to CSO/EE females spending more time in the periphery compared to the CSO/EE male counterparts (*p* = .012; See Figure 3b).

3.2.3 Frequency of entries in the centre and peripheral zones

Analyses of centre entry frequencies indicated a supplementation*environment interaction [*F* (1,56) = 4.105, *p* = .048, η^2_p= .068], attributable to CSO/EE (*p* = .043) and FO/RC (*p* = .018) groups entering the centre zone more frequently compared to FO/EE rats (See Figure 3c). For entries in the periphery, analysis revealed a supplementation*environment interaction [*F* (1,56) = 4.553, *p* = .037, η^2_p= .075], related to rats in the CSO/EE and FO/RC conditions making more entries in the periphery compared to FO/EE rats (*p* = .044 and *p* = .027, respectively; See Figure 3d).

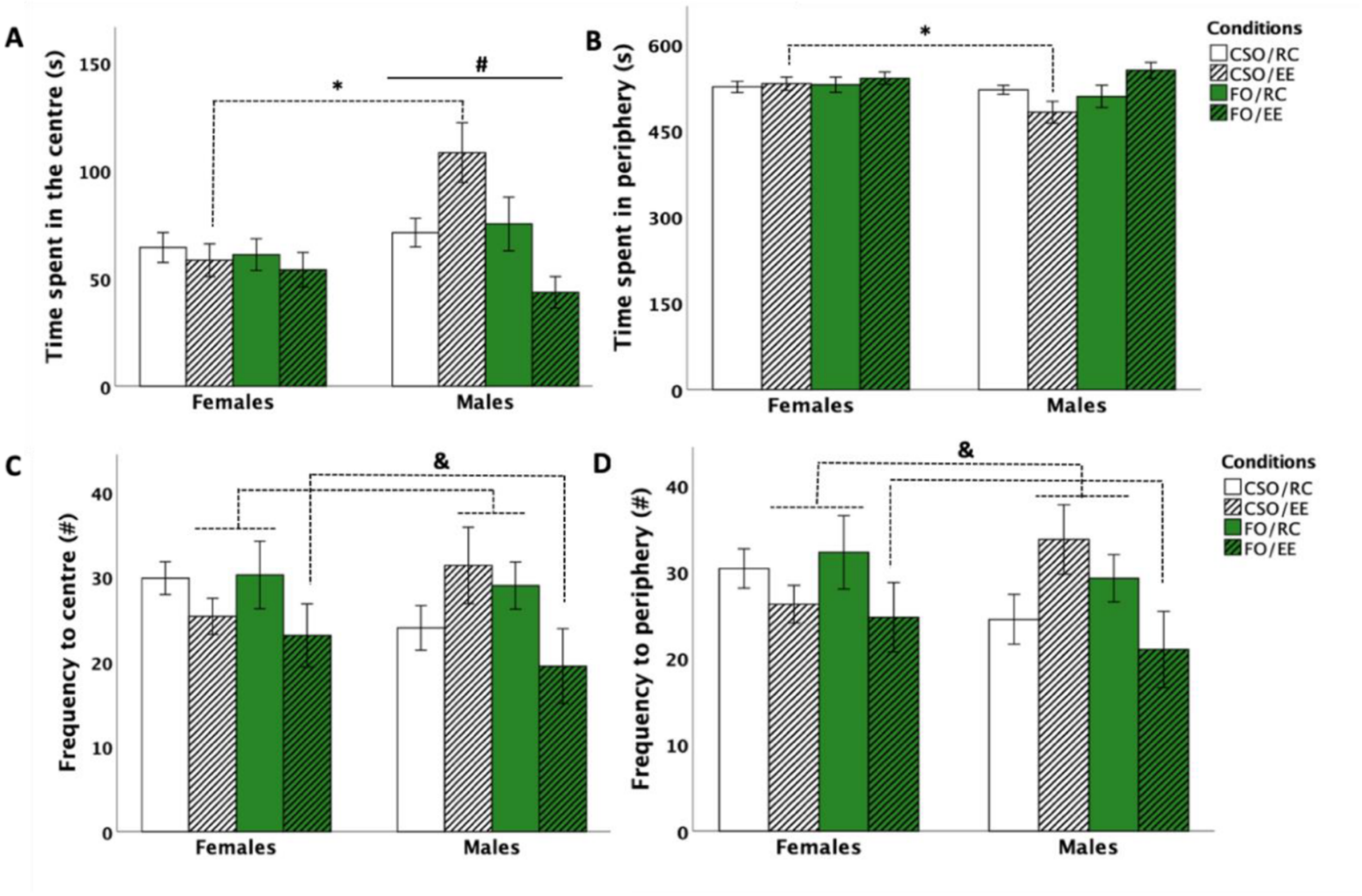

Figure 3. Effect of supplementation, sex, and environment in the Open Field Test (OFT) for Time spent in the centre (Fig 3a), time spent in the periphery (Fig 3b), frequency to centre (Fig 3c) and frequency to periphery (Fig 3d). Male rats spend more time in the centre zone compared to females (*p* <.05; #). CSO/EE males spent increased time in the centre zone (3a) while females in the same condition spent increased time in the periphery (3b) (*p* <.05; *). Rats in the CSO/EE and FO/RC groups also entered more frequently the centre (3c) and peripheral zones (3d) compared to the FO/EE condition (*p* <.05; &). Data are presented as mean ± S.E.M. * and # Indicates statistically significant difference between groups at *p* < .05. & Indicates significant impact of the condition (supplementation and housing only) without an influence of sex at *p* < .05.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.3 Elevated Plus Maze (EPM)

EPM data analyses indicated no significant changes in time spent in open or closed EPM arms, or number of risk assessments (all *p* > .05). For arm entries, main effects of supplementation for open [*F* (1,56) = 11.721, $p = .001$, $\eta^2_p = .173$] and closed [*F* (1,56) = 17.759, $p = .001$, $\eta^2_p = .241$] were found. CSO-fed rodents tend to enter the open arms more compared to their FO-fed counterparts ($p < .001$; See Figure 4a and b).

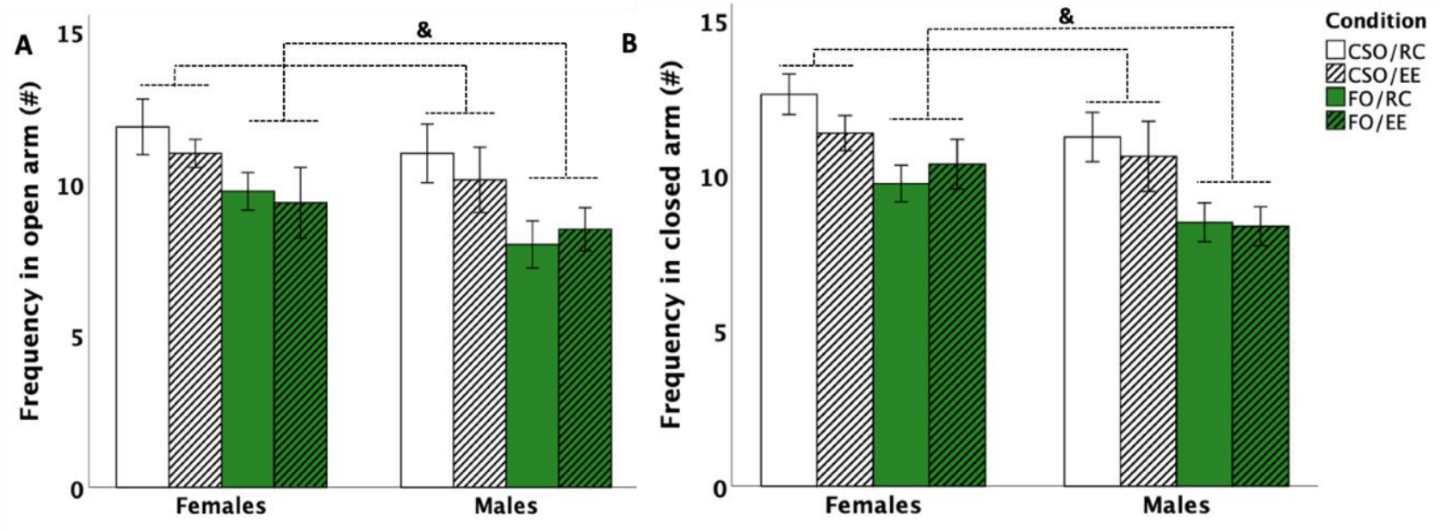

Figure 4. Effect of supplementation, sex, and environment in the Elevated Plus Maze (EPM) for frequency in the open (Fig 4a) and closed (Fig 4b) arms. CSO-supplemented rats entered the open (4a) and closed (4b) arms more frequently than FO supplemented rats ($p<.001$; &). Data are presented as mean ± S.E.M. & Indicates significant impact of the condition (supplementation only) without an influence of sex or housing at $p < .05$.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.4 Social Approach Test (SAT)

3.4.1 Social Approach

SAT data was analysed using chamber exploration time ratios. Changes in sociability involved a ratio calculated from time spent interacting with S1 versus total exploration time (S1 and empty cup exploration) [i.e., $T_{S1} / (T_{S1} + T_{EC})$]. Statistical analyses revealed main effects of supplementation [*F* (1,56) = 7.546, $p = .008$, $\eta^2_p = .129$] and environment [*F* (1, 56) = 4.005, $p = .050$, $\eta^2_p = .090$]. Overall, post-hoc comparisons showed FO diet to promote exploration of S1 compared to EC ($p =.05$). No main effect of sex emerged. However, due to an a priori interest, planned comparisons were performed, which showed increased time exploring S1 versus EC in enriched environment and FO-fed females compared to CSO-fed counterparts (p=.013). In males, no significant changes in sociability were noted (See Figure 5a).

3.4.2 Social Preference

Changes in social recognition were assessed using a ratio between time spent interacting with S2 versus total exploration time (S1 and S2) [i.e., $T_{S1} / (T_{S1} + T_{EC})$]. Analyses revealed main effects of supplementation [F (1,56) = 8.98, p = .004, η^2_p = .138], sex [F (1,56) = 11.52, p = .001, η^2_p =.171], and supplement*sex [F (1,56) = 8.674, p = .005, η^2_p = .171] and environment*supplement*sex [F (1,56) = 8.578, p = .005, η^2_p = .151] interactions. Post-hoc comparisons revealed CSO to promote social recognition in male rats through increased S2 exploration compared to FO counterparts (p = .004), independent of housing conditions. CSO-fed males also interacted more with S2 compared to CSO-fed females (p = .001) (See Figure 5b).

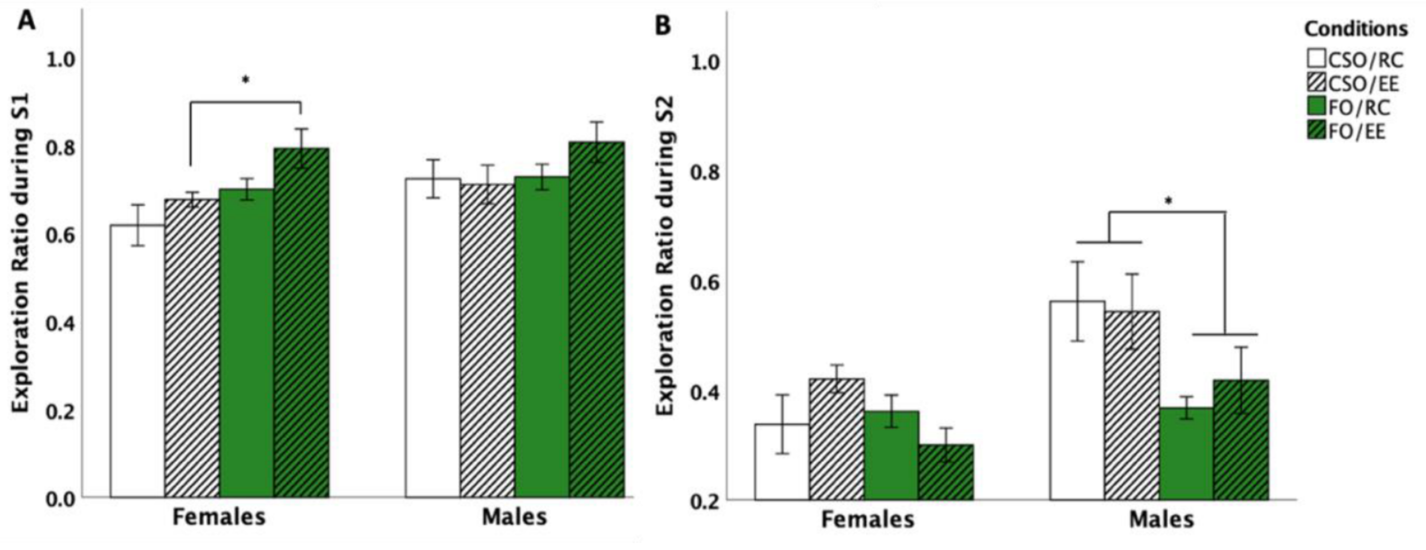

Figure 5. Effect of supplementation, sex, and environment in the Social Approach Test (SIT) for exploration in session 1(Fig 5a) and session 2 (Fig 5b). FO/EE females spent more time interacting with S1 than the empty cup compared to CSO/EE counterparts (p<.05; *), supporting increased sociability (5a). CSO in males promoted social recognition through increased interaction time with S2 compared to FO supplementation (p<.05; *), notwithstanding housing conditions (5b). Data are presented as mean ± S.E.M. * Indicates statistically significant difference between groups at p < .05.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.5 Y-Maze Avoidance Test

3.5.1. Latency to Aversive Arm Entry

Analyses revealed a main effect of sex [F (1,56) = 7.385, p = .005, η^2_p = .132], as well as sex*environment [F (1,56) = 4.719, p = .034, η^2_p = .078] and environment*supplementation [F (1,56) = 5.889, p = .018, η^2_p = .095] interactions. Post-hoc comparisons indicated reduced latency for the aversive arm in females compared to males (p =.005). Interestingly, reduced latencies (i.e., fear conditioning) in FO fed females compared to males was observed in EE housed animals (p=.028), while a similar sex difference was observed in CSO/RC housed females compared to males (p=.008). In CSO fed males, but not females, EE housing significantly reduced latencies to re-enter the aversive arm compared to RC housing (p=.033) (See Figure 6a).

3.5.2 Risk Assessment Behaviour

Analyses indicated a main effect of sex (F (1,56) = 9.092, p = .004, η^2_p = .128), and environment*supplement (F (1,56) = 3.7, p = .016, η^2_p = .156) and sex*environment (F (1,56) = 3.378, p = .018, η^2_p = .158) interactions. Post-hoc comparisons indicated reduced risk assessments in females compared to males (p = .03 for RC- and p = .04 for EE-housed rats). Within male groups, CSO/RC rats made significantly more risk assessments compared to CSO/EE and FO/EE conditions (p =.014 and p=.033 respectively; See Figure 6b).

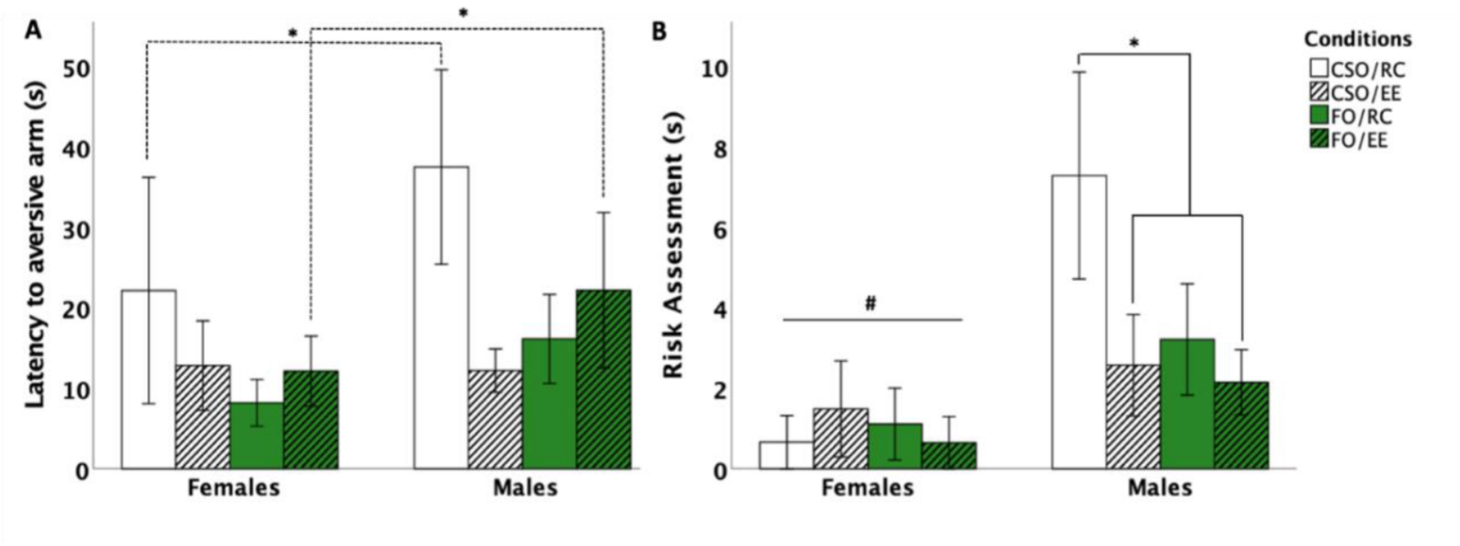

Figure 6. Effect of supplementation, sex, and environment for Latency to arm re-entry (Fig 5a) and Risk Assessment behaviour (Fig 5b) in the Y Maze passive avoidance test. Findings show that males CSO/RC and FO/EE took more time to re-enter the aversive arm compared to females in the same conditions ($p< .05$; *) (5a). In males, the CSO/RC condition increased their risk assessments, compared to the CSO/EE and FO/EE ($p < .05$; *). Females performed less risk assessments compared to males ($p < .05$; #) (5b). Data are presented as mean ± S.E.M. *and # Indicates statistically significant difference between groups at $p < .05$.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.6 Corticosterone Levels

A repeated measure ANOVA revealed a sex*time [F (1,53) = 10.781, $p = .035$, $\eta^2_p = .655$] interaction, attributable to higher CORT levels in females compared to male rats. Pairwise comparisons revealed CORT levels to be significantly elevated on DAY21 compared to DAY1 ($p < .001$), DAY7 ($p < .001$), and DAY14 ($p < .001$). No significant effects of dietary supplementations or housing conditions were found (See Figure 7).

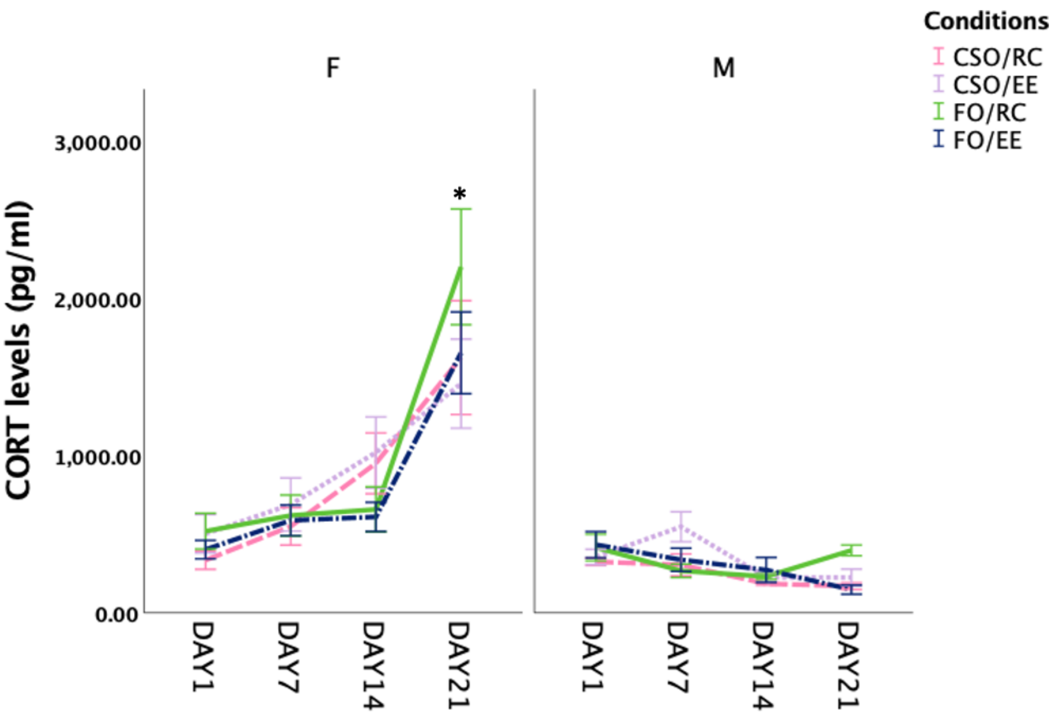

Figure 7. Corticosterone levels (pg/ml) assessed on Experimental Days 1, 7, 14 and 21. On DAY21, analyses revealed increased CORT levels in females compared to males ($p < .001$). Females showed higher CORT level on day 21 compared to days 1, 7 and 14 ($p <.001$ for each day). Data are presented as mean ± S.E.M. * Indicates statistically significant difference between groups at $p < .05$.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage

3.7 Glucocorticoid Receptor Immunoreactivity

Three-way ANOVA on CA1 GR-ir counts revealed no group differences in GR-ir ($p > .05$). At the CA3, analyses revealed a main effect of sex [F (1,42) = 5.577, p = .023, η^2_p = .117], supplementation [F (1,42) = 4.574, p = .038, η^2_p = .098] and a supplementation*environment [F (1,42) = 5.519, p = .024, η^2_p = .116] interaction. Pairwise comparisons indicated reduced GR-ir expression in FO- compared CSO-fed rats (p = .038). The effect of sex is attributable to reduced GR-ir in male compared to female counterparts (p = .023). Finally, CSO/RC groups showed elevated CA3 GR-ir compared to CSO/EE (p = .019) and FO-RC (p = .005) groups (See Figure 8).

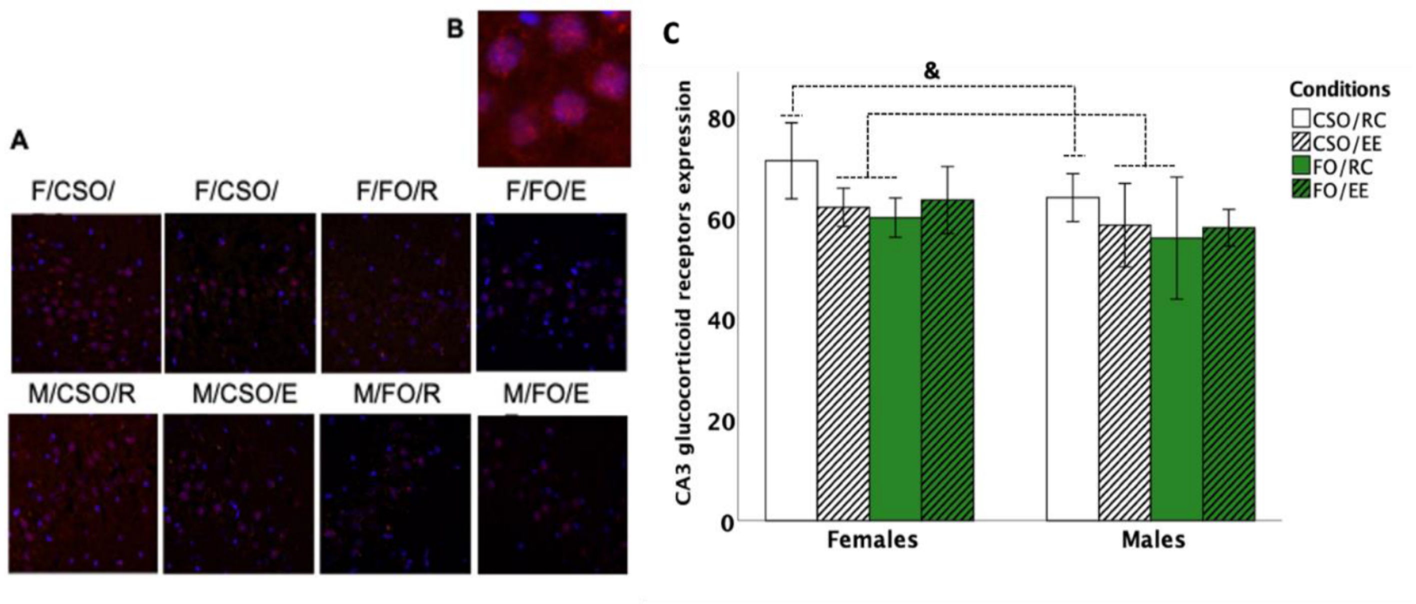

Figure 8. GR-ir in the CA3 region of the hippocampus. CSO/RC rats showed significant increase in CA3-GR-ir compared to CSO/EE and FO/RC rat groups (*p* = .019 and *p* = .005 respectively). Data are presented as mean ± S.E.M. & Indicates significant impact of the condition (supplementation and housing only) without an influence of sex at p < .05.

FO: Menhaden fish oil; CSO: Control Soybean oil; EE: Enriched Environment; RC: Regular cage; GR: Glucocorticoid Receptors

4. Discussion

The aims of this study were to assess the sex-specific effects of n-3 dietary supplementation and environmental enrichment exposure during adolescence on delayed adulthood coping mechanisms, stress response, and sociability. Our findings support differential effects of treatments administered individually or in combination on studied parameters across both sexes.

4.1 Sexually dimorphic influence of FO- supplementation and housing on coping response

On PND 90-91, all rats were exposed to the FST. Although often used to infer depressive-like behaviour in rodents exposed to various experimental paradigms (Porsolt et al., 1977), several studies have supported the possibility for immobility in the FST to reflect an adaptative strategy promoting energy conservation (Holmes, 2003; reported in review by

McCormick and Green, 2013). Thus, de Kloet and Molendijk (2016) demonstrated that elevated corticosterone secretion and hippocampal GR activation seen upon rescuing rats from the cylinder facilitate storage of this event in memory, and lead to adoption of a preferred, mostly passive, coping style during re-exposure to the FST (see Monlendijk & de Kloet, 2019 for a review). This perspective provides an interesting scope to evaluate sex-, housing-, and diet-related effects in the FST.

Our findings revealed FO supplementation in females to be associated with a distinctive increase in immobility (i.e., passive coping) compared to all other conditions. Colom-Lapetina et al. (2017) identified heightened immobility to be related to learning and memory consolidation, especially in a familiar environment. In other words, using additional resources to escape the apparatus (active coping) is deemed unnecessary, as experience indicates the swim session will end shortly. Interestingly, increased immobility was more frequently detected in female compared to male rats. In contrast, soybean oil-supplemented, regular cage-housed males showed heightened activity marked by a ~ 2-3.5-fold increase in time spent climbing compared to all other rats, promoting active coping. Several studies have demonstrated a strong association between FO- supplementation and memory consolidation, especially in females (Sidhu et al., 2011; Lassek et al., 2011). Although brain circuitries remain to be fully characterized, passive coping responses, such as tail-supported immobility or tail support-free floating, have been shown to depend on efficient dorsolateral striatal and limbic learning circuits and to involve hippocampal GRs as essential parts of prior FST experience consolidation (Kloet and Molendijk, 2017; Campus et al., 2015). Warden et al. (2012) have also shown medial prefrontal cortex (mPFC) activation to act as an on/off switch for active versus passive behavioural selection in the FST. They demonstrated that triggering a cluster of mPFC neurons that project to the brainstem's serotonergic dorsal raphe nucleus (DRN) induced a profound, rapid, and reversible selection of active behavioural state in the FST (Warden et al., 2012). Future research should address potential sex-specific differences resulting from activation of such pathways in coping responses. In our study, FO/RC females showed a trend for highest CORT elevations as adolescents, which may have had an impact in differentially priming adulthood FST-induced GR activation, enabling FO/RC females to better attend cues linked to the stressful swim exposure, and by extension, enhancing consolidation of this experience (Molendijk and de Kloet, 2019). Importantly, EE housing prevented FO-induced immobility in females, supporting synergistic actions of diet and environmental conditions. This is a particularly interesting finding, supporting that upon acute exposure to a potent stressor, adolescent experience has a significant impact on modelling response patterns. Considering that groups in this study were not exposed to commonly used stress paradigms leading to depressive-like behaviour in the FST, more studies are required to understand the singular impact of EE in FO-fed females. Interestingly, male rats-maintained immobility mainly using tail support, an alternate strategy that was only privileged by the CSO/RC treated females. The use of distinct strategies when both are accessible in the FST (i.e., using floating versus tail support) emphasize the need to look closer and be cautious when interpreting reduced floating in males as equivalent to reduced depressive-like behaviours, considering than having the choice between strategies for immobility the males did not select floating.

4.2 Diet and housing altered global activity with minimal effects on anxiety-like behaviours.

In the OFT, male rats showed increased time exploring the centre zone compared to female counterparts. In particular, CSO males exposed to EE spent increased time in the centre zone and performed increased frequencies of entries in the centre and peripheral OFT zones, which support increased locomotor exploration and reduced anxiety-like behaviour. Consistent with observation, Teixeira et al (2011) found combined exposure to CSO-supplementation and exercise in mice to reduce anxious behaviour, as demonstrated by greater head-dipping frequency and longer time spent in open arms, whereas exercise did not improve these parameters in lard and hydrogenated vegetable fat-fed rats, or rats fed CSO alone. Anxiolytic effects in CSO-exercised mice were associated with higher activity of Na+K+-ATPase in the cortex and hippocampus, favoring cell membrane fluidity. Interestingly, soybean oil supplementation has been shown to enhance serotonin or tryptophan availability (Sahakian, Sarna, Kantamaneni & Curzon, 1986; Crane and Greenwood, 1987; Kita et al., 2010; Friedman, 2018), and to increase tryptophan and serotonin secretion in cortical tissues (Choi et al, 2009). Such effects could contribute to combined CSO/EE reducing anxiety-like behaviour in the OFT. Additional studies on biochemical effects of juvenile FO and CSO supplementation in males and females are warranted to confirm mechanisms involved.

In the EPM, increased frequencies of arm entries were observed in CSO-fed male and female rats compared to FO fed rats, notwithstanding the housing conditions. EPM exploration through arm entries was also increased in female compared to male rats. However, groups did not differ in terms of time spent in the open arms. These observations were not expected considering effects of diet enriched with n-3 PUFA to reduce anxiety symptoms (Ikemoto et al., 2001). Several research have also associated n-3 PUFA deficiency with increased anxiety-like behaviours (Anisman, 2002; Autry and Monteggia, 2012; Ginty & Conklin, 2015; Rice and al., 2015). Of interest, findings from previous studies have proposed the effect of soybean oil on locomotion to be linked to modulation of serotonergic and dopaminergic brain pathways (Yitmit et al., 2012; Teixera et al., 2012). For instance, Yimit and colleagues (2012) reported increased serum dopamine level sassociated with daily soybean peptide supplementation in healthy subjects (Yimit et al., 2012), and effect that could influence locomotion. Additionally, a study by Teixeira et al. (2012) demonstrated increased locomotion and exploratory behaviour in male rodents fed soybean oil compared to hydrogenated vegetable fat or lard, which they hypothesized was directly linked with trans-fat rich diet affecting the dopaminergic circuitry. Therefore, it is possible that CSO supplementation enhanced exploration in part through regulation of the serotonergic and dopaminergic pathways.

Our findings support that juvenile FO supplementation, when provided to non-deficient rats, does not alter anxiety levels. Notably however, frequencies of entries in the open and closed zones of the OFT was reduced in FO/EE treated male and female rats, while FO alone reduced the number of EPM open and closed arm entries in both male and female rats compared to CSO, possibly indicating reduced novelty-induced arousal. In this context, Thesing et al. (2018) reported HPA axis dysregulation to be associated with reduced plasma DHA levels, leading them to propose effects of DHA to regulate physiological stress and improve stress-related behaviours. Consistent with this, Rice and al. (2015) reported omega 3deficiency to be associated with elevated glucocorticoid levels and depression-like symptoms, while Nemets et al. (2006) reported supplementation of omega 3to reduce depressive symptomsin young adults. Considering these findings, reduced novel open field and EPM exploration observed in juvenile FO supplemented rats could in part be attributable to FO effects to regulate novelty-induced arousal and/or HPA reactivity.

4.3 FO and EE foster sociability in female rats while CSO promotes social recognition in males

Akin to other measures assessed in this study, n-3 PUFA supplementation and environmental enrichment exposure resulted in sex- and context-specific effects on sociability and social recognition. In the SIT, our findings support FO to foster sociability in females, increasing time exploring the stranger rat rather than the empty cage compared to CSO supplementation (*p*=.013), with no significant impact of housing. This observation is consistent with studies showing a key role for n-3 PUFA in regulating prosocial behaviour, impulsive mood, and negative interaction in distinct species (Hamazaki & Hamazaki, 2008), including effects to reduce violent and aggressive behaviour in rodents, dogs, and humans (Augier et al., 2003; De Vriese et al., 2004; Re et al., 2008). However, we were unable to observe an effect of EE by itself, we reported an increase on sociability in female exposed to the FO/EE, illustrating a potential synergic effect of both conditions combined. Our observations are consistent with Hendershott et al. (2016), which reported EE housing in male and female mice to induce bordered significance in the social approach test. Interestingly, the social and physical components of enrichment may play distinctive roles in promoting sociability, as increased social enrichment itself does not always alter social behaviour, while housing in larger cages equipped with physical stimuli has been shown to promote social affiliation (Pietropaolo et al., 2004).

Social recognition revealed a differential impact of diet and housing conditions, where soybean supplementation and regular housing acted to increase novel congener (S2) interaction in male, but not female, rats. A recent review by Aspesi and Choleris (2021) supports social recognition to be sex-specifically regulated by a panel of steroids and mediated in males by direct interaction of the androgen metabolites 3α-diol and 3β-diol with oxytocin and vasopressin, affecting other downstream systems and behaviours (e.g., $GABA_A$ and anxiety-related behaviours; Handa et al., 2012). In this context, Su et al. (2021) noted a 20% dietary CSO supplementation to significantly increase luteinizing hormone (LH) and testosterone levels in male mice, possibly driving observed effects in our study. In addition, Choi et al. (2009) showed increased cortical tryptophan and serotonin secretion in males rats supplemented with soy protein, proposed to regulate enhanced cognitive performance associated with a 4-week soybean oil supplementation (Crane and Greenwood, 1987). In contrast, Watanabe et al. (2010) demonstrated a significant reduction in serotonin turnover in the hypothalamic region following a PUFA-supplemented diet, related to fish oil's hypophagic effects. Determination of biochemical effects of adolescence-targeted supplementation in males and females are warranted to confirm mechanisms involved.

4.4 CSO supplementation in adolescent males accentuated adulthood conditioned fear responses.

In the YMPAT, CSO increased conditioned fear memory (through increased risk assessments and latency to enter the aversive arm) in male rats, an effect abolished by EE housing. EE also similarly attenuated fear conditioning in FO-fed female rats, more so than in male counterparts. This is consistent with studies showing DHA-supplementation and EE to promote stress resilience and adaptation to aversive environment (van Praag et al., 2000; Takeuchi et al, 2016). These results are concordant with the risk assessment findings, where CSO/RC males tended towards increased risk assessment behaviours compared to all other male and female groups, possibly indicative of increased stress resilience in rat groups fed with FO and exposed to EE. Noteworthy, Barbelivien et al, (2006) reported that EE exposure in female rodents reduced fear conditioning upon exposure to aversive stimuli. In other words, female rodents in an EE were able to process contextual information faster, which led to a reduced fear conditioning (Barbelivien et al., 2006). On the same note, Mora-Gallegos et Fornaguera (2019) reported that social contact and EE at a young age was able to reduce anxiety and fear conditioning, suggesting that early exposure to EE play a role in identifying contextual cues and assessment of an environment.

4.5 CSO/EE and FO/RC reduced adulthood GR-ir at the CA3, while having no impact on CA1 expression

GR-ir expression measured in adulthood supports lasting impact of diet and housing conditions on hippocampus CA3 region, while CA1 receptor expression was not significantly affected. Notably, individual adolescent exposure to CSO or EE led to a downregulated CA3 GR-ir expression in adult male and female rats, contrasting elevated GR-ir concurrent to CSO/RC adolescent exposure. de Quervain et al. (2009) showed elevated hippocampal GR expression to be beneficial for memory consolidation, while it may impair memory retrieval and working memory. Although the role of observed changes cannot be confirmed in this study, GR changes could play a role in coping strategies observed in the FST, which could involve change in the ability to cope with stress (i.e., develop resilience) (de Koeht et al., 2018). In other words, reduced GR-ir at the CA3 related to adolescent CSO/EE and FO/RC exposure could support increased resilience to stress as they reach adulthood, a response that is not observed in CSO/RC rats. Such proposition remains to be ascertained. Interestingly, in humans, a recent study has shown a beneficial impact of n-3 PUFA-rich diet in mitigating CORT-induced apoptosis in the hippocampus (Borsini et al, 2020). This is intriguing and interesting in relation to decreased CA3 GR-ir observed in FO/RC rats. In terms of EE-induced effects, our findings at the CA3 contrast reports of elevated GR expression associated with adolescent or adulthood EE exposure (Gergerlioglu et al., 2016; Sampedro-Piquero et al., 2014; Shilpa et al., 2017) or those showing EE to down regulate GR levels (Fan et al., 2021). Together, our findings illustrate complex effects of environment and diet. As EE was found to mainly benefit most CSO- than FO-fed rats, more studies are warranted to better understand the specific impact of diet or environmental enrichment on functional regulation of hippocampal circuitry, as changes affecting the ventral CA3 regions may have more decisive impact on adulthood regulation of socio-affective states.

4.6 Limitations

A few noteworthy observations have been made throughout the study, the first relating to supplement palatability. We noticed that FO supplementation elicited a more aversive response in rats exposed to this diet. This could result in increased stress in the FO groups, possibly affecting responses to additional stressors and/or delayed anxiety-like behaviour in the OFT, EPM, and the SAT. While the gavage procedure itself has been widely used to administer supplements, Brown et al. (2000) demonstrated that giving oil supplements such as soybean, sesame or peanut oils by gavage can increase CORT levels up to 4 h following the procedure, although habituation appears to be achieved in a few days (Brown et al., 2000). Here, it is assumed that FO worked in a similar way, and that animals gradually adapted to supplements. Using the same feeding protocol, we have demonstrated sex-specific changes in CORT secretion over supplementation period marked by increased CORT levels measured on the last supplementation day, which reached significance only for female rats. Male rats showed no impact of gavage on CORT secretion measured over days (blood droplets were collected on supplementation days 1, 7. 14, and 21) (Raymond et al., 2020). As per the FST, future studies could introduce additional measures, such as physiological fluctuation of CORT, adrenaline, and/or noradrenaline following testing (Koolhaas et al., 1999). This could provide stronger connection between behavioural and physiological coping and further validate both immobility strategies observed during our experiment. Lastly, with the goal to principally focus on the cognitive benefits of overall EE experience, we attempted to control for effects that could be attributable to increased physical exercise. Thus, we provided RC rats intermittent exposure to a large, emptied Morris Water Maze pool arena where they could freely locomote. The resemblance of this open space environment to the open field could have influenced OFT behavioural responses of the RC-housed rat groups. However, our findings do not support this to have played a decisive role in RC exploration or anxiety-like responses in the OFT.

5. Concluding Remarks

This study is the first to assess long-term, combined effects of dietary omega 3 supplementation and exposure to environmental enrichment during the adolescent period. The contrasting, non-synergistic and/or sex-specific effects of dietary supplementation and environment on the different observed parameters support distinct physiological mechanisms. Importantly, our results support the use of passive coping strategies associated with adolescent n-3 PUFA supplementation in females and males, as well as a sex-dependent effects of EE exposure. Additional observations illustrate that CSO supplementation benefited males' emotional response profile, which was not expected. Literature however supports that CSO may serve a better high energy diet, more enjoyable to eat. Outside palatability, serotonin receptors are greatly influence by soy supplemented diets in male rodents. Generating an increase of serotonin could play a role in the beneficial effect of CSO supplementation rather than FO supplementation in male rodents, which requires a more in-depth exploration in future studies. While our findings failed to confirm exposure to an enriched PUFA diet to show enhanced benefits on social and emotional behaviour in male rodents, they globally supported positive effects in female rodents. Finally, assessing adolescent CORT secretion over the supplementation period led to show a differential CORT secretion profile in male and female rats as supplementation days elapsed; females developing a sensitivity to repeated oral gavage, manifest through significantly increased basal CORT secretion detected on the last supplementation day (PND47). We could not address any remote impact of this sex difference. However, this growing sensitivity to stress exposure in females could become important when exposed to more chronic, invasive, or emotionally toned stressors. Together, our findings highlight the intricate effects of dietary and environmental influence experienced at a critical developmental stage on immediate and delayed HPA activation and stress coping strategies.

6. Acknowledgements

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Study 4

Short-term fish oil supplementation during adolescence supports sex-specific impact on adulthood visuospatial memory and cognitive flexibility.

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Abstract

Introduction: Numerous studies have supported beneficial effects of omega 3 supplementation using Menhaden fish oil (FO) to attenuate cognitive impairments in aged rodents or following pathological conditions. Notably, FO supplementation also promotes brain maturation and plasticity during critical developmental periods, although prophylactic effects on cognitive performance and attention have not been determined. **Objectives**: The goal of this study was to observe sex-specific immediate and adulthood impact of adolescent omega 3 supplementation on visuospatial performance and cognitive flexibility. **Methods:** 64 post-weaning Wistar rats (n = 32 males; n = 32 females) were randomly assigned to one of four groups. Daily menhaden fish oil (FO) or soybean oil (CSO) were supplemented via oral gavage (0.3ml/100g body weight) to experimental or control groups rats during the early and mid-adolescence period (postnatal day 28-47). The Barnes Maze Test (BMT) was used to measure visuospatial memory and reversal learning trials (RL) determined cognitive flexibility. Half of the experimental and control groups underwent behavioural testing immediately after the gavage period (adolescents), while the other half were tested during adulthood (postnatal day 90). **Results:** Notwithstanding supplementation, adult rats showed reduced working memory errors (WME) and gradual decrease in latencies to reach the escape box over the 6 testing days compared to adolescents. As adults, FO-supplemented females displayed fewer WME than male counterparts, while CSO benefited males' performance on discrete testing days. In the RL, females tended to quickly reduce the distance travelled to reach the hidden box compared to males, as well as a gradual decrease in WME as testing progressed. **Conclusion:** A clear discrepancy in BMT performance was observed between adolescent and adult rat groups, supporting improved visuospatial memory in adult rodents, and cognitive flexibility to require brain maturation to be expressed. Overall, sex- and supplementation-dependent effects supported a positive impact of FO in female rats, while a FO- supplemented males failed to improve. While such pattern was not observed throughout the study, it nonetheless demonstrates the potential for dietary supplementation limited to the early adolescence period to exert influence of spatial learning and cognitive flexibility.

Keywords: n-3 PUFA, docosahexaenoic acid, eicosapentaenoic acid, Sex-dependent, Age-dependent, Barnes Maze Test, Visuospatial memory, Cognitive flexibility

Introduction

Dietary habits have significantly evolved over the last century, mainly due to the industrialisation which has revolutionized agricultural practices (Simopoulos, 2016), leading to important dietary changes, and a switch from consumption of untransformed foods including nuts, fish, and vegetables to industrialized grain-based diets with higher fat and reduced fibre content. Expansion of the so-called Western diet led to a substantial increase in omega 6 polyunsaturated fatty acid (n-6 PUFAs), saturated fat, and *trans*-fat dietary intake at the expense of essential fatty acids (EFA) such as omega 3 polyunsaturated acids (n-3 PUFAs) (Mani & Kurpad, 2016). N-3 PUFAs are mainly found in plants and nuts, with highest n-3 PUFA levels being metabolised from oily substances like, sunflower oil, salmon, herring, and mackerel (Simopoulos, 2002; Gomez-Pinilla, 2008). The disproportionate consumption of n-6 fatty acids in the Western diet compromises the n-6 to n-3 ratio, which can then reach a 20:1 ratio (Simopoulos, 2002; Marion-Letellier, Savoye & Ghosh, 2015), significantly overpassing the optimal 4:1 ratio (Simopoulos, 2016).

A major concern from societal dietary shifts comes from the fact that n-3 PUFAs cannot be synthesized by the human body and therefore need to be acquired through diet (Jump, 2002). In this context, dietary deficits have generated negative impacts on brain maturation processes and associated functional outcomes (Jump, 2002). Indeed, raised endogenous levels of docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids brought by omega 3 consumption is related to a variety of essential roles in humans and animals, notably by promoting membrane fluidity and synapse formation in the brain, favoring vesicle formation and protein transport (Kitajka et al., 2001; Shahidi & Ambigaipalan, 2018), and improving cognitive and attentional capacities (Jump, 2002; Woo et al., 2014, Gow & Hibbeln, 2014; Ayee et al., 2020). Discrete studies have supported DHA supplementation to improve cognitive development in children (Helland et al., 2003; Richardson et al., 2012; See Weiser et al., 2016 for a review), and beneficial effects on cognitive tasks performance in adolescent rats (Gamoh et al., 1999; Catalan et al., 2002; Fedorova et al., 2007; See Gharami et al., 2015 for review). Evaluating the impact of daily DHA enriched diet for underperforming children, Richardson et al. (2012) showed supplementation between the age of 7 and 9 to improve overall reading performance. Likewise, Dalton et al. (2009) highlighted n-3 PUFA dietary intake to enhance verbal learning and memory in children (between 7 and 9 years old). Studies also reported maternal omega 3 intake to influence toddlers IQ scores (Lassek & Gaulin, 2011; Karr et al., 2012). Notably, a study by Lassek and Gaulin (2011) provided compelling sex-dependent effects of an n-3 PUFA enriched diet, reporting measurable impact of n-3 PUFA intake on cognitive test scores in both sexes, although greater advantage was observed in females. In rodents, similar observation patterns were noted, with enriched n-3 PUFA diet promoting cognitive performance. Consistently, Fedorova et al. (2007, 2009) have shown that maternal PUFAs deficiency during the gestation and lactation periods (7-8 weeks) led to impaired performance on the Barnes Maze test (BMT) in Long Evans rats and in mice. Rats also demonstrated reduced reversal learning (RL), validating the influence of omega 3 on cognitive flexibility. Later life cognitive abilities are also influenced by the consumption of omega 3, with DHA consumption protecting the hippocampus from degeneration and delaying cognitive decline in both humans and non-human mammals (Külzow et al., 2015).

Although several studies have supported the influence of in utero, early developmental, and adult omega 3 dietary intake on cognition, the impact of n-3 PUFA supplementation during the adolescent period on cognitive processes remains to be established (Karr et al., 2011;

Fuhrmann et al., 2015, Gerhard et al., 2021). This developmental stage represents a critical time window marked by extensive brain maturation, affecting the “late blooming” prefrontal cortex (PFC) for which the course of maturation is prolonged well after puberty (Akirav et al., 1999; Petrovich et al., 2001; Ghashghaei et al., 2002; Naneix et al., 2012; Calabro et al., 2020). The crucial role of neural outputs from the frontal cortex to areas such as the hippocampus, basal ganglia, cerebellum, thalamus, and other association cortices in cognitive processes, executive function, and reversal learning is well acknowledged (See Park & Moghaddam, 2016 for review; Reinert et al., 2021). Importantly, the PFC is the region which stores the highest DHA concentrations. Indeed, of the 35-40% DHA concentrations stored in the brain, more than 15% accumulates in the frontal cortex (Carver et al., 2001; Gharami et al., 2015). Several studies have explored the association between n-3 PUFA supplementation and improved cognition. For instance, n-3 PUFA has been shown to improve hippocampal-dependent learning, adulthood neurogenesis, and facilitate learning processes (Pravosudov et al., 2005; Bos et al., 2016). Not only has DHA supplementation been shown to improve cognitive abilities, but researchers also report its importance in the attention process itself. Yoshida et al. (1997) demonstrated that reduced α-Linolenate consumption, a precursor of DHA, led to a decrease of nearly 30% of synaptic vesicle density in the terminals of the hippocampal CA1 region and created learning impairments (e.g., increased error rate) in the brightness-discrimination learning test. On a similar note, Ahmad et al. (2002) demonstrated a decrease of cell density, volume and total cell counts in the CA1 and CA3 regions of the hippocampus associated with a high dietary intake of linoleic acid (LA), likely to contribute to observed spatial memory deficits.

Interestingly, effective n-3 PUFA conversion is influenced by several factors, notably gonadal hormones. Testosterone has been found to inhibit adequate conversion of n-3 PUFAs while estradiol promotes the separation of free fatty acids toward oxidation and away from triglyceride storage (Huang et al., 1987; D'Eon et al.,2005). Elevated levels of testosterone during development have been proposed to influence negatively attentional abilities and sociability in children (Tammam et al., 2016), whereas estrogen is known to exert beneficial effects on cognition, although the relationship with n-3 PUFA metabolism has not been established (Huang et al., 1987; Marra et al., 1989; Schuchardt et al., 2009). Sexually dimorphic responses to omega 3 supplementation have been previously demonstrated, which could be of particular importance when supplementation occurs during the adolescence period (Tamman et al., 2016).

1.1 Goal and Hypothesis

Thus, the goals of this study are to: 1) characterize visuospatial memory performance in adolescent and adult rodents; 2) monitor sex-dependent effects of diet on such cognitive abilities; and 3) determine the short and long-term effects of omega 3 on visuospatial memory and cognitive flexibility. As literature identifies a sex-dependent effects of omega 3 on cognition and visuospatial performance, we expect females to show increased spatial memory performance [i.e., find the escape box faster with fewer errors], both in the adolescent and adult testing periods.

2. Methods

2.1 Subjects

Wistar rats (n = 64; 32 females and 32 males) arrived at the animal facility on postnatal day (PND) 23 and were acclimated for a period of 5 days prior to initiation of the gavage procedure. Animals were daily supplemented with a high source of omega 3 through Menhaden fish oil or a control soybean oil from PND28 to PND47, covering the early and mid-adolescence period (see Figure 1 for experimental timeline). Half of each supplementation groups underwent behavioural testing immediately following diet (n = 8 females; n = 8 males per condition), while the other half groups were kept in the animal facility, on a regular diet, until behavioural testing during adulthood (PND 90; n = 8 females; n = 8 males per condition). Rats were kept on a 12h light/dark cycle (lights on at 7 a.m.) and had free access to regular food chow and water throughout the experiment. Vaginal cytology was performed on adult females from PND 56 to PND 69 to determine stages of the estrus cycle. Microscopic analysis was performed using MacLean et al. (2012) and Goldman et al. (2007) for the assessment of each stage. All procedures were carried out in accordance with the Canadian Council of Animal care (CCAC) and approved by the University of Ottawa Animal Care Committee. Experimentation complied with the ARRIVE guidelines and was in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

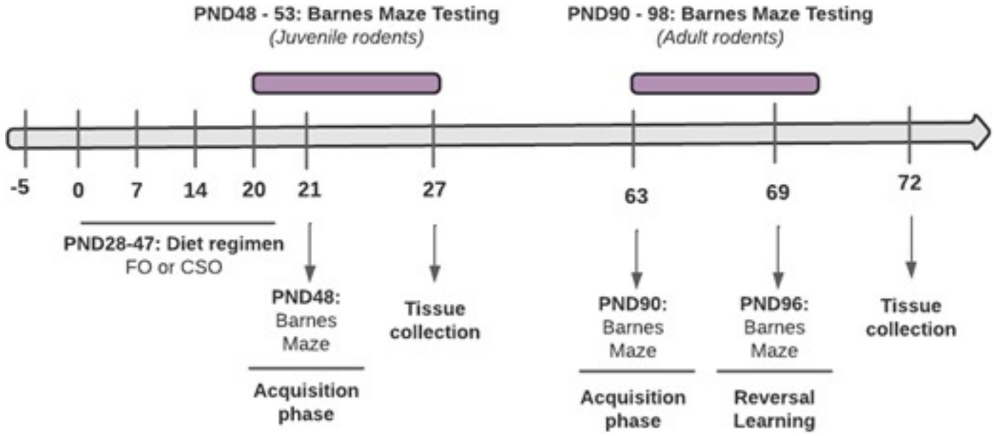

Figure 1. Experimental Timeline. Animals arrived at the facility on PND 23 and acclimated for 5 days prior to the start of the experiment. Dietary supplementation occurred daily from PND 28 to PND47 (20-day period). Half the cohort underwent Barnes Maze testing immediately following the supplementation period (n = 32), while the other half was tested as they reach adulthood (PND90). Barnes maze acquisition was evaluated for 6 days in adolescent and adult rats, while reversal learning was assessed for 3 days in adults only.

FO: Menhaden fish oil; CSO: Control soybean oil.

2.2 Supplementation and Weight Monitoring

Menhaden fish oil (FO; Sigma-Aldrich Canada) and soybean oil (CSO; Sigma-Aldrich Canada) were administered via oral gavage at a concentration of 0.3 mL of supplement per 100 g of body weight (Raymond et al., 2020). Supplementations were composed of the following: 1) Control-Soybean oil (CSO), a non-hydrogenated omega 6 rich supplement with a 7.4/1 n-6/n-3 ratio and 2) Fish oil from menhaden (FO), an omega 3 rich supplement (Sigma Aldrich; 20.0 - 31.0% omega 3 fatty acids content; Eicosapentaenoic Acid (EPA - 10-15%)/ Docosahexaenoic Acid (DHA 8-15%), and administered daily between 7 and 9 a.m. To facilitate the procedure and limit gavage-induced stress, the tip of gavage syringes was slightly dipped in condensed milk (Eagle Brand® Low Fat Sweetened Condensed Milk). Weight was monitored daily during oral gavage, and once per week until euthanasia to ensure optimal weight gain and development.

2.3 Behavioural Testing

On testing days, rats were moved from the vivarium to an acclimation room near the testing area at 7 a.m. for a minimum of 30 min prior to testing. Females' estrus cycle was assessed at least 1 h before testing. The testing rooms was kept between 21-23ºC with 40-60% humidity and brightly lit with 400 lux overhead lights. A black curtain was used to separate the testing area and the researcher. The testing apparatus was cleaned with 70% ethanol between each animal. Every test was recorded using a ceiling-mounted camera (Panasonic® Analog Camera, Model: WV-CP284) and coded using ODlog™ (trademark of Macropod Software) and Noldus Ethovision video tracking (Noldus, Leesburg, Va).

2.3.1 Cognitive testing: Barnes Maze Test (BMT) and Reversal Learning (-RL)

The BMT was used to assess spatial memory in the adolescent and adult rats (Barnes, 1979). The apparatus consisted of a plastic circular slab (d = 122 cm; c = 376.8 cm) with 18 equidistant holes (d = 10 cm). An escape box was attached under one hole using a drawer-like system. Geometrical shapes placed on a black curtain surrounding the maze acted as visuospatial cues. Two bright lights (400 lux) illuminated the arena, increasing the rats' motivation to find the escape box. Trials were conducted over the course of 6 to 9 days [three additional days were required when reversal learning was assessed], with 2 daily trials lasting a maximum of 5 min each. Rats were placed under an opaque plastic container in the middle of the maze and the container was lifted via a pulley system to start the exploration period. Each time the rat found the escape box, it was kept in the box for a period of 90 s before being placed back in its cage. The second trial took place on average 3 h following the first trial. If the rat did not enter the escape box within 5 min, the experimenter gently guided the animal to the hidden box and encouraged entry. The location of the escape box remained the same throughout the 6 days of acquisition. In adult groups, three additional daily sessions evaluated reversal learning. In those sessions, the testing procedure was identical with the exception that the escape box was placed under a hole which position was different to that used during acquisition.

Latency to find the escape box (s) and the numbers of working memory errors (WME; poking their nose in hole not associated with the escape box) were measured using ODlog^{TM} software. Distance travelled (cm) before entry in the escape box was recorded and analysed using Noldus EthoVision video tracking system (Noldus, Leesburg, Va).

2.4 Statistical Analyses

Five-way mixed analyses of variance (ANOVA) was performed using IBM SPSS Statistics 28.0 software with between-group factors Supplementation (FO vs CSO), Age (adolescent vs adult), and Sex (female vs male) as well as within-group factors Day (1-6) and Trial (1 vs 2). The outliers were corrected by using the group's now most extreme value plus or minus

1, for upper- and lower-end data, respectively. All assumptions were met prior to analyses. Shapiro-Wilk's test assessed normality of data, homogeneity of variance was confirmed by using Levene's test, while Mauchly's test was used to measure sphericity. In case of violation of sphericity, Greenhouse-Geisser correction was used. Effect size was computed alongside alpha value (α = < .05). For all measures, significance was set at $p < .05$ and Bonferroni correction was applied for all pairwise comparisons.

3. Results

3.1 Acquisition Phase: Latency to Escape Box Entry

For latencies to reach the hidden box, five-way mixed ANOVA revealed main effects of testing Days [F (2.935,164.34) = 32.893; $p < .005$; $\eta^2_p = .37$] and Trials [F (1,56) = 41.911; $p < .0005$; $\eta^2_p = .44$] as well as Day*Age [F (2.935,164.34) = 3.824; $p = .012$; $\eta^2_p = .064$] and Trial*Sex [F (1,56) = 7.696; $p = .008$; $\eta^2_p = .121$] interactions. No impact of dietary supplementation was observed. Pairwise comparisons for Day*Age analysis revealed adolescent rats to require increased latencies to find the escape box compared to adult rats, apparent on DAY4 ($p = .044$), 5 ($p = .014$), and DAY6 ($p = .003$; See Figure 2A). Furthermore, the adolescent rats showed reduced latencies on DAY1 compared to DAY4 ($p = .038$), 5 (p = .013) and 6

($p = .003$), which contrasted the gradually reducing latencies observed over days in adult rats, and evidenced between DAY1 and 3 ($p = .002$), 4, 5, and 6 (all $p < .0005$), DAY2 and 4 ($p = .01$), 5, and 6 (both $p < .0005$), DAY3 and 5 ($p = .004$) and 6 ($p < .0005$) as well as between DAY4 and 5 ($p = .039$) and 6 ($p < .0005$). Post-hoc comparisons examining the Trial*Sex interaction highlight both female and male rats to show increased latency during Trial 1 than during Trial 2 ($p = .004$ for females; $p < .0005$ for males).

3.2 Acquisition Phase: Number of Working Memory Errors (WME)

For the number of WMEs, five-way mixed ANOVA revealed main effects of Day [*F* (3.37,192.113) = 3.795; $p = .009$, $\eta^2_p = .062$] and Trial [*F* (1,57) = 31.235; $p < .0005$, $\eta^2_p = .354$] and significant interactions for Day*Age [*F* (3.37,192.113) = 23.095; $p < .0005$, $\eta^2_p = .288$], Day*Supplementation*Sex [*F*(3.37,192.113) = 2.933; $p = .029$, $\eta^2_p = .049$], Day*Sex*Age [*F*(3.37,192.113) = 3.097; $p = .023$, $\eta^2_p = .052$], Trial*Age [*F*(1,57) = 7.073; $p = .01$, $\eta^2_p = .110$], Day*Trial [*F*(5,285) = 2.841; $p = .016$, $\eta^2_p = .047$], Day*Trial*Age [*F*(5,285) = 3.904; $p = .002$, $\eta^2_p = .064$], and Day*Trial*Supplementation*Sex [*F*(5,285) = 3.036; $p = .011$, $\eta^2_p = .051$]. Pairwise comparisons for Day*Age showed that adults made more WME than the adolescents on DAY1 ($p = .003$), while thereafter the adolescents made more WME compared to adults [DAY3, DAY4, Day 5 and DAY6 - all $p < .0005$]. The adolescents made fewer WME on DAY1 compared to DAY2 ($p = .006$), DAY3, 4, 5, and 6 (all $p < .0005$). As Figure 2B illustrates, post-hoc analyses related to the Day*Supplementation*Sex interaction showed reduced WME in CSO- compared to FO-fed males ($p = .017$), and reduced WME in CSO males compared to female counterparts ($p = .05$). On DAY4, CSO males made less WME than FO males ($p = .05$) while FO- females made less WME than FO-fed males ($p = .034$).

An effect of age emerged from post-hoc comparisons assessing the Trial*Age interaction, which supported reduced WME in adult than adolescent rats during Trial 1 and Trial 2 (both $p < .005$). The adolescent and adult cohorts made more WME during Trial 1 than Trial 2 ($p = .044$ for adolescents; $p < .0005$ for adults). For the Day*Trial interaction, pairwise comparisons showed increased WME during T1 on DAY5 than on DAY6 ($p = .011$). There were more WME during T1 than T2 on DAY4 ($p = .016$) and DAY5 ($p < .0005$) (See Figure 2C). Post-hoc analysis of the Day*Sex*Age interaction was attributable to adolescent females making less WME than adolescent males ($p = .011$) on DAY4, and to adult (females and males) making less WME than adolescent counterparts (*p* = .002 and *p* < .0005, respectively) (See Figure 2D). The Day*Trial*Age interaction is related adult cohorts making less WME over days for both Trial 1 and 2 compared to adolescent cohorts (*p* < .025).

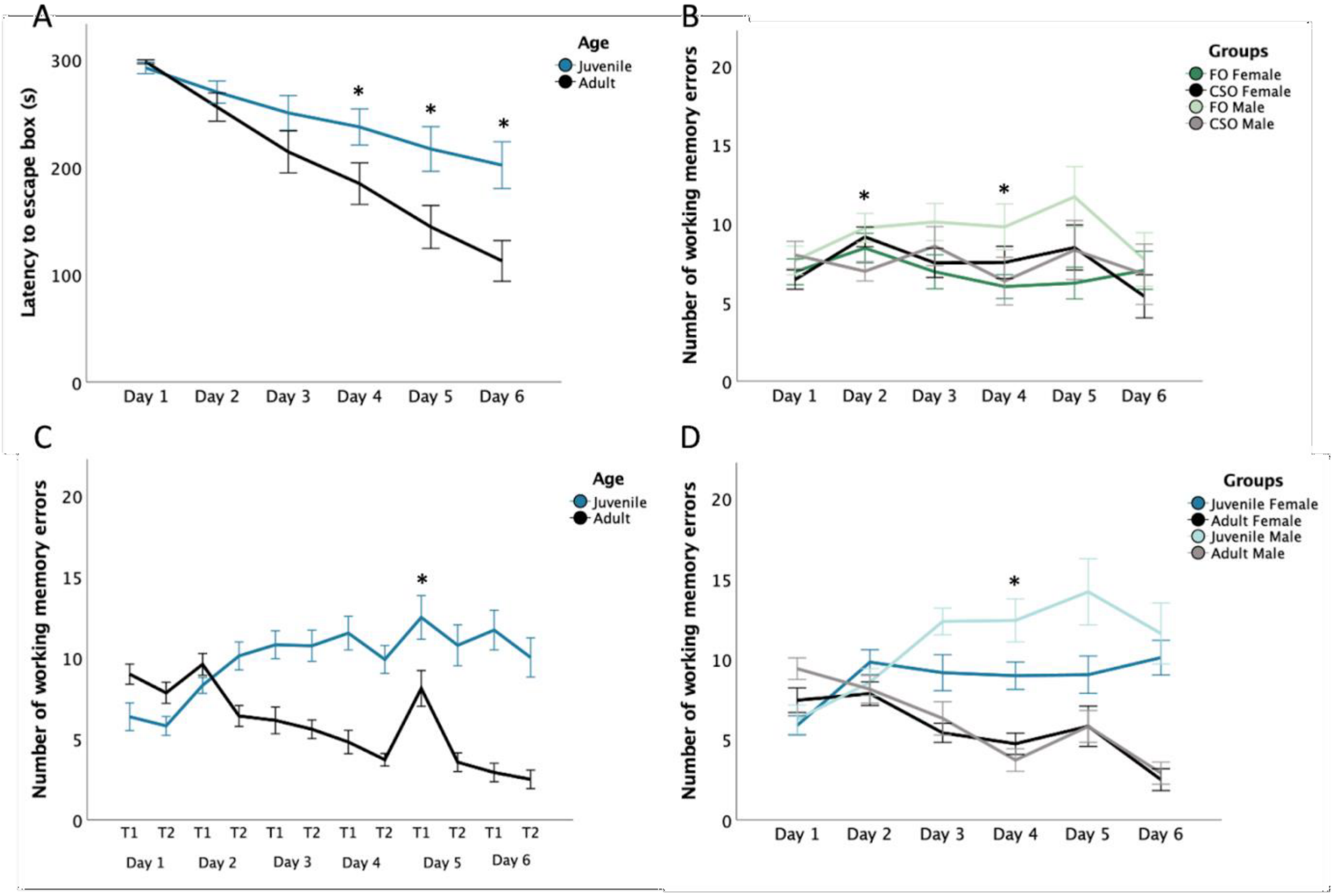

Figure 2. Latency to Escape Box and Number of Working Memory Errors (WME) in the Barnes Maze Test. Panel A shows the latency (s) to the escape box in adolescents and adults. Panel B shows the number of WME made by FO- and CSO-supplemented males and females. Panel C shows the number of WME made by adolescent vs adult rats. Panel D shows the number of WME made according to rodents' sex and age. Data are presented as mean ± S.E.M. * Indicates statistical significance at p < .05.

CSO: Control soybean oil; FO: Menhaden fish oil; T1: Trial 1; T2: Trial 2

3.3 Acquisition Phase: Distance travelled

Five-way mixed ANOVA revealed significant main effects of Day [*F* (3.424,188.297) = 51.542; *p* < .0005; η^2_p = .484] and Trial [*F* (1,55) = 120.475; *p* < .0005;

$\eta^2_p = .687$]. Significant interactions were found for Day*Sex [F (3.424,188.297) = 2.989; $p = .026$; $\eta^2_p = .052$], Day*Age [F (3.424,188.297) = 4.617; $p = .002$; $\eta^2_p = .077$], Trial*Supplementation [F (1,55) = 9.281; $p = .004$; $\eta^2_p = .144$], Day*Trial [F (4.351,239.319) = 4.687; $p < .0005$; $\eta^2_p = .079$], and Trial*Supplementation*Sex*Age [F (1,55) = 13.655; $p < .0005$; $\eta^2_p = .199$] (See Figure 3). Two rats were omitted from this analysis due to technical issues and their data points were replaced using multiple imputation.

Pairwise comparisons for the Day*Sex interaction supported increased distance traveled by females compared to males on DAY1, 2 ($p < .0005$ for each comparison), and 4 (marginal $p = .05$). However, both sexes showed reduced distance travelled as testing days progressed ($p < .05$) (See Figure 3A). The Day*Age interactions was attributable to longer distance travelled in adolescents compared to adults on DAY4 ($p < .0005$), 5 ($p = .007$), and 6 ($p = .001$). Adolescent rats also travelled more on DAY1 ($p = .002$), 2 ($p = .003$), and 4 ($p < .0005$) than on DAY5 and more on DAY1 through 4 than 6 (all $p < .007$). Adult rats similarly travelled more on DAY1 and 2 than on DAY3, 4, 5, and 6 (all $p \leq .001$) (See Figure 3B).

Pairwise comparisons for the Trial*Supplementation interaction supported increased distance travelled in both FO- or CSO- rats during Trial 1 and Trial 2 (both $p < .0005$). As for the Day*Trial interaction, post-hoc comparisons supported rats to travel longer distance during the first than second daily trials from DAY2 through DAY 6 (all $p < .002$). Similarly, travelled distance for both Trials 1 and 2 gradually decreased over testing days (all $p < .035$). Three-way interactions of Age, Sex and Supplementation during the first trial F (1,55) = .12, p = .728; or during the second trial F (1,55) = 1.30, $p = .259$ were not significant.

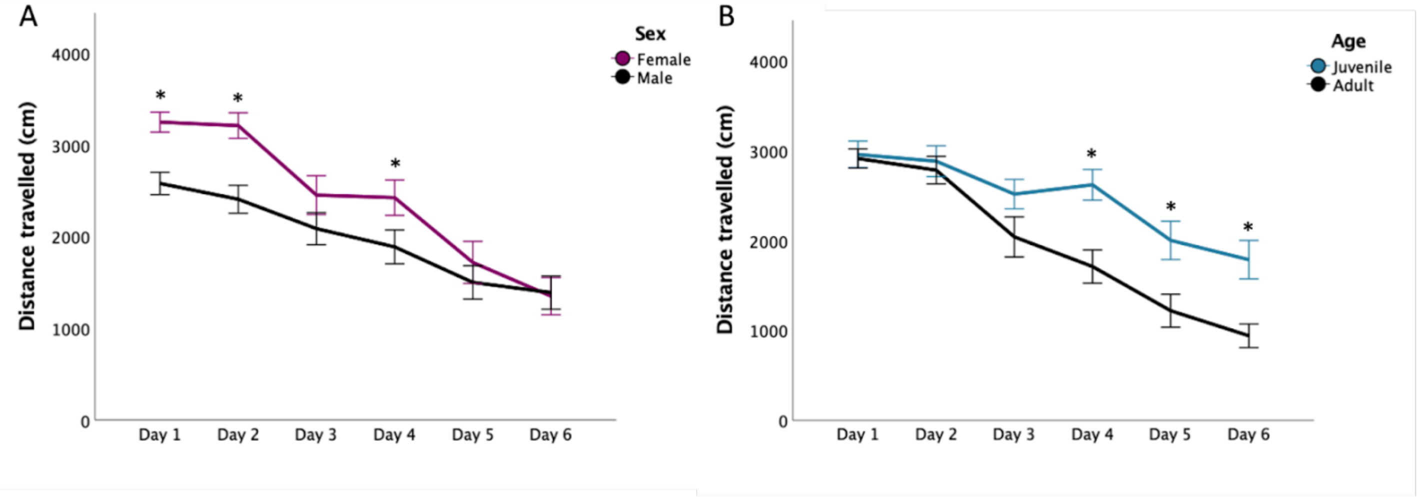

Figure 3. Distance Travelled to reach the escape box in the Barnes Maze Test. Panel A shows the distance travelled (cm) by females and males, whereas panel B depicts the distance travelled by adolescents and adults. Data are presented as mean ± S.E.M. * Indicates statistically significant difference between groups at $p < .05$.

T1: Trial 1; T2: Trial 2.

3.4 Reversal Learning: Latency to Escape Box Entry

Mixed ANOVAs on adult rats' latencies to enter the escape box during the reversal learning trials revealed a main effect of Day [F (2,58) = 24.926; $p = .001$; $\eta^2_p = .462$], attributable to all rats showing increased entry latencies on DAY1 compared to DAY2 and 3 (both $p < .0005$). In addition, a trend was found for Trials [F (1,29) = 4.077; $p = .053$; $\eta^2_p = .123$] related to a tendency for all rats to enter the escape box quicker during the second than the first trial ($p = .053$) (not shown in figure).

3.5 Reversal learning: Distance travelled

In adults rats, four-way mixed ANOVA revealed significant main effects of Day [F (2,54) = 15.019; $p < .0005$; $\eta^2_p = .357$] and Trial [F (1,27) = 5.734; $p = .024$; $\eta^2_p = .175$] as well as significant interactions for Day*Sex [F (2,54) = 10.731; $p < .0005$; $\eta^2_p = .284$] and Day*Trial*Sex [F (2,54) = 7.113; $p = .002$; $\eta^2_p = .209$] (See Figure 4A). No effect of supplement was observed (p□ .05). The Day*Sex interaction is related to males travelling longer distance than females on DAY2 ($p = .011$). Females travelled longer distances on DAY1 compared to DAY2 ($p = .002$) and 3 ($p < .0005$) while for males, distance travelled was longer on DAY1 ($p = .003$) and 2 ($p < .0005$) compared to DAY3. On the other hand, pairwise comparisons for Day*Trial*Sex showed females to have travelled more during the first trial on DAY1 when compared with DAY2 and 3 ($p < .0005$) while males exhibited such effect between DAY2 and 3 ($p = .044$). During Trial 2, males travelled longer distance on DAY1 than on DAY2 ($p = .04$) and 3 ($p = .005$). On DAY1 and 2, females travelled longer distances during Trial 1 than Trial 2 (both $p < .05$). In sum, travelled distance was decreased over days [the longer distances being travelled on DAY1 compared to DAY2 ($p = .02$) and 3 ($p < .0005$)] and from the second compared to the first trial ($p = .024$).

3.6 Reversal Learning: Working Memory Errors

A four-way ANOVA conducted in adult rats showed significant a main effect of Day [F (2,56) = 13.818; $p < .0005$; $\eta^2_p = .33$] and an interaction of Day*Trial*Sex [F (2,56) = 3.195; $p = .049$; $\eta^2_p = .102$]. No effect of supplement was observed (p□ .05). For the Day*Trial*Sex interaction, post-hoc analyses revealed that, during Trial 1 and 2, females made more WME on DAY1 than on DAY2 and 3 (all $p < .04$). Males, on the other hand, committed more WME on DAY1 than DAY3 ($p = .022$) during Trial 2. Overall, WME decreased over days, with rats exhibiting more on DAY1 than 2 ($p = .04$) and more on DAY1 and 2 than on DAY3 (both $p < .02$). Interactions for 4-way ANOVA. (See Figure 4B).

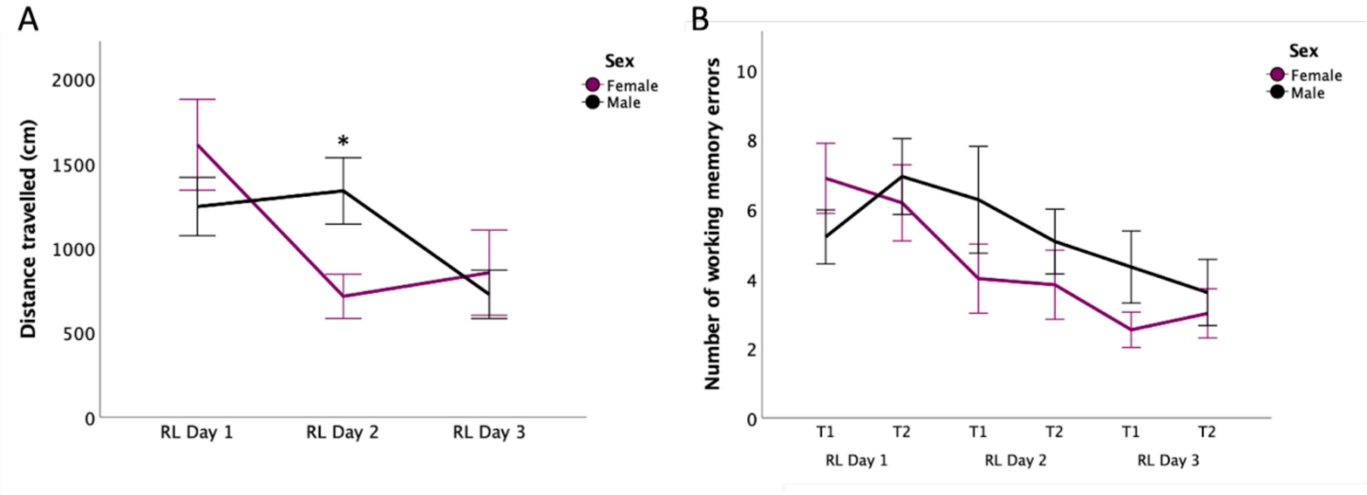

Figure 4. Reversal Learning Trials in the Barnes Maze Test (Adult rats only). Panel A shows the distance travelled (cm) for males and females during the 3 days of reversal learning. Panel B presents the number of working memory errors in male and female rats. Data are presented as mean ± S.E.M. * Indicates statistically significant difference between groups at p < .05. No effect of the supplementation was observed.

RL: Reversal learning; T1: Trial 1; T2: Trial 2

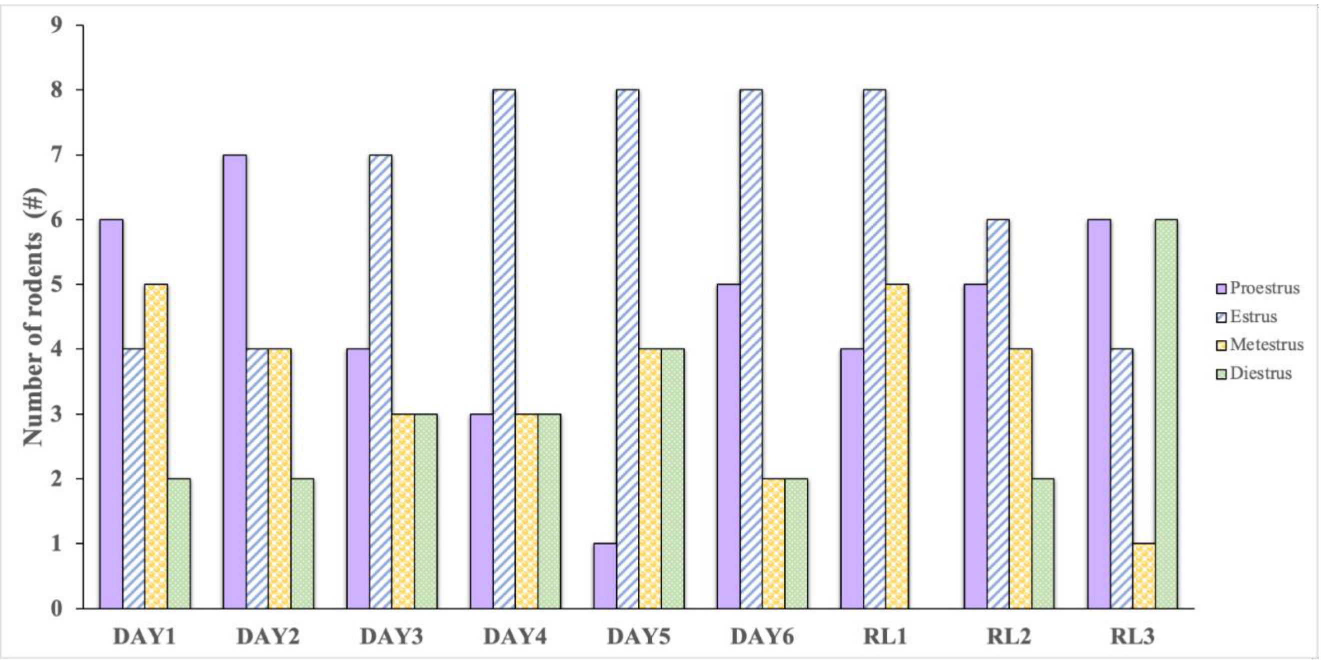

Figure 5. Estrus Cycle Stages. Vaginal cytological evaluation analysis was performed, and stages were visually identified for each day of the Barnes Maze Test, for adult rodents only (n=17).

RL: Reversal learning

4. Discussion

The present study characterized sex-specific and time-limited effects of adolescence omega 3 supplementation on visuospatial memory performance and cognitive flexibility during different developmental periods. To date, researchers have reported notable impact of omega 3 supplementation during pregnancy, adulthood or aging on cognitive function, leaving other critical developmental periods largely unassessed. Furthermore, the determination of differences in nutritional effects associated with sex, including omega 3 supplementation, has been neglected. Our study uncovered a limited impact of fish oil supplementation on visuospatial performance although it revealed age and sex as important factors regulating visuospatial memory formation and adulthood cognitive flexibility.

4.1 Contrasting visuospatial learning abilities in Adults and Adolescent rodents.

Our study indicated no significant impact of a 20-day supplementation on distance travelled or latency to escape box entry in adolescent and adult rodents. Nonetheless, our data suggest age-dependent differences regarding latency and distance travelled to escape box entry, with adolescents exhibiting significantly poorer performances on both measures. One could argue that the complexity level of the BMT may exceed the abilities of adolescent rats' hippocampal networks to process and remember such visuospatial information (Weinhard et al., 2017), explaining the observed differences between adolescents and adults. Notably, in 2018, a group of researchers developed a smaller version of the BMT aimed at assessing visuospatial learning in adolescents, better accounting for stages of brain development (McHail et al., 2018). In this modified BMT, they exposed adolescent rodents to a smaller testing area, with fewer options (8 holes rather than 16). McHail et al (2018) reported that both adults and adolescents tested in this modified BMT were able to find the escape box and learn the spatial task within a similar timeframe. Interestingly, they indicated that adolescents tended to use more efficient spatial search techniques from the second day compared to adult rodents who were able to achieve such behaviour on the first day. They concluded that the test would be appropriate for younger rats aged from PND17 to PND26 (McHail et al., 2018). In our study, the rats were several weeks older than in McHail et al.'s (2018) study, and according to Blair et al.'s (2013) findings, adolescent rodents reach similar hippocampal maturation as adults towards the end of the third postnatal week, which falls several weeks prior to the first day of testing in our own study. Additionally, Wills et al. (2013) demonstrated adult-like directional firing of grid cells from the hippocampal region linked to spatial representation. They indicated that adolescents rapidly achieve adult-like maturity of this system, often around PND28-29, sometimes even earlier. As such, adolescents most likely passed the critical period, and therefore, an incomplete hippocampal maturation might not be responsible for the age differences observed in our sample.

Contrasting the inability of the adolescents to remember the task over days, both the adolescent and adult rats significantly reduced escape latencies from Trial 1 to trial 2. This observation supports the ability of the adolescents to acquire the rule and apply it within a short presentation delay. although not being able to maintain active such learning to consolidate it and show improved performances over days Supporting these findings, Brown, and Kraemer (1998) found that adolescent rodents tend to forget faster compared to adults, and to show significant increase in latencies when testing session were irregularly presented. Together, these observations support adolescents' memory consolidation pathways involving hippocampal long-term potentiation and prefrontal cortex activation to remain functionally immature, a hypothesis consistent with their inability to perform the reversal learning task, which require prefrontal activation.

4.2 Steady Improved Working Memory Performance in Adult rats while learning difficulties persist in Adolescent Male rats.

As adults, spatial memory performances were comparable in males and females, both sexes showing reduced WME as daily testing progressed. Interestingly, while adolescent rats experienced learning difficulties as a group, this was particularly notable in male cohorts, which showed the highest numbers of WME compared to all other groups (significantly different from testing days 3-5; see Figure 2D), indicating a spatial learning deficit in adolescent males. These finding contrasts other studies showing male rodents to achieve spatial tasks more efficiently than female counterparts (Sandstorm, Kaufman et Huettel, 1998; Jonasson, 2005). Precisely, Jonasson (2005) reported male rodents to solve spatial problems quicker and perform better overall compared to females. Many studies have linked this performance gap between males and females to differences in sex hormones (Roof, 1993; McCarthy and Konkle, 2005). For instance, McCarthy and Konkle (2005) demonstrated that high secretion of androgens, such as testosterone, can lead to improved learning and memory in male rodents around PND45, which falls a few days prior to testing in our study.

Thus, the reasons pertaining to increased WME in males compared to females remain uncertain. Interestingly, this effect was particularly salient in FO-fed males (See Figure 2B). Reduced performance related to n-3 PUFAs was unexpected, research having however observed supplementation with unsaturated fat to impair performance in male but not female guinea pigs, the males showing reduced WME and latencies in a spatial memory test (radial Y-maze) (Nemeth et al. (2015). Omega 3 PUFAs are known to be differentially metabolised in male and female rodents, testosterone reducing the conversion of PUFAs following its consumption, an effect related reduced attention and social behaviour in boys (Huang et al., 1987; D’Eon et al., 2005, Tammam et al., 2016). Considering these differences, males may require more important FO concentrations than females to achieve similar benefits. In this context, a previous study from our laboratory showed that males fed the same FO concentration as that used in this study elevated anxiety-like behaviour and reduced OFT and EPM exploration (Raymond et al., unpublished). Additionally, we reported soybean oil supplementation in male rats to foster increased sociability compared FO supplementation. Notably, Teixeira et al. (2011) reported soybean oil supplement in male rodents to improve BMT performance compared to lard or hydrogenated vegetable fat supplemented groups. On a similar note, Crane and Greenwood (1987) observed that a 4-week soybean oil supplementation favours cognitive performance in male mice. These findings concur with our observations to support differential benefit from FO supplementation in male and female rodents. It must be noted that studies showing positive effects of FO on memory in humans and rodents have used longer periods of supplementation, supplementation often being offered over months (Burckhardt et al., 2016; see Singh, 2020 for a review; Fedorova et al., 2009; Prusceddu et al, 2015). For instance, long term gestational DHA supplementation has been associated with increased neurite outgrowth and synaptogenesis in infants having a positive impact on learning and memory (Sidhu and his colleagues, 2011). A limited period of FO supplementation, even performed during the critical adolescence maturation period, was not sufficient to significantly impact cognitive abilities in the male rodents.

4.3. Sex-Dependent Effect on Working Memory Errors in adults

In adult rats, our findings support sexual dimorphism manifest by a more rapid learning of the BMT's reversal learning task rules, manifest through reduced latencies and number of WME over the 3 testing days in females compared to males. Although mechanisms linked to these observations remain to be examined, research indicates that 17β-estradiol (E_2) enhances working memory performance in the Radial Arm Maze (Frick & Berger-Sweeney, 2001) and spatial memory consolidation in the Morris Water Maze (Packard & Teather, 1997). Studies further suggest rises in E_2 levels in maturing female rats. From PND28 up to PND48-75, secretion of sex hormone gradually increases to finally reach adult levels (Vetter-O'Hagen and Spear, 2012). While gonadal hormone levels were not measured, we observed that 65% of the females started the reversal learning trials in the proestrus or estrus stages (See Figure 5). As research indicates, females experience high estradiol levels at the start of the estrus cycle, a slight increase in progesterone in the proestrus and estrus phase as well as enhanced luteinizing hormones in the late proestrus (van Goethem et al.,2012; McLean et al., 2012; Biswal, 2014). These peaked levels are linked to improved memory retention and tend to promote functional connectivity of the hippocampus (Contreras et al., 2000; van Goethem et al., 2012; Ismail et Blaustein, 2013; Riordan et al., 2018). Therefore, it is plausible that elevated levels of circulatory E_2, progesterone and LT hormone in females could have positively impact on learning and cognitive flexibility in the BMT. Furthermore, facilitating effects of E_2 on spatial learning may also contribute to improved performance of adolescent females in the acquisition phase of the BMT. To confirm this, it would appear important for further studies addressing adolescents' learning to formally link E_2 concentrations in females during testing days (through monitoring or manipulation of E_2 concentrations), as well as characterize the impact of FO supplementation on E_2 concentrations and bioavailability.

4.4 Changes in Search Strategy Under Stressful Conditions in Male Rats

An interesting observation was noted on DAY5, Trial 1 for male rodents, specifically those fed with FO. A significant increase in WME was found in this specific group compared to other days and trials. This testing day was characterized by increased unforeseen noise, which could have had an acute impact on corticosterone secretion. Several studies have shown that spatial memory can be influenced by acute stress exposure (Schwabe et al, 2008; Tropp and

Markus, 2001, Gawel et al., 2019), and effect reported in several spatial tests including the Y-Maze, Radial Arm Maze and Morris Water Maze. On the other hand, it is interesting to note that learning has been shown less affected by stress in the BMT (Gawel et al, 2009), potentially creating an isolated impact of stress on WME as seen in our study. Moreover, Schwabe et al. (2008) have reported a tendency for male rodents to modify their spatial strategies following an acute or chronic stressor, an observation not visible in females. In fact, following stress, males tend to rely more on intra-maze cues compared to extra-maze ones (such as the different shape sign placed on each wall) (Schwabe et al., 2008; Bettis and Jacobs, 2009), possibly leading males to make increased WME on DAY5 initial trial.

5. Concluding Remarks

In summary, our findings indicated that, during acquisition of a Barnes Maze task, adult rodents make reduced WME, show reduced travelled distance and box entry latencies compared to adolescent counterparts. In the reversal learning trials, adult female rats showed superior performance, travelling less distance, and making reduced WME compared to male rats, suggesting improved cognitive flexibility. Although impact of dietary supplementation targeting the adolescent period had minimal impact on performance, slight behavioural changes were apparent, including opposite trends in the impact of FO supplementation on spatial memory in male and female rats. Thus, females appeared to benefit most from omega 3 supplementation than males, an effect manifest through reduced WME in FO-fed females, although supplementation for this duration did not translate into significant effects. These findings support the possibility for nutrition status during the adolescence to be one contributor to sexual dimorphism observed in behavioural responses. This can play an important role in terms of modulating responses through the synergistic actions of panoply of environmental factors.

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General Discussion

The overarching goal of this thesis was to investigate the effect of short-term nutritional supplementation and environmental conditions during the adolescence period. We designed our studies to identify changes in physiological, emotional, and cognitive functions. In this section, we will discuss the contributions of our findings and how they refined our knowledge of the impact of diet and environment in shaping responses of male and female rats.

The first study was designed to test the effects of distinct dietary supplements in male rats, during the targeted adolescence developmental period. This study aimed to determine a feeding procedure enabling to best evaluate the impact of the supplementation itself on behaviour and physiological response, with a minimal impact of other external variables (i.e., stress induced by the procedure). This study enabled to compare the influence of three different supplements delivered using two methods on behavioural and physiological stress response. Assessing the impact of the three supplements, our findings indicated the high fat Hydrogenated Vegetable Fat (HVF) [18% of saturated fat and 0% of trans fat in 12g of HVF] supplementation to promote anxiety-like behaviour assessed in the OFT and EPM, an effect that was observed in FO and CSO supplemented male rats. Notably, upon the last supplementation day, FO-fed male rats tended to show reduced CORT secretion, indicating that 21-day menhaden fish oil supplementation could positively influence stress reactivity in male rodents. Characterization of the effects of the delivery methods supported oral gavage to be less stressful compared to restricted feeding for adolescent rats, independently of the provided supplement. Interestingly, FO supplementation attenuated the impact of restricted feeding on CORT secretion on the last supplementation day, further highlighting a possible role of n-3 PUFA enriched diet at adolescence in regulating anxiety. Together, findings from this study confirmed distinctive effects of delivery methods and helped informing our group and other researchers on the selection of dietary supplementation regimen minimally impacting adolescent rodents' stress reactivity. Moreover, this first study supported potential benefits from short-term dietary supplementation targeting the early/mid adolescent period in male rodents.

The main objective of the second study aimed at a better understanding of the influence of a regulation of omega 3 and 6 dietary intake on brain fatty acids profile. More specifically, we provided male and female rats soybean or fish oil supplementation during the adolescent period and explore the immediate and delayed effects on the expression of essential fatty acids in brain tissues [with an emphasis on ensuing alteration of EPA and DHA brain concentrations]. Our findings demonstrated that notwithstanding the diet, DHA levels were higher in females compared to males, both for adolescent and adult rats. Most interestingly, contrary to the impact of both supplementations on DHA levels in adolescent females, males' DHA concentrations immediately post supplementation were significantly reduced (by 50%) compared to females when supplemented a DHA-rich FO supplementation. Furthermore, in the adolescent males the CSO diet showed an inability to significantly alter brain DHA concentrations. Interestingly, upon reaching adulthood, male and female rats showed similar DHA brain concentrations, adolescent FO only having a marginal benefit over CSO supplementation at this delayed interval.

Examination of the literature opened some discussion avenues for the sexually dimorphic EFAs expression profiles in brain tissues of adolescent rats. Meta-analyses have confirmed sex differences in the DHA status in plasma lipids, phospholipids, and erythrocytes (Lohner et al., 2013). However, a placebo-controlled study assessing the effects of 8-week ALA supplementation followed by 8-week wash-out period in metabolically healthy men and women revealed similar supplementation effects on fatty acid composition of serum phospholipids and erythrocytes (Burak et al., 2017). To our knowledge, our study is the first to assess and demonstrate sex-specific effects of short-term dietary supplementation occurring during the adolescence period on fatty acid brain concentrations. Although mechanisms need to be determined, the biosynthesis of long-chain PUFA from essential fatty acid consumption is in part regulated by gonadal hormones (Child et al., 2008; Schuchard et al, 2010; Gonzalez-Soto et al., 2021). In this context, testosterone’s mitigating effects on metabolic activities could contribute to age-related changes in fatty acid brain content (Gitay et al., 2004; Childs et al., 2008; Schuchardt et al., 2010). Additionally, this study revealed that CSO-supplementation in adolescent females translated into higher DHA levels, a singular effect that was not observed in male counterparts. This needs to be further characterized, but as discussed in study 2 could be related to maturation of the gonads leading to raising estradiol levels combined to low testosterone concentrations, which acted to favor DHA conversion rates in females (Alessandri et al., 2008; Decsi & Kennedy, 2011). Additional studies are required to evaluate other endogenous mechanisms that could be at play. It must be noted that reduced number of brain samples in certain groups prevent firm conclusions, as analysis was completed with a reduced sample size for adult FO supplemented male rats. While we understand the limitations, the results are intriguing and in line with human studies showing increased benefits from adolescent omega-3 supplementation in girls versus boys (Lassek & Gaulin, 2011). These observations are providing a compelling and informative steppingstone for our future research projects characterizing the impact of environmental conditions, including fish oil supplementation on behavioural responses observed in male and female adolescent rats.

These two studies lead to a third one aimed at characterizing sex-specific effects of a 20-day n-3 PUFA supplementation during the adolescence period on socioemotional responses, coping strategies upon stress exposure, corticosterone secretion and neurochemical responses. Furthermore, our study determined effects of combined exposure to environmental factors, which we believed likely to induce synergistic actions. This question was examined by combining dietary supplementation (CSO or FO) and housing, enabling to investigate environmental enrichment versus standard housing conditions. We provided daily supplementation with soybean (CSO) or menhaden fish (FO) oils from PND28-47, and environmental enrichment (EE) or standard pair housing (SC) from PND28 to PND58. Then, both male and female rodents from all experimental conditions were exposed to repeated sessions of FST stress and the first FST session served to evaluate coping strategies (i.e., energy conservation). Assessment of swimming patterns in rats following an acute stressor (first 15-min swimming session) enabled us to interpret findings using a different angle, and we considered immobility in this context as a strategy promoting energy conservation (Kloet and Molendijk, 2017; Campus et al., 2015). Following repeated exposure to the FST, we compared the groups on measures of social approach/social recognition, and fear avoidance to determine if diet and housing conditions responses would differentially impact these discrete responses. Our findings revealed a sexually dimorphic influence of fish oil supplementation on coping mechanisms, marked by increased immobility (floating) in FO/RC females compared to all other groups. Interestingly, although males did not use floating, they minimized energy consumption using tail support, significantly more than all female groups, except the CSO/RC female group which also used tail support. Another notable effect was related to increased climbing in CSO/RC males, an active coping strategy. Our results provided interesting differences of stress coping strategies linked to living conditions. If stress reactivity has been proposed to enhance consolidation of the FST experience and be associated with increased immobility, it is interesting to note that FO/RC females showed the highest basal CORT secretion upon repeated gavage sessions as adolescents. As CORT secretion was not assessed in relation to FST sessions, it is not possible to validate this hypothesis. When GR-ir was measured at end of the study, we could not find any differences in GR-ir at the CA1 between the groups and although there were group differences at the CA3, these are difficult to directly reconcile with observations in the FST days earlier.

For anxiety-like behaviour, we observed that CSO/EE males tend to explore more the central anxiogenic zones in the OFT. On the other hand, CSO fed rodent, both male and female, increase frequency in the open arms in the EPM. Considering proposed beneficial effects of FO reported under different supplementation regimen and other developmental periods (Ginty & Conklin, 2015; Rice and al., 2015), these observations were certainly interesting. The fact that CSO-fed males ventured more in the anxiogenic zones could in part be attributable to soybean's effects on serotonin levels (Choi et al., 2009; Friedman et al., 2018). However, considering that groups did not significantly differ in the time spent in the open arms, these effects could also be related to other neurochemical networks regulating locomotor exploration. For instance, enhanced exploration of the mazes in CSO fed rodents could be associated with increased dopamine as stated in research by Teixeira et al. (2012). In fact, while soybean was demonstrated to heighten serotonin levels (Choi et al., 2008), it was also shown to enhance dopamine (Yimit et al., 2012) and could have favoured exploratory behaviour.

One great interest that we had in manipulating environmental conditions was related the well characterized role of play behaviour at the adolescence on ensuing behaviour and brain responses. In this spirit, we assessed sex-specific changes on adulthood sociability and social recognition. Our findings supported beneficial impact of n-3 PUFA supplementation and EE on sociability in female rodents, with FO and EE exposed females displaying increased socioexploratory behaviours with a familiar congener. On the other hand, males exposed to the CSO showed heightened interest toward a stranger then a familiar congener. These findings further support soybean to be more influential in regulating male behaviour, with than fish oil having less favourable impact in males. One can reconcile the CSO-related effects observed in the EPM and SIT tests in male rats, both being marked by reduced inhibition to explore novelty. Although serotonin secretion promoted through soybean supplementation could play a role, regulation of other factors such as hormones could be involved. Finally, upon assessment of conditioned fear, RC-housed CSO males showed increased latencies to re-enter the aversive arm, making increased risk assessments, a response prevented by EE housing.

The fourth and final study determined the immediate and delayed sex-related effects of adolescent dietary supplementation on visuospatial performance, and further explored adulthood cognitive flexibility. One interesting observation was related to maturation of cognitive circuitries rendering the task quite challenging for the adolescent rodents, which made significantly more WME, required more trials to learn the placement of the escape box and struggled completing the task. Nonetheless, adolescent rats improved from same day trial exposure (T1 vs T2), supporting learning acquisition but an incapacity to consolidate and use this experience to perform better on following testing days. The ongoing maturation of the meso-corticolimbic brain circuitry, including prefrontal and hippocampal bidirectional projections, likely contributed to additional learning challenges in adolescents. Importantly, our findings showed supplementation to exert a minimal impact on performance in the Barnes Maze Test, in both adolescent and adult rats. Nonetheless, our study pointed to interesting results, which caught our attention. Indeed, on specific days, FO-supplemented male rats showed increased working memory errors compared to FO-fed females and CSO-fed males. On the other hand, the performance of FO- supplemented females improved as testing progressed. These alterations in spatial memory performance could indicate a 20-day supplementation period to be too short to induce tangible effects. In fact, most studies evaluating dietary supplements have reported changes in cognitive performance associated with much longer ingestion periods (8-12 weeks are commonly used; ~~(~~Burckhardt et al., 2016; Singh, 2020; Pusceddu et al, 2015) or evaluated changes over multiple generations (Lassek et al., 2011; Karr et al., 2012; Fedorova et al., 2009). Finally, assessing cognitive flexibility in adults, our findings revealed statistical differences between males and females' performances in the reversal task. The females made less WME and took reduced time to complete the reversal learning sessions compared to males. We proposed oestradiol, luteinizing hormone and progesterone to promote cognitive flexibility in females by enhancing memory consolidation and recall. In sum, this study indicated that notwithstanding supplementation types, adolescent rats although able to use visuospatial cues to find the escape box more rapidly in T2 than T1 on a precise testing day, they failed to show improved performance over the course of a 6-day session, supporting an inability to properly consolidate learning experiences.

4.1 Overall beneficial influence of n-3 PUFA in female rodents

The set of findings of this thesis revealed complex sex differences that sometimes can be hard to interpret. This is in part due to increased number of studies having only assessed male rodents, resulting in numerous variables being not assessed in females. However, studies in humans and animals that have included both sexes support brain and behavioural responses to present many differences (McCormick, 2011; see review by Blaustein, Ismail and Holder, 2016; McEwen and Milner, 2017) including in studies addressing responses to nutrition (Chen et al., 2020; Child, 2020). Regarding findings from our studies assessing adolescent supplementation, short term n-3 PUFA supplementation induced sex-specific changes, which overall led to more positive outcomes in female rodents. One important difference that was noted from assessment of DHA/AA/ALA brain concentrations in adolescent male and female rats is related to a significantly reduced ability for adolescent males to metabolise nutrient, which resulted in significantly reduced EFAs levels in brains assessed immediately post supplementation. In contrast, FO- and CSO- supplementation in females both resulted in steady elevated brain DHA concentrations in the adolescent and adulthood periods (Study 2). Behaviourally, FO-supplemented females showed a preference for passive coping in the FST (Study 3), showed heightened sociability (Study 3), reduce GR-ir in the CA3 region of the brain (when combined with RC) (Study 3), and sporadically reduce working memory errors (Study 4). As stated in the thesis introduction, several studies identified factors promoting optimized nutrient metabolism in females, leading to differential DHA content in brain tissues, plasma and erythrocytes compared to that observed in males (Childs, 2020). Both female rodents (Child et al., 2012) and humans (Lohner et al., 2013) have shown higher levels of DHA in their bloodstream compared to males/men counterparts.

Future studies evolving from our observations shall implicate more precise assessment of gonadal hormone levels during the targeted dietary period. Establishing correlations between the maturational statuses of gonadal hormone secretion in the male and female adolescents and current results, including stress response, cognitive performance and DHA concentration, would provide rich and insightful background data on the sex-dependent impact of n-3 PUFA. Additionally, based on our current data on DHA concentrations, whole brain sample should be analysed at multiple standpoints. An evaluation of gonadal maturation and associated hormone production related to the targeted adolescent period (assessing the pre-, mid-, and late-adolescence period) would enable to better examine the relationship of hormonal secretion with DHA brain levels. It could also inform as to the period where DHA levels appear to stabilize over the passage from adolescence to adulthood. By extent, this could provide a better-tailored omega-3 supplementation based on dietary absorption. Furthermore, such data would also pave the route to assessment of more specific mechanisms that may contribute to sexual dimorphism in FO supplementation-related behavioural responses.

As we overview our results, our study provided insights on the beneficial impact of n-3 PUFA supplementation in females, even when exposed to a short-term adolescence-targeted diet. Our studies helped appreciate how, even when restricted in time, environmental conditions can induce enduring impact. Our results also contribute other studies having explored longer-term dietary supplementation, sometimes expanding over multiple generations.

4.2 General positive impact of soybean over fish oil supplementation in male rats

As data collection progressed, we observed that male rats displayed quite different responses to fish oil supplementation compared to females, an effect we partially, but not fully anticipated from available literature on this subject. In fact, CSO-fed males tended to react more positively in certain behavioural tests than the FO-fed counterparts. For instance, CSO-fed males ventured more into the anxiogenic spaces in the EPM and OFT compared to HVF fed rodents (Study 1) and showed decreased anxiety-like behaviour in the OFT following CSO/EE exposure (Study 3). CSO also enhanced sociability and heightened social recognition in the SIT (Study 3). When housed in RC (but not EE), the CSO fed males also showed significantly increased climbing compared to all other groups. These interesting results may support soybean oil and environment conditions to differentially regulate brain responses according to sexes and have beneficial impact on emotionality in males.

Our observations are consistent with that of others showing a 4-week diet supplementation of soybean oil to increase sociability (Choi et al. 2009), favours cognition and serotonin bioavailability (Crane & Greenwood, 1987). Using insights provided from assessment of fatty acids in brain tissues performed in Study 2, we could hypothesize differential response profiles in male and female rats exposed to CSO or to FO to somewhat be related to differential translation of nutrient into brain fatty acid, which appeared to drastically affect DHA content in adolescent male and female brains. As was observed in Study 2, adolescent males exposed to CSO or FO during 20-days had significantly lower DHA levels when we collected tissue immediately post supplementation. This could have created the discrepancy in behaviour and biomolecular results observed in the CSO versus FO-fed male rats in Study 3. A clear shift in fatty acid brain composition was observed in male rats as they reached adulthood. While testosterone bioavailability has been presumed to interfere with the metabolization of dietary n-3 PUFA into DHA (Schuchardt et al, 2010; Giltay et al., 2004), as adults, male rats showed equivalent metabolic capacities to extract fatty acid content from nutritional sources as suggested through comparable adulthood DHA brain concentrations in both sexes. Our observations are consistent with studies that have demonstrated drastic increases in DHA brain levels in male rats from 40- 50 days of age (Zanato et al.,1994; Zemunik et al., 2001). The role of such difference in fatty acid metabolization is an interesting avenue to explore, considering more consistent and beneficial impact of FO supplementation in female rodents [from passive coping strategies to increased sociability and heightened spatial working memory performance].

Additionally, we observe that CSO supplementation (when combined with enriched environment) in males was associated with reduced GR-ir in CA3 region of the brain in males (and females), an effect that may underlay differential regulation of emotional behaviour. As stated by Han et al. (2017), reduced GR level in the hippocampus can be linked to resilient behaviour (Han et al., 2017), often presented as an adaptative process mediating stress coping strategies (Charney, 2004; Feder et al., 2009). Likewise, additional beneficial impact of CSO was observed in male rodents in the Barnes Maze test (Study 4). For instance, we observed that CSO-supplemented male rodents were able to reduce their WME in the acquisition phase compared to male fed with FO.

While not always consistent, effects in females were globally more readily observed. Our studies lack examination of various endogenous sex-specific factors, including the ones mentioned (i.e., gonadal hormones), which could contribute to our observations. Future studies need to decipher determinant factors supporting sex differences in behavioural and brain responses observed in male rats, immediately and at remote intervals, including housing conditions [discussed in the following section]. For instance, further investigation of altered serotonin and dopamine levels in relation to dietary supplementation could help ascertain a proposed relationship between soybean supplementation in males and increased activity in the FST (climbing) and in the EPM (increased frequencies of arm entries), reduced anxiety-like behaviour in the OFT and heightened cognitive performance. Overall, our results regarding supplementation effects suggest mixed response patterns in male and female rats, highlighting complex interplay. Several behavioural, cognitive, and physiological effects supported short-term adolescence targeted FO supplementation to be itself insufficient to positively benefit male rats.

4.3 Divided effects of Enriched Environment in female and male rodents

Our global aim being to better understand how extrinsic factors influence the adolescent brain and subsequent behavioural responses, we believed important to consider synergic effects of environmental factors. We studied two that appeared central to proper development, diet and environmental enrichment. As they both shown influences on cognition, sociability, and stress reactivity, combining diet and environment in our study would likely exert decisive impact. In general, both females and males appeared to largely benefit from the EE, although exceptions were noted.

Results indicate that when exposed to both conditions (FO/EE), females tend to increase social behaviour in the SIT and expressed attenuated fear responses in the Y Maze (Study 3). As previous studies supported, adolescence exposure to EE facilitates social behaviour through social play and mutual grooming, increases oxytocin levels and promotes cognitive performance (Neal et al., 2018; Kempermann, 2019). In addition, Whitaker et al. (2016) showed combined EE and exercise to increase sociability in females. Beneficial impact of EE has also been reflected in females through changes in stress coping styles, EE exposure promoting a more active coping style in females, shown to 'switch in' social engagement with others. To this effect, Eisenberg and al. (1995) have shown low physiological stimulation/reactivity to be associated with lower sociability. In contrast, Schaack et al. (2021) recently highlighted that stress exposure, leading to increased physiological response, tend to impair social recognition and behaviour, especially in females. Such findings are insightful in relation to our own experimental observations. For instance, in females, exposure to EE could have created an environmental milieu prone to increase familiar social seeking/encounters rather than new interactions, considering that EE housing was associated with modest reductions in corticosterone secretion profile compared to that observed in females exposed to RC. However, it must be noted that females that favoured passive coping strategies were the RC- and not the EE-housed FO females (Study 3). Although mechanisms need to be confirmed, this behavioural strategy in RC females could be related to slightly elevated corticosterone level noted at a younger age in this group, which if associated with increased FST-induced CORT secretion may have later favouring passive coping mechanism, though enhanced consolidation of the FST experience. Such contention is supported by de Kloet et Molendijk's (2016) demonstration of an association between increased CORT secretion and increased FST immobility, which they proposed to be associated with increased resilience.

It should be noted that the literature has supported mitigated results concerning beneficial effects of EE exposure in female rodents. For instance, while multiple studies identified EE as beneficial, others have reported enhanced baseline CORT in females exposed to grouped versus isolated housing (Martin & Brown, 2010; for a review see Girbovan & Plamondon, 2013). A review on this topic by Girbovan and Plamondon (2013) demonstrated several conflicting results of EE on anxiety-like behaviour and stress reactivity in females. Our findings further contribute to observed difficulties in extracting consistent observations; we found no significant impact of EE in the OFT or EPM in female rodents (Study 3). Considering the difficulties in assessing the sole impact of EE exposure in females, it is plausible that combined environmental conditions in our study accentuated the continuum of possible effects. Interestingly, behavioural responses in male rats demonstrated increased exploration and decreased anxiety like behaviour in the OFT in CSO/EE exposed males, which support synergistic actions fostering benefits in male rats. We also observed EE in CSO male and female rats to reduced CA3- GR-ir expression, potentially promoting consolidation, another positive impact of EE exposure (de Quervain et al., 2009; Sampedro-Piquero et al., 2014; Ashokan et al., 2018).

The complex response profiles that have arisen from our studies command further investigations. As an example, it would appear interesting to investigate EE-related alterations in oxytocin and vasopressin, these two hormonal signals being strongly associated with social recognition, non-social anxiety, and aggression (Veenema et al., 2012; Paul et al., 2014). This could provide important insights on the sex-dependent changes that were apparent following 31 days of enriched environment exposure.

4.4. Short term n-3 PUFA supplementation exerts a small influence on visuospatial performance and cognitive flexibility.

A large cohort of USA children aged 6–16 years of age revealed cognitive benefits of higher n-3 PUFA consumption in both boys and girls, but the effects were twice as prominent in girls (Lassek and Gaulin, 2011). At present however, there is a fair number of inconsistencies between studies examining the effects n-3 PUFA supplementation on cognitive performance, especially in humans (Weiser et al., 2016; Brainard et al., 2020). In rodents, the impact of PUFA supplements has mainly been demonstrated in PUFA-deficient or memory-impaired conditions (aging, neurodegenerative models, etc.) (Butler et al., 2021; Wen et al., 2021). Thus, it is an interesting question to evaluate the impact of PUFA supplementation in the maturing healthy brain in both sexes. We assessed spatial memory performance in adolescent FO-supplemented male and female rats using the Barnes Maze Test immediately post supplementation and during adulthood. While a selective beneficial impact of diet on visuospatial performance was observed in adult females, our results could not demonstrate this pattern to remain consistent throughout testing trials. Adult FO-fed females made fewer WME compared to FO- and CSO-fed males. Although, limited to certain days, observable changes using such a limited supplementation period remains an interesting observation. Additionally, during the reversal learning aimed at testing cognitive flexibility, females made significantly less WME errors and learned the task faster compared to males, most likely due to heightened oestradiol and progesterone levels during testing.

Another interesting difference in performance was related to age at testing, the adolescent rodents struggling more to find the escape box compared to adults, and this regardless of diet or sex. Together, our findings could not support a decisive impact of n-3 PUFA supplementation on cognitive performance. Omega-3 supplementation has been shown associated with increased IQ score, school performance, physical mobility in children (Helland et al., 2003; Kidd, 2007; Richardson et al., 2012; Gharami et al., 2015). In rodents, studies showed long-term PUFA exposure to benefit learning and memory (Karr et al, 2011). Importantly, several studies have reported these changes following extended periods of supplementation. Importantly, literature often reflects on how omega-3 fatty acid supplementation may exceedingly benefit when provided early during ontogeny and how the positive effect of n-3 PUFA may be transmitted in utero from mother to progenies (Daniels et al., 2004; Sidhu et al, 2011; Sidhu et al., 2016). As for our study, the exposure to dietary omega-3 was limited in time and insufficient to achieve consistent statistical results for each day. Consequently, a longer dietary supplementation could provide better understandings of overall impact, especially for CSO- supplementation in males as they benefitted from the soybean supplementation rather than n-3, as it's typically observed. In conclusion, short-term supplementation of n-3 PUFA during the adolescence period, is insufficient to promote enhance visuospatial performance or cognitive flexibility throughout the testing phase but display slight improvement in females.

4.5 Final thoughts

The compendium of findings that composed this thesis clearly support the importance of studying convergent environmental factors to better illustrate life situations. However, such a goal brings with it the complexity of possible interactions between factors. Our studies indicate that there is not a particular combination that appeared beneficial for all, and as life as already informed us, no singular solution will act on all to reduce stress, improve cognition and modify behavioural responses. Only a combination of multiple factors can promote healthier and adapted behaviour. Our studies showed evidence that a short term 3-weeks supplementation of fish oil supplementation during the adolescence period can create beneficial impact on stress reactivity, coping, sociability and working memory, and this, mostly for female rodents. The trends for positive effects observed in different tests and encourage to pursue investigation, as well as clear differences in responses associated with sex, which fully support male and female brains to respond differentially to similar environmental conditions. This is certainly a fascinating constatation, which continues to emerge as studies assessing sex differences are spreading. Furthermore, as much as literature supports the positive effect of omega-3 supplementation on stress reactivity and cognition, there is limited information on the adolescence period. However, studies have shown this critical maturation period to be highly responsive to environmental changes, which have decisive effects in modelling neurophysiological and behavioural responses in a sex-dimorphic manner (Burke et al., 2017; Kane et al., 2017; Calabro et al., 2020; Bangasser & Cuarenta, 2021). At this stage, research would benefit from exploring discrete neurochemical pathways mediating sex differences. It would also be extremely interesting to use this critical and sensitive developmental period as a tool to better understand epigenetic changes related to prolonged or generational exposure to discrete diet and environmental conditions. Together, the present work has contributed to increase knowledge on the impact of changes in environmental conditions occurring during adolescence. Our findings complement that of other studies in rodents and humans addressing persisting effects of childhood and/or adolescent exposure to stress or life adversity, and the vulnerability to stress exposure and development of emotional disorders described in adulthood.

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