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# Effect of Prenatal Tryptophan Diet On Mouse ASD-like Behavior

Dissertação no âmbito do Mestrado em Química Medicinal, orientada pela Doutora Joana Gonçalves e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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# LIST OF ABREVIATIONS

| | |
|---|---|
| ADHD | Attention deficit/hyperactivity disorder |
| AKT | Protein kinase b |
| ANOVA | Analysis of variance |
| ASD | Autism spectrum disorder |
| BBB | Blood-brain barrier |
| cAMP | Cyclic adenosine monophosphate |
| CTR | Control diet |
| CNS | Central nervous system |
| DNA | Deoxyribonucleic acid |
| dB | Decibel |
| EDTA | Ethylenediamine tetraacetic acid |
| ELISA | Enzyme-linked immunoassay |
| ENS | Enteric nervous system |
| EPM | Elevated plus maze |
| FMT | Fecal microbiota transplantation |
| G | Gestational day |
| GABA | Gamma-aminobutyric acid |
| GDP | Guanosine diphosphate |
| GEFs | Guanine nucleotide exchange factors |
| GTP | Guanosine triphosphate |
| HMO | Human milk oligosaccharide |
| HPA | Hypothalamic-pituitary-adrenal |
| IDO | Indoleamine 2,3-dioxygenase |
| KYNA | Kynurenic acid |
| LNAA | Large neutral amino acids |
| MAPK | Mitogen-activated protein kinase |

| | |
|---|---|
| MGB | Microbiota-gut-brain |
| mTOR | Mammalian target of rapamycin |
| NF1 | Neurofibramatosis type 1 |
| P | Postnatal day |
| PCR | Polymerase chain reaction |
| PKCζ | Protein kinase C-ζ |
| QUIN | Quinolinic acid |
| Ras | Rat sarcoma |
| RNA | Ribonucleic acid |
| RTKs | Receptor tyrosine kinases |
| SCFAs | Short-chain fatty acid |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of the mean |
| TDO | Tryptophan 2,3-dioxygenase |
| TE | Tris-ethylenediamine tetraacetic acid |
| TPH | Tryptophan hydroxylase |
| Trp | Tryptophan |
| TRP+ | Tryptophan-rich diet |
| USVs | Ultrasonic vocalizations |
| WT | Wild-type |
| 5-HIAA | 5-hydroxyindole acetic acid |
| 5-HIA | 5-hydroxyindole acetaldehyde |
| 5-HT | 5-hydroxytryptamine |
| 5-HTP | 5-hydroxytryptophan |

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# RESUMO

O Transtorno do Espectro do Autismo (TEA) é um distúrbio do neurodesenvolvimento caracterizado por défices na capacidade de socialização, comunicação e exibição de interesses restritos e comportamentos repetitivos, observados na primeira infância.

Devido ao facto de os indivíduos com TEA apresentarem uma elevada prevalência de distúrbios gastrointestinais, foi colocada a hipótese de uma possível ligação entre a disbiose da microbiota intestinal e a modulação da função cerebral e do comportamento nos sintomas e na gravidade desta perturbação do neurodesenvolvimento.

Considerando o eixo microbiota intestino-cérebro (MGB), esta tese de mestrado tem como objetivo investigar o efeito de uma dieta enriquecida em triptofano (Trp) num modelo animal caracterizado com TEA. É também importante compreender o efeito desta dieta em indivíduos saudáveis. O triptofano é um aminoácido essencial obtido apenas através da dieta e é o único precursor da serotonina (5-HT), um neurotransmissor crucial para a emoção e cognição. Com isso, este aminoácido é uma chave no eixo MGB. Além disso, tem sido relatado em vários estudos a deficiência de Trp e níveis periféricos elevados de serotonina em indivíduos com TEA.

Este estudo longitudinal foi dividido em 2 hipóteses experimentais. A primeira foca-se na importância do Trp durante a gestação em fêmeas saudáveis, ou seja, sem nenhuma mutação associada ao TEA. Para isso, utilizou-se murganhos C57BL6/J e formou-se 2 grupos experimentais: mães *wild-type* (WT, estirpe selvagem) com dieta de controlo (CTR), mães WT com dieta enriquecida em Trp (TRP+). Dado que a disponibilidade do Trp, na gestação em fêmeas com mutação associada ao TEA, é crucial para a sintomatologia e severidade do TEA na descendência. Assim, utilizou-se um modelo animal estabelecido de TEA, murganhos com neurofibromatose tipo 1 (NF1), que apresentam uma mutação no gene *Nf1*, e formou-se 2 grupos experimentais: mães $Nf1^{+/-}$ com dieta CTR e mães $Nf1^{+/-}$ com dieta TRP+. Em ambos os casos, foram efetuados testes maternos assim como testes comportamento na descendência na infância e na idade juvenil. Os níveis de Trp, serotonina e quinurenina no hipotálamo foram medidos para compreender como os sistemas moleculares podem estar correlacionados com os resultados comportamentais observados.

Curiosamente, os nossos resultados revelaram que uma dieta suplementada com Trp tem efeitos comportamentais distintos na descendência de mães WT, ou seja,

em indivíduos neurotípicos, ou na descendência de mães com mutação associada ao TEA. Assim observamos, que animais WT apresentam dificuldades de aprendizagem e défices de memória assim como um aumento no comportamento restrito/repetitivo, principalmente nos machos. Por outro lado, murganhos *Nf1*$^{+/-}$ mostram um melhor neurodesenvolvimento neonatal, aumento na interação social, diminuição de comportamentos repetitivos e restritos, bem como melhor performance de aprendizagem e memória.

Embora, uma dieta suplementada com Trp possa ser perigosa para indivíduos saudáveis que têm o metabolismo deste aminoácido regulado, este estudo demonstrou que a mesma poderá ser uma abordagem promissora no início da vida para reduzir/melhorar a gravidade dos sintomas de TEA.

Globalmente, este estudo realça a importância de uma dieta pré-natal adequada para a regulação do metabolismo da Trp e pode contribuir para aliviar/prevenir os sintomas e/ou a sua gravidade nas doenças do neurodesenvolvimento.

**Palavras-chave** | Transtorno do espetro do autismo; Neurofibromatose tipo 1; Triptofano; Serotonina; Suplementação de triptofano; Dieta pré-natal.

# ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by deficits in the ability to socialize, communicate and exhibit restricted interests and repetitive behaviors, observed in early childhood.

Due to the fact that individuals with ASD have a high prevalence of gastrointestinal disorders, it has been hypothesised that there is a possible link between gut microbiota dysbiosis and the modulation of brain function and behavior in the symptoms and severity of this neurodevelopmental disorder.

Considering the microbiota-gut-brain (MGB) axis, this master's thesis aims to investigate the effect of a diet enriched in tryptophan (Trp) in an animal model characterised by ASD. It is also important to understand the effect of this diet on healthy individuals. Trp is an essential amino acid obtained only through diet and is the only precursor of serotonin (5-HT), a neurotransmitter crucial for emotion and cognition. As such, this amino acid is a key player in the MGB axis. In addition, several studies have reported Trp deficiency and elevated peripheral serotonin levels in individuals with ASD.

This longitudinal study was divided into 2 experimental hypotheses. The first focuses on the importance of Trp during pregnancy in healthy females, i.e. without any mutation associated with ASD. For this purpose, C57BL6/J mice were used and 2 experimental groups were formed: *wild-type* (WT) mothers with a control diet (CTR), WT mothers with a diet enriched in Trp (TRP+). Since the availability of Trp during gestation in females with a mutation associated with ASD is crucial for the symptomatology and severity of ASD in the offspring. Therefore, an established animal model of ASD was used, mice with neurofibromatosis type 1 (NF1), which have a mutation in the *Nf1* gene, and 2 experimental groups were formed: $Nf1^{+/-}$ dams on a CTR diet and $Nf1^{+/-}$ dams on a TRP+ diet. In both cases, maternal tests were performed as well as behavioral tests on the offspring in early life and at juvenile age. The levels of Trp, serotonin and kynurenine in the hypothalamus were measured to understand how the molecular systems might be correlated with the behavioral results observed.

Interestingly, our results revealed that a diet supplemented with Trp has different behavioral effects on the offspring of WT dams, i.e. neurotypical individuals, and on the offspring of dams with the mutation associated with ASD. Thus, we observed that WT animals show learning difficulties and impairment memory as well as an increase in restricted/repetitive behavior, especially in males. On the other hand, $Nf1^{+/-}$ mice show better neonatal neurodevelopment, increased social interaction, decreased repetitive and restricted behavior, as well as better learning and memory performance.

Although a Trp-supplemented diet could be harmful for healthy individuals whose metabolism of this amino acid is regulated, this study has shown that it could be a promising approach early in life to reduce/improve the severity of ASD symptoms.

Overall, this study emphasizes the importance of an adequate prenatal diet for the regulation of Trp metabolism and may contribute to alleviating/preventing symptoms and/or their severity in neurodevelopmental disorders.

**Keywords** | Autism spectrum disorder; Neurofibromatosis type 1; Tryptophan; Serotonin; Tryptophan supplementation; Prenatal diet.

# CHAPTER 1 | **STATE OF THE ART**

## 1.1. AUTISM SPECTRUM DISORDER

### 1.1.1. Autism spectrum disorder: symptoms and therapies

Autism spectrum disorder (ASD) is a set of heterogeneous neurodevelopmental conditions characterized by deficits in social communication, social interaction and unusually restricted and repetitive patterns of behavior, interests, or activities presented during early periods of development that negatively impact social, occupational, or other domains of their daily lives (American Psychiatric Association, 2013) (Figure 1).

These core symptoms are characterized by affected social and non-social communication, such as speech delays, poor diction, monotony in speech, poor eye contact, difficult in evaluating facial expressions/gestures, difficulties in understanding the conversation overall and, finally, repetitive physical behaviors, such as hand flapping, repetitive speech or use of the same object frequently (Hyman et al., 2020).

The Center for disease Control and Prevention (2019) estimated that 1 in 44 children is diagnosed with ASD and affects 4 times more males than females. However, this ratio has been decreasing to 3:1 due to a sex bias in the criteria for the diagnostic of ASD (Figure 1). It has been noticed that some girls who have severe autistic traits fail to meet diagnostic criteria for this disorder because there is a lack sensitivity to the female phenotype (Loomes et al., 2017). This difference in the ratio between the genders suggests that genes that participate in sexual development and/or sex hormones, particularly testosterone, may modulate the effects of genetic variations in the autistic phenotype (Werling & Geschwind, 2013).

The etiology is very complex and heterogeneous, there are several causes that increase susceptibility and influence the development and prognosis of ASD (Figure 1). One of them is genetic anomalies that include genes involved in brain development (Yoon et al., 2020). Altered epigenetic mechanisms such as abnormal DNA methylation, abnormal histone modification and deregulated micro-RNAs, affecting synaptic function are also a key contributor for the development of this disease (Hodges et al., 2020). Finally, environment factors also mediate ASD etiology while exposed to heavy metals and air pollution during neurodevelopment (Fett-Conte et al., 2015), as well as in utero exposure to medications and others prenatal factors (short interpregnancy interval, multiple gestation, maternal obesity, gestational bleeding, advanced parental age), maternal infection and immune activation (Hyman et al., 2020; Landrigan, 2010).

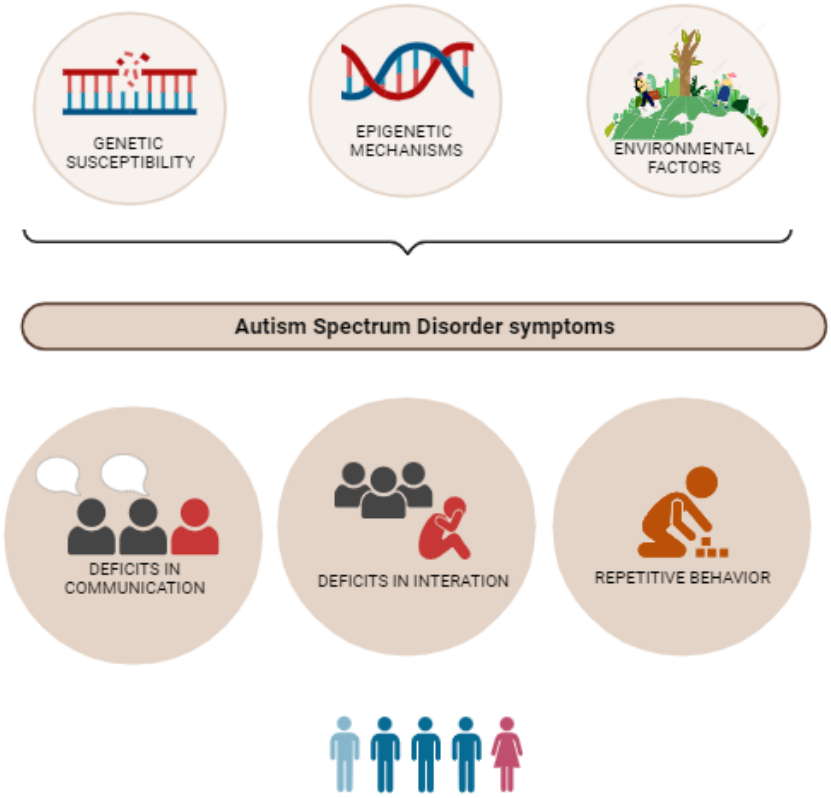



**Figure 1 | Autism spectrum disorder's main symptoms, causes and ratio**
Autism spectrum disorder (ASD) is a neurodevelopmental disorder and it seems to be caused by vary factors such as characterized abnormal genetic and epigenetic mechanisms, and/or from environmental risk factors. This leads to deficits in social interaction and in social communication, and by repetitive, stereotyped behaviors and interests. This disorder affects in a ratio of 4 males:1 female, however this ratio has been decreasing to 3:1 owing to a sex bias in the diagnostic criteria for ASD

Around 18 and 24 months of age, ASD symptoms, appear and start to challenge their limitation in social communication. These symptoms can have a great variability in severity and extent of this disability. As a young child, problems related to social communication and interaction can be seen in delay or regression in development and/or speech, difficulties in understanding the intent of others, reduced visual contact, and inappropriate patterns of behavior and play. As an older child, these problems can affect academic performance, social constraints and behaviors that also disturb family life (Hyman et al., 2020; Mukherjee, 2017).

Children with ASD disease can have an aggressive and destructive behavior, self-injuries, tantrums, difficulty in recognizing danger, adaptive skills, transitioning activities and in their daily routines. Furthermore, they can also carry cognitive limitations, inappropriate emotional responses, or repetitive behaviors (Kodak & Bergmann, 2020; Mukherjee, 2017).

Usually, people with ASD can have psychiatric and cognitive comorbidities as intellectual disability, anxiety, depression, mood disorders, attention

deficit/hyperactivity disorder (ADHD) as well as gastrointestinal (GI) problems, eating problems, immune system abnormalities, epilepsy and sleep deficits (Hodges et al., 2020; Masi et al., 2017).

Despite the advances in understanding the neurobiology and genetics of ASD, the diagnosis of this disease continues to be based on identifying and reporting behaviorally defined clinical symptoms. The need for consistency in clinical diagnosis of a very heterogeneous disorder are a challenge in determining accurate prevalence rates. Even if symptoms of ASD are neurologically based, they still manifest as behavioral characteristics that present differently depending on age, language level, and cognitive abilities.

Diagnostic of ASD should include domain-specific information regarding level of functioning and severity. Firstly, these diagnostics should start with identification of "red flags" very representative of this condition, such as reduced eye contact and name response, regression of social and language skills, rigidity, extremely focused interests, or difficulty in understanding emotions. Then, in a more comprehensive evaluation, it should be important a parent/caregiver interview to obtain a medical/developmental report of current behavior and abilities, a direct observational assessment where its procedures a medical evaluation, cognitive or developmental testing, language testing, and structured assessment in social interaction and finally a complete physical exam (Hodges et al., 2020; Huerta & Lord, 2012; Sharma et al., 2018). Also, pediatricians can use algorithms and toolkits to obtain an earlier diagnosis and design a management strategy (Johnson et al., 2007).

Due to the fact that the disease in heterogeneous, the treatment maintains limited and the main focus is the symptom management. Various classes of drugs are included on the pharmacological treatment including. psychostimulants (methylphenidate and amphetamines), atypical antipsychotic drugs (risperidone, aripiprazole, quetiapine, ziprasidone), antidepressants (Fluoxetine, sertraline, citalopram, escitalopram, and fluvoxamine), alpha-2 adrenergic receptor agonists (Aman et al., 2008; Masi et al., 2017; Sharma et al., 2018). Although, the current pharmacological therapies demonstrate varying levels of efficacy and only provide partial symptomatic relief, instead of provide modification of the underlying basis, the disease process itself. Otherwise, there are non-pharmacological interventions, that have already shown evidences in reducing aggression, increasing cognitive skills and improving social interaction and verbal communication of ASD patients, which include cognitive behavioral therapy and social behavioral therapy (Aman et al., 2008; Reichow et al., 2018; Sharma et al., 2018).

As a result of the limited treatment for ASD, this condition affects the different aspects of people daily lives and their day-to-day continues to face challenges. The difficulties in social relationships, education, professional life and mental health are still, in long-term, poor outcomes. In adult ASD people, the improvement of social and

emotional skills, with the creation of empathy for example, is a good therapy that can help them in their condition (Howlin & Magiati, 2017).

### 1.1.2. Sex dimorphism in autism spectrum disorder

Over the years, researchers have discovered that sex dimorphism in ASD is not due to the fact that this disorder is more prevalent in males. Instead, it has been noticed that females might be underdiagnosed or that the presentation of ASD in females may differ from that of males, leading to potential challenges in identification (Harrop et al., 2019; Lai et al., 2015). Indeed, females with ASD may exhibit different social and behavioral patterns or have more subtle symptoms that are not as well-captured by the current diagnostic criteria, leading to underdiagnosis or misdiagnosis (Harrop et al., 2019; Lai et al., 2015). It is known that girls with ASD present less social and communicative impairments (Stacy et al., 2014), less repetitive behaviors (Hiller et al., 2014; Supekar & Menon, 2015), however they are better ability to maintain reciprocal conversation or combine verbal with non-verbal interactions and scoring higher on visual perception. (Carter et al., 2007). In addition, female with ASD have shown more likely to deal with sleep problems, depression and anxiety or other emotional problems (Hartley & Sikora, 2009; May et al., 2014). On the other hand, boys show increased language and motor skills (Carter et al., 2007), as well as hyperactivity, aggression and impulsivity (May et al., 2016).

Yet, ASD girls display a camouflaging effect (Dean et al., 2017; May et al., 2019; Napolitano et al., 2022), developing adaptive strategies to mimic social norms, making it harder to recognize their ASD traits compared to men who may exhibit more noticeable and stereotyped ASD behaviors (Harrop et al., 2019; Ratto et al., 2018). Furthermore, as stated earlier, girls with ASD may be more likely to have co-occurring conditions (anxiety, depression) that overlap with the core traits of ASD. Consequently, ASD symptoms will be overshadowed or overlooked during evaluation, complicating the diagnostic process (Hartley & Sikora, 2009; May et al., 2014).

The reasons behind the sex dimorphism in ASD are not entirely clear, but researchers have proposed several theories suggests the action of sex-differential risk factors for ASD that might act to either increase males' vulnerability (Extreme Male Brain theory) (Baron-Cohen, 2002; Baron-Cohen et al., 2005; Napolitano et al., 2022) and/or protects females (Female Protective Effect) (Jacquemont et al., 2014). An alternative explanation for this ASD sex dimorphism is based on differences in sex chromosomes, where on one hand the Y chromosome can be a risk in itself and on the other hand the additional X chromosome in females protects them in some way (May et al., 2019).

So, it is important to recognizing the sex dimorphism in ASD to improving diagnostic accuracy and providing appropriate support and interventions for both genders (Santos et al., 2022).

Since mice and rats are highly active and social species, the study of ASD-like behaviors and their underlying pathways related to this disease relies heavily on animal experimentation. With that, several animal models of ASD were created, which leads to a translation into the clinical domain and the investigation of therapeutic targets (DiCicco-Bloom et al., 2006; Ey et al., 2011; Moy et al., 2006). One of these animal models is mouse *Nf1*$^{+/-}$, which will be used during this study.

## 1.2. NEUROFIBROMATOSIS TYPE 1

### 1.2.1. Neurofibromatosis type 1: disease overview

Neurofibromatosis type 1 (NF1), which affects 1 in 2.700 births, is a single-gene neurodevelopmental disorder that is caused by a mutation in the *Nf1* gene located on chromosome 17 (17q11.2). This mutation encodes its protein product neurofibromin, which is caused by germline loss-of-function mutations of the *Nf1* tumor suppressor gene (Bernardino et al., 2022; Plasschaert et al., 2015).

The *Nf1* gene encodes the protein neurofibromin that is predominantly expressed in neurons, glial cells and immune cells (Molosh & Shekhar, 2018; Trovó-Marqui & Tajara, 2006). This protein is a negative regulator of Rat Sarcoma protein (Ras) pathway (Jett & Friedman, 2010) and can activate signaling cascade by turning guanosine diphosphate (GDP) into guanosine triphosphate (GTP) (Figure 2) (Ballester et al., 1990; Buchberg et al., 1990; Maloney et al., 2018; Xu et al., 1990). Because of this protein has various biochemical functions, including activation of Ras GTPase, negative regularization of Ras protooncogene, modulation of adenylyl cyclase and microtubule association. With that, it is relevant in cell division, differentiation, proliferation, growth, and apoptosis (Costa & Silva, 2003; Garg, Green, et al., 2013a; Gutmann et al., 2017). Ras signaling can be activated by the association of growth factors to RTKs (receptor tyrosine kinases). This binding leads to the action of GEFs (guanine nucleotide exchange factors), that allow Ras to bind GTP, becoming active and communicating the signal to other downstream effectors (Gutmann et al., 2017; Diggs-Andrews & Gutmann, 2013). This activated via results to an increased AKT/MEK (protein kinase B/Mitogen-activated protein kinase) activity and, consequently, to a better cell proliferation and cell survival (Gutmann et al., 2017). Furthermore, Ras signaling regulate the levels of cyclic adenosine monophosphate (cAMP) through protein kinase C-ζ (PKCζ) and this cAMP is responsible for cell proliferation and differentiation (Diggs-Andrews & Gutmann, 2013; Gutmann et al., 2017).

The mutations on *Nf1* gene results to the loss of neurofibromin function leads to an up-regulation of Ras/MAPK signaling and, consequently, its downstream pathways MAPK/ERK and AKT/mTOR, leading to neurofibroma formation (Molosh & Shekhar, 2018).

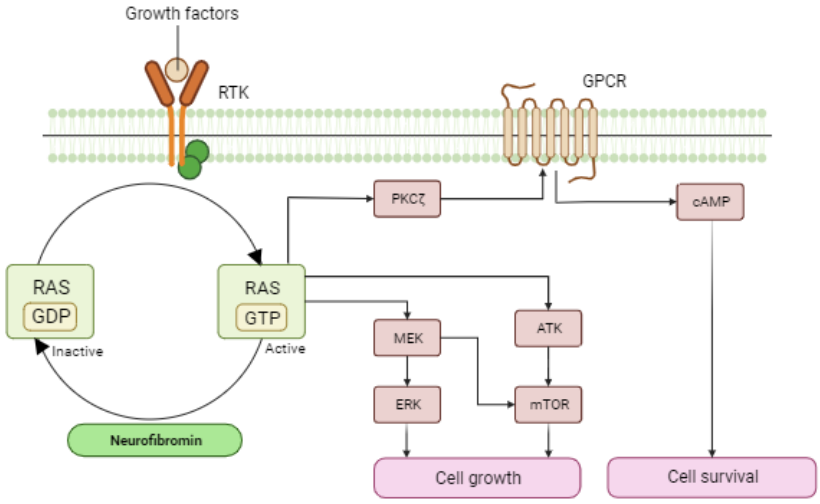



**Figure 2 | Signaling pathway of neurofibromin**
Neurofibromin regulates cell growth and survival through several downstream signaling effectors by convert of active GTP-bound RAS to its inactive GDP-bound form. Adapted from Gutmann et al., 2017.

As observed by McKeever et al., (2008), around 50% of the cases are inherited and the other 50% happens due to spontaneous genetic mutation (uninherited) of the *Nf1* tumor suppressor gene. The average global prevalence of NF1 is ~1 case per 3.000 individuals, although prevalence estimates a variety between countries (Gutmann et al., 2017).

The first appearances of this clinical manifestations and their severity can vary between individuals. The clinical manifestations of this multisystem neurodevelopmental disorder are characterized by clinical manifestations affecting the eyes, skeleton, skin (café-au-lait macules), neurofibromas and behavioral manifestations (Gutmann et al., 2017; Nix et al., 2020).

Studies have already shown that 80% of children with NF1 also exhibit cognitive symptoms, including learning and memory disabilities, attention deficit disorder, social and communications deficits, motor delays (Champion et al., 2014; Erdoğan-Bakar et al., 2009; Hyman et al., 2005, 2006). In addition, comorbid diagnoses of ASD are becoming more recognized and it can be seen that the prevalence of ASD among these NF1 patients ranges between 15% and 30%, and an additional 30% showing partial ASD symptomatology (Garg, Green, et al., 2013b; Garg, Lehtonen, et al., 2013; Walsh et al., 2013).

### 1.2.2. Animal model of neurofibromatosis type 1

It is required to understand that the pathophysiology of NF1 and to identify the cell types, cellular processes, and molecular pathway that are altered by *Nf1* mutations.

For that, it is necessary use animal models, such as a *Nf1*$^{+/-}$ mouse (Costa & Silva, 2003; Molosh & Shekhar, 2018; Tamura, 2021). There are two recognized groups of various well-characterized *Nf1*$^{+/-}$ mouse models: mouse models with growth and cell differentiation abnormalities and mouse models with learning disabilities (Costa & Silva, 2003).

Studies in mice revealed that the exhibited defects in hippocampal spatial learning are due to the hyperactivation of Ras activity, which leads to an increased level of gamma-aminobutyric acid (GABA) neurotransmission (Gonçalves et al., 2017; Santos et al., 2023), as observed in individuals with NF1 (Ribeiro et al., 2015; Violante et al., 2016). This contributes for the special learning and memory deficits present in mutant mice (Costa et al., 2002).

These models are so important for the study of cellular and intracellular mechanisms of NF1 because they show a pattern of cellular and molecular change very similar to that of human individuals with NF1 phenotype. These studies, allows the development of treatments for the different pathological processes associated with NF1 (Costa et al., 2002).

Both human and mice with NF1 have an increased propensity to develop tumors, skin pigmentation problems, brain abnormal function and cognitive and behavioral deficits such as defects in hippocampal spatial learning and deficits in attention and social behavior (Costa et al., 2002). As seen by Silva et al. (1997), *Nf1*$^{+/-}$ mice can overcome hippocampal learning and memory deficits with appropriate training. One thing important to notice in these studies of both NF1 human and mice phenotypes is that a complete loss of neurofibromin (homozygous mutation) is lethal, due to the fact that they are heterozygous (Brannan et al., 1994).

Besides that, our group (Santos et al., 2023) detected an sexual dimorphism in *Nf1*$^{+/-}$ mice regarding hippocampal neurochemistry and autistic-like behaviors. This study led to the identification of "camouflage" behavior in females for the first time in an animal model of ASD.

## 1.3. AUTISM SPECTRUM DISORDER AS A MICROBIOTA GUT BRAIN AXIS DISORDER

The real causes of ASD-related neurodevelopmental disorders are unclear and the diagnosis of ASD is based on the presence and severity of stereotypic behavior and deficits in language and social interaction (Hsiao et al., 2013; Mukherjee, 2017). ASD are also often associated with many medical comorbities that occur in much higher prevalence that in neurotypical children, among which GI problems are one of most common (Saurman et al., 2020).

This GI disorders include malabsorption, maldigestion, microbial overgrowth (fungal, bacterial and viral) and abnormal intestinal permeability. All of these mentioned before can cause symptoms including diarrhea, chronic constipation, increased intestinal permeability, abdominal pain, and disturbed intestinal microbiota (Mukherjee, 2017; Sorboni et al., 2022).

Various studies have reported discrepancies in the microbiota between children with ASD and neurotypical controls, which are significantly reduced in autistic children (Cowan et al., 2020; Sorboni et al., 2022). GI disturbances are approximately four times more prevalent in children with ASD than in the neurotypical population (McElhanon et al., 2014). Furthermore, GI symptoms appear to highly correlate with ASD severity, showing an important role of the gut microbiota in the brain (Beopoulos et al., 2022; Sorboni et al., 2022).

Gut microbiota imbalances occur not only in ASD, but also in Alzheimer's disease, Parkinson's disease, epilepsy, and major depressive disorder (Chen et al., 2021). It has been shown that GI problems alter the composition and, consequently, the function of the microbiota, leading to a relationship between gut microbiota dysbiosis and neurodevelopmental disorders (Figure 3). All of this is related to the fact that there is a high prevalence of GI dysfunction in ASD and its significant correlations with challenging behaviors and psychiatric comorbidities (Cryan & Dinan, 2012; Saurman et al., 2020; Sorboni et al., 2022).

Due to the increasing amount of data regarding the role of gut microbial dysbiosis in ASD, researchers are currently turning on strategies for treating such a disease by modulating the microbiota-gut-brain (MGB) axis as a potential therapeutic approach. The most common is the use of oral prebiotic, probiotic, dietary, fecal microbiota transplantation (FMT) as well as microbiota transfer therapy (Kang et al., 2017; Santocchi et al., 2016). These methods are an important part of modulating microbial composition, regulating their essential metabolites, improving neurological complications by increasing neurochemicals and short-chain fatty acid (SCFAs) and reducing intestinal permeability, regulating neural, metabolic and immune pathways (Sorboni et al., 2022). Recently, FMT study reported that fecal microbiota from a small

number of autistic individuals into germ-free mice is sufficient to induces hallmark autistic behaviors, especially social deficits and increased repetitive behavior, which confirmed the importance of gut microbiota on modulation of ASD behavior (Sharon et al., 2019). This and others human and animal studies suggests that gut microbial-targeting therapy may be a tractable strategy for developing novel therapeutics not only in ASD patients but also in another complex central nervous system (CNS) disorders (Alharthi et al., 2022; Cowan et al., 2020; Cryan & Dinan, 2012).

The MGB axis is a complex biochemical network of two-way communication which occurs between the GI tract and the CNS via chemical transmitters, neuronal pathways and the immune system that creates such communication (Damiani et al., 2023). This axis is essential for the maintenance of homeostasis and the regulation of hormonal and immunological levels. The stress-response, GI motility and overall behavior are mainly affected by perturbations of these systems. (Cryan & O'Mahony, 2011).be

The signaling behind MGB axis is extremely complex and its modulation can affect both, normal healthy state and pathological state. Despite the mechanisms are not yet completely known, several evidences suggest that this bidirectional interaction relies on four main communication pathways: neuronal pathway (vagus nerve), endocrine pathway (neurotransmitters), immunological pathway (cytokines) and metabolic pathway (SCFA) (H.-X. Wang & Wang, 2016).

The gut microbiota is a complex ecosystem of $1\times10^{13}$ to $1\times10^{14}$ microorganisms that resides in the human GI tract, consisting mainly of bacteria but also fungi, viruses and protozoa, which varies between individuals (Carlson et al., 2018; Cowan et al., 2020; Cryan & Dinan, 2012). Despite the intestine contains a wide diversity of microorganisms, the majority of the studies focus on anaerobic bacteria belonging to two main fila: Bacteroidetes and Firmicutes (Carlson et al., 2018; Cowan et al., 2020).

It is recognized that gut microbiota represents the first protection system of the GI apparatus and have a crucial role in the development and functionality of innate and adaptive immune responses, maintenance of gut homeostasis, nutrient absorption and fat distribution, metabolites production and transportation and regulation of the CNS and enteric nervous system (ENS) (Cryan & Dinan, 2012; Mangiola et al., 2016; Sorboni et al., 2022). Therefore it is essential to maintain a healthy microbiota (Sorboni et al., 2022; Taniya et al., 2022).

Under physiological conditions, the continuous stimulation of the immune system by the gut microbiota provides a rapid and effective mechanism of defense against pathogens. On the other hand, the flora exerts its protective role competitively, metabolizing those nutrients needed for pathogens survival, and producing molecules that inhibit the growth of such microbes (Mangiola et al., 2016).

This microbiota colonization of the intestinal tract begins during the prenatal period due to the existence of microbial communities in the placenta, amniotic fluid and

the blood of the umbilical cord (Alharthi et al., 2022; Damiani et al., 2023). So, there are some prenatal risks that need to be taken in account, such as maternal infection, maternal physical health, the health condition of pregnant women (unhealthy diet, metabolic stress), and drug use in pregnancy. During this time a maternal microbiome dysbiosis can cause abnormal neurological development of offspring, leading to lifelong behavioral deficits (Alharthi et al., 2022; Taniya et al., 2022).

After de birth, the baby is exposed to bacteria during breastfeeding, through the ingestion of food, and from the surrounding environment (Yassour et al., 2016). Some studies have demonstrated that the postnatal risk factors include formula feeding, where breastfed infants have greater diversity and faster microbiota development relative to formula-fed, air contamination, antibiotic intake (antibiotics influence gut homeostasis), and nutrition factors (Ghozy et al., 2020; C. Wang et al., 2017). We concluded that there are risk factors for the development of psychological and neurodevelopmental disorders as well as GI problems and/or microbiota alterations are highly influenced by both prenatal and postnatal stress (Cowan et al., 2020).

In adolescence, microbial diversity and functional capabilities are similar to an adult, however, due to different dietary habits that will influence the microbiota's composition, each individual has a unique microbial community (Nagpal et al., 2018; Yatsunenko et al., 2012).

Many aspects of daily life cause variations in the microbiota, just spending time in natural environment, changes in diets, pet ownership and increasing use of antibiotics and disinfectants (Figure 3) (Ayeni et al., 2018; De Filippo et al., 2017; Tun et al., 2017). Medical causes such as bacterial or viral infections, GI problems, interventions of medical procedures in the early stages of life and the overuse of antibiotics and drugs exhibit microbiota alterations (Figure 3) (Maier et al., 2018; Mortensen et al., 2018). So, because of the factors stated above, it is tricky to find a cause of ASD-associated gut bacterial dysbiosis due to the high inter-individual heterogeneity microbiota (Damiani et al., 2023).

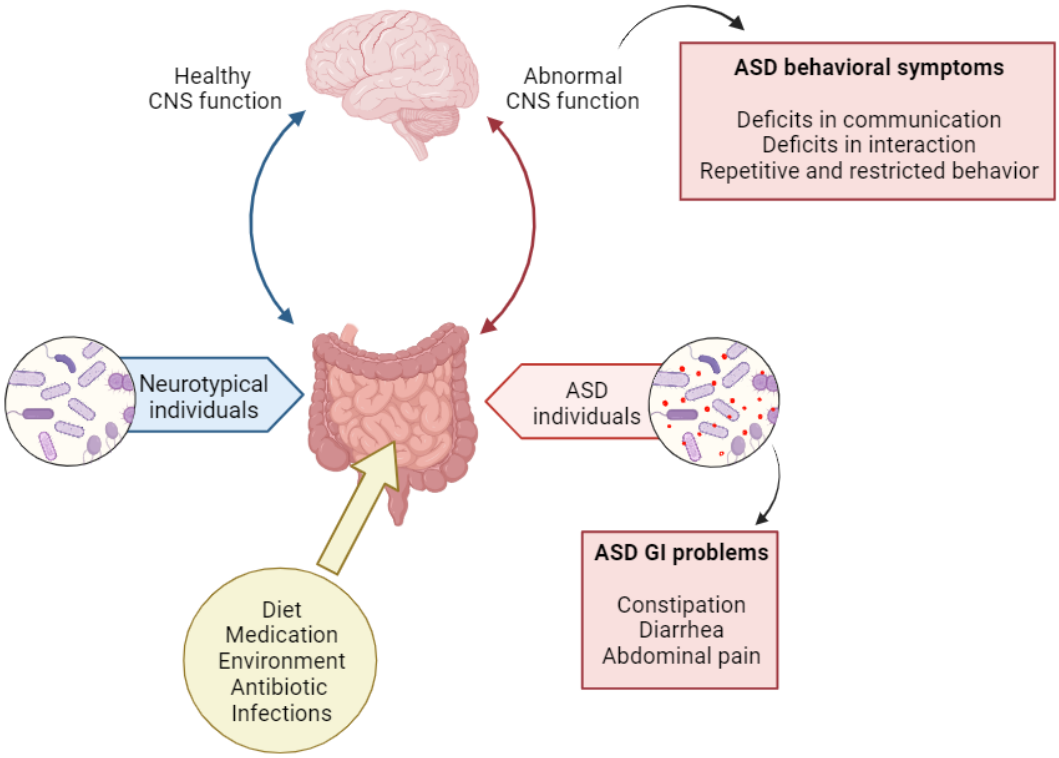



**Figure 3 | The microbiota-gut-brain axis plays a critical role in autism spectrum disorder**
There are differences in the gut microbiome of typically developing children compared to children with autism spectrum disorder (ASD). However, various factors can influence the gut microbiome and their including diet, medication regimens, medical comorbidities, environment, antibiotic and infections symptoms. In addition, the brain-gut-microbiome axis is bidirectional with regard to changes in both the gut [gastrointestinal (GI) symptoms and microbial profile] and behavior and therefore changes in microbiota composition in children with ASD will influence their behavioral symptoms.

The most sensitive period to disruption appears to be the early life of the microbiota. These sensitive periods (also known as critical periods/windows) are defined as specific developmental periods in which a system exhibits heightened plasticity and is particularly responsive to external factors (Cowan et al., 2020). Notably, the significant periods of microbial colonization and maturation overlap with the period of brain development, which neural circuits are highly plastic and potentially vulnerable to the external factors (Alharthi et al., 2022; Damiani et al., 2023). Due to this parallel development in both microbiota and brain, was hypothesized the existence of a microbiota-gut-brain axis (Borre et al., 2014). Further, about 40% of all human metabolites are generated by the gut microbiome, an intestinal dysbiosis can have a profound impact on CNS and ENS, which compromise the brain function and result in disease states. In turn, stress at the level of the CNS can affect gut function and lead to perturbations of the microbiota (Alharthi et al., 2022; Cryan & Dinan, 2012).

In the healthy status of the individual a well-balanced gut microbial composition with an adequate diversity is essential not only to maintain microbial homeostasis, but also for normal physiological functioning in other organs, especially the brain (Cryan & Dinan, 2012; Dinan & Cryan, 2017).

## 1.4. INFLUENCE OF MATERNAL DIET ON OFFSPRING NEURODEVELOPMENT

Since the focus of this study is on shaping the microbiota of the offspring through a maternal diet, this factor will be discussed further.

### 1.4.1. Microbiome at pregnancy

Microbiota is a fundamental source of essential nutrients and energy for the growing brain and this modulation can have effects on neurodevelopment (Cowan et al., 2020). Microbial colonization and development in early life are very important, and microbes transmitted by mothers help in the normal succession of the microbiome and promote the maturation of the neonatal immune system (Xiao & Zhao, 2023).

During pregnancy, maternal microbial colonization, that included gut, vaginal and uterine, placental and oral microbiota, is vertically transmitted to the offspring (Codagnone et al., 2019; Ferretti et al., 2018). It is not surprised that changes in the maternal microbiota composition due to diet/nutrition will have a significant impact in infant health (Yao et al., 2021). So, it is important to understand the interplay of maternal diet and the influences it will have on the offspring (Codagnone, Spichak, et al., 2019; Linehan et al., 2022).

Pregnancy is a unique period in human life, where it have been observed significant female microbiota composition changes (Codagnone, Stanton, et al., 2019; Xiao & Zhao, 2023). Indeed, during early pregnancy, the gut microbiome is similar to that in healthy non-pregnant women characterized by a high abundance of Bacteroidetes and Firmicutes. However, over the pregnancy, large alterations occur in the richness and diversity of the microbiota. In contrast to the oral microbiome variation, microbial diversity in the gut and vagina decreases significantly during pregnancy (Xiao & Zhao, 2023). Even if there is a decrease in the diversity of the gut microbiota, it is important to notice that occurs an enrichment in Proteobacteria (Koren et al., 2012). Several studies have demonstrated that this natural change in the population of bacteria, particularly proteobacteria, can help the body with the energy demands of the developing fetus in the third trimester (Shin et al., 2015). The vaginal microbiota composition during pregnancy is characterized by a decrease in diversity and an increase in Lactobacillus spp (Romero et al., 2014). This bacteria play an important role in maintain a healthy vaginal microenvironment by lowering its pH and inhibiting the growth of other harmful bacteria), preparing to a normal vaginal delivery (Codagnone, Stanton, et al., 2019; Xiao & Zhao, 2023).

Disruption of the maternal microbiota can cause negative pregnancy outcomes, including late miscarriage, premature rupture of membranes, hyperemesis gravidarum, preterm birth, intrauterine growth restriction and stillbirth (Romero, 2009). It is known by Ishimwe et al. (2021), that women with pregnancy complications have a reduced diversity of their gut microbiota, which is detrimental to the health of both mother and fetus. These findings indicate that these changes in the microbiota composition during pregnancy have an adaptive role for maternal and promote the correct development and health of the fetus (Figure 4) (Codagnone, Stanton, et al., 2019; Linehan et al., 2022).

Imbalances in the gut microbiome early in life predispose infants to be colonized by opportunistic pathogens such as Enterococcus, Enterobacter, Clostridium and Klebsiella species. In addition, and especially in preterm newborns, gut dysbiosis in the infant can also lead to poor growth and an increased risk of sepsis and necrotizing enterocolitis (Fundora et al., 2019). There are also long-term consequences of disrupted microbial colonization, which include allergies, asthma, metabolic syndrome, diabetes and inflammatory bowel disease (Milani et al., 2017).

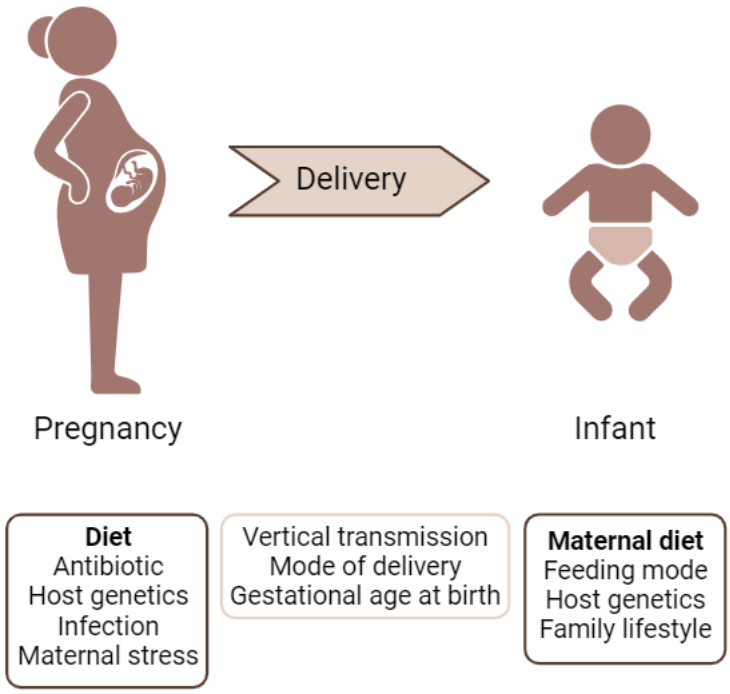



**Figure 4 | The factors affecting maternal or infant microbiota modulation**
The microbiome of the mother is altered by diet, drugs and stress. The mode of delivery determines the first colonizers of the newborn's microbiota. Infant microbiota composition is also affected by diet, drugs and stress.

Similar to humans, the gut and vaginal microbiota of mice and rats change their composition during pregnancy, constituting a strong preclinical model to investigate the connection between the maternal gut environment and the offspring's brain development (Gohir et al., 2015; Hallam et al., 2014). Furthermore, several studies have

demonstrated that maternal exposure to the previously mentioned factors during pregnancy can alter the offspring's microbiome and immunity (Bodnar et al., 2022; J. Wang et al., 2018; Yu et al., 2018).

### 1.4.2. Maternal diet

Since the composition of maternal microbiome has been linked to maternal metabolism. Thus, maternal habits during pregnancy and lactation, for example through diet, can be beneficial or detrimental for both maternal mental health and offspring neurodevelopment (Codagnone, Spichak, et al., 2019).

“Psychiatric nutrition" is a new concept based on nutrition as a brain modulator (Horn et al., 2022). It has been reported that deficiencies of specific nutrients and overall poor diet quality are associated with long-term negative impacts on developing cognition (Freeman et al., 1980; Tandon et al., 2016). On the other hand, nutritional interventions have been demonstrated to reduce the severity of symptoms of neurodevelopmental disorders and improve childhood cognitive outcomes in vulnerable populations (Freeman et al., 1980; Isaacs et al., 2009). Palladino et al. (2021) reported that dietary changes in pregnant mice impact the growth and development of the fetus through gut microbes. During the gestation, maternal vital substances reach the fetus through blood circulation to placenta. The gut microbiota can digest carbohydrates in the gut to produce vitamins, amino acids, and SCFAs, that are essential for fetal development, immunity and metabolism.

Dysbiosis of the gut microbiome are often associated with unhealthy lifestyles (Xiao & Zhao, 2023). Numerous studies have emphasized associations between microbial dysbiosis and unhealthy diet which, in addition to leading to obesity and poor cardiovascular health, also have impact in behavior and inflammatory phenotypes. The most common models of unhealthy diet include high-fat diet (~60 % kcal from fat), western diet (high fat and high sugar) and diet with specific nutrients deficits (Codagnone, Spichak, et al., 2019; Yao et al., 2021). These types of diets showed metabolic dysfunction of fetal growth (Linehan et al., 2022; Yao et al., 2021).Both high-fat and western maternal diet showed significant increased Firmicutes/Bacteroidetes ratio in the offspring’s microbiota (Koliada et al., 2017; Serino et al., 2012; Steegenga et al., 2017). In rodents, consumption of a high-fat diet before and during pregnancy impairs the trajectory of maternal and offspring’s microbiota, resulting in juvenile impaired social behavior and anxiety-like phenotype (Buffington et al., 2016; Gohir et al., 2015; Steegenga et al., 2017). Through FMT of malnutrition children in rodents, it was possible to demonstrate that a change in the microbiota due to poor nutrition leads to metabolic problems and growth retardation. This study shows that it is possible to reverse these microbiota-dependent problems by supplementation with sialylated milk

oligosaccharides, typically found in breastmilk, which will act as prebiotics for the microbiota (Charbonneau et al., 2016). Several nutrients, such as Omega-3, can have an impact in neurodevelopment and microbiota maturation. A high-fat diet supplemented with omega-3 polyunsaturated fatty acids can contribute to an increase diversity of microbiota, an enrichment of Bifidobacterium at a species view, a regularization of the hypothalamic-pituitary-adrenal (HPA) axis activity, reconstitution of the composition of the maternal stress-induced gut microbiota and, finally, confers resilience to stress later in life. However, a lack in omega-3 polyunsaturated fatty acids can affect the metabolome, impair communication and social behavior, while increasing depressive-like behavior (Pusceddu et al., 2015; Robertson et al., 2017; Robertson et al., 2017).

Breastmilk is a dynamic and complex nutrition source for the developing infant which changes based on maternal diet and the resultant human milk oligosaccharide (HMO) and bacterial composition in human milk (Seferovic et al., 2020). HMOs regulate the immune system and prevent pathogenic bacteria from adhering. Thus, modulation of the HMO composition of breast milk through dietary manipulation may represent a method for manipulate the establishment of microbial species in the infant gut (Sakanaka et al., 2019). Therefore, breastfeeding mothers should have a careful nutrition to proportionate the best health to the baby (Yao et al., 2021).

Through a healthy maternal diet throughout the gestational period, it is possible to achieve optimization and acquisition of a rich and diverse gut microbiota in newborns and an improvement in long-term health outcomes. Although there is no known "ideal" diet, it is generally recommended that pregnant women consume "a balanced diet with adequate distribution of the basic groups of the food pyramid" (Yao et al., 2021). Indeed, consumption of a vegetarian diet during early pregnancy has been associated with a distinct microbial composition, rich in fatty acid, lipid and folate biosynthesis pathways (Barrett et al., 2018).

In conclusion, there is a need to better understand how diet during pregnancy can influence the microbial connection between mother and baby, so optimize the health and behavior of mother and child in the perinatal period, as well as informing the appropriate type and timing of therapeutic interventions (Yao et al., 2021).

## 1.5. TRYPTOPHAN METABOLISM: FOCUS ON SEROTONIN

Tryptophan (Trp) is an essential amino acid obtained exclusively from the diet and the precursor of neuroactive compounds within the CNS and so, is gaining in interest relative to dietary and nutritional sciences (Kałużna-Czaplińska et al., 2019; Pais et al., 2023; Roth et al., 2021).

This amino acid can be found in several protein-based foods and dietary proteins including dairy products, meats, dried fruit, fish, eggs, bananas, seeds (pumpkin and sesame), chocolate, soy and tofu. Thus, the availability of Trp is highly dependent on dietary ingestion (Jenkins et al., 2016; Nayak et al., 2019).

Trp plays an important role in protein formation, and is also a precursor to several bioactive molecules needed for the nervous and immune system functions, such as kynurenine and the neurotransmitter, serotonin (Jenkins et al., 2016; Nayak et al., 2019). In fact, Trp obtained from diet is the only precursor for serotonin, which is crucial for the processing of emotional regulation, appetite, sleep, and pain, as well as colonic motility and secretory activity in the gut (Roth et al., 2021). Thus, as a precursor of molecules that act at the interface between the host and the microbiota, Trp is essential in the modulation of microbiota-gut-brain axis (Bosi et al., 2020).

The Trp enters in peripheral circulation after intestinal absorption and it will be released at target sites, either peripherally or centrally. Furthermore, the resident gut microbiota produces Trp-specific metabolites and indirectly influences host physiology (Comai et al., 2020; Gheorghe et al., 2019). It important to notice that this amino acid is also the precursor of multiple biologically crucial neuroactive compounds that are generated through two critical pathways: hydroxylation to produce serotonin and oxidation to produce kynurenine and its metabolites (Figure 5) (Pais et al., 2023).

Kynurenine synthesis occurs 90% via tryptophan 2,3-dioxygenase (TDO) in liver and 10% via indoleamine 2,3-dioxygenase (IDO) in brain, GI tract and liver (Boadle-Biber, 1993). Thus, kynurenine is catabolized into two neuroactive inflammatory compounds, kynurenic acid (KYNA), and quinolinic acid (QUIN), which have neuroprotective and neurotoxic effects respective. QUIN is further catabolized into NAD+, which are active in other critical cellular metabolic processes (Roth et al., 2021). Kynurenine plays a key role in intestinal homeostasis and in regulating immune responses (Figure 5) (Pais et al., 2023). In the serotonin pathway, Trp is catalyzed by tryptophan hydroxylase (TPH) to be converted to 5-hydroxytryptophan (5-HTP), more specifically by TPH1 in enterochromaffin cells, or by TPH2 in the CNS and ENS. In turn, the latter decarboxylates to form 5-hydroxytryptamine (5-HT), also known as serotonin. Subsequently, serotonin can be metabolized into melatonin, the main endogenous regulator of sleep onset and circadian rhythms, or it is catabolized by monoamine oxidase into 5-hydroxyindole

acetaldehyde (5-HIA) and then by aldehyde dehydrogenase into 5-hydroxyindole acetic acid (5-HIAA) which is excreted in the urine (Figure 5) (O'Mahony et al., 2015; Roth et al., 2021).

Since the metabolism of Trp leads to the production of various neuroactive molecules with crucial neurological role in the organism, such as nutrient sensing, metabolic stress response, and immunity, it is important to regulate concentrations of Trp in order to keep systemic homeostasis (Gostner et al., 2020; Pais et al., 2023).

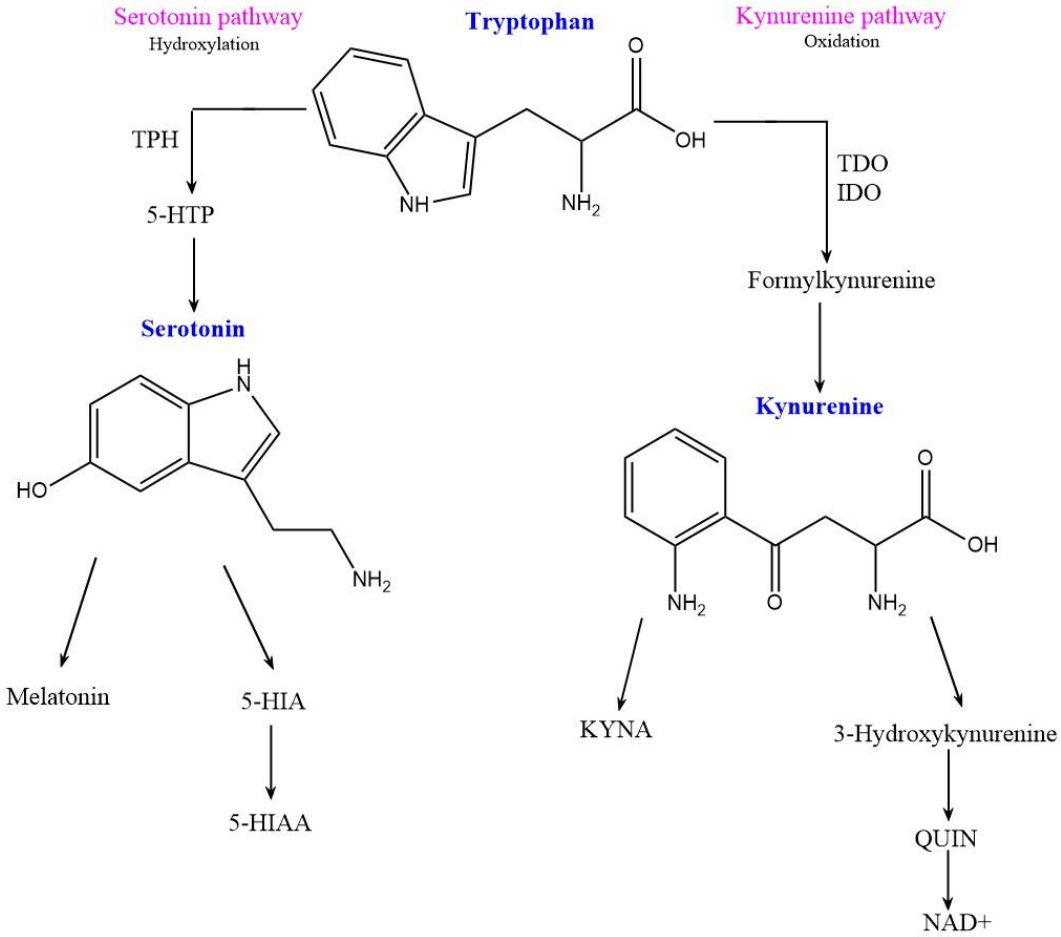



**Figure 5 | Tryptophan metabolic pathways**

Tryptophan metabolism occurs via the kynurenine pathway or the serotonin pathway to produce bioactive metabolites. 5-HIA - 5-hydroxindole acetaldehyde; 5-HIAA - 5-hydroxyindole acetic acid; 5-HTP - 5-hydroxytryptophan; IDO - indoleamine 2,3-dioxygenase; KYNA - kynurenic acid; NAD+ -nicotinamide adenine dinucleotide; QUIN - quinolinic acid; TDO - tryptophan 2,3-dioxygenase; TPH - tryptophan hydroxylase. Created with ChemDraw.

Serotonin has long been associated with several fundamental aspects of humor and behavior, including sleep, appetite, cognition (especially learning and memory) and social and emotional behaviors such as anxiety, depression, empathy and aggression (Gyurak et al., 2013; Steenbergen et al., 2016). Unlike Trp and kynurenine, is not capable of crossing the blood-brain barrier (BBB) (Gostner et al., 2020). Therefore, the central

production of this neuromodulator is completely dependent on the availability of Trp in the CNS (Roth et al., 2021).

Trp has to compete for uptake across the BBB with other amino acids essential for brain function, known as the large neutral amino acids (LNAA) (Gibson, 2018). These LNAA consist of Trp, tyrosine, phenylalanine, and the branched-chain amino acids (leucine, valine and isoleucine) (Fernstrom, 2013). The transporter is competitive, thus increasing the circulation concentration of one LNAA increases the brain absorption of that LNAA and decreases the uptake of the others. In this case, the ratio of Trp to LNAA in plasma or serum (Trp:LNAA) is considered as the best biomarker of TRP uptake in the brain (Fernstrom & Fernstrom, 1995). Thus, this concept of competitive transport is the foundation of some Trp supplementation or depletion diets, to increase or decrease, respectively, serotonin synthesis in the brain (J. D. Fernstrom, 2012; J. a. J. Schmitt et al., 2006; Silber & Schmitt, 2010).

Adequate intake of Trp is essential for children's neuronal development and growth (Nayak et al., 2019). Both deficiency and excessive intake are harmful to human health, its recommended daily dose for adults is in the range of 250 mg to 425 mg, corresponding to a dietary intake of 3.5 to 6.0 mg/kg body weight per day (Richard et al., 2009).

Under normal conditions, a sexual dimorphism in Trp metabolism has been suggested. Animal studies have revealed higher Trp circulating levels in females than in males, which also reflects in increased serotonin production. Furthermore, female rats exhibit higher Trp as well as serotonin levels in multiple brain regions (Pais et al., 2023).

Impaired Trp metabolism or abnormal metabolic pathways has been consistently associated with the pathogenesis of several neuropsychiatric disorders, such as depression, ASD, ADHD, and obsessive-compulsive disorder, and consequently, abnormalities of serotonergic function (Chugani, 2004; Nayak et al., 2019; Pais et al., 2023). Central and peripheral serotonergic signaling pathways are disrupted in GI diseases, such as inflammatory bowel disease and irritable bowel syndrome, and also in neurodevelopmental disorders with GI comorbidities (Gheorghe et al., 2019).

A hyperserotonemia (elevated whole blood serotonin), has been reported from several studies, thus have been considered as an biomarker for ASD (Gabriele et al., 2014). Furthermore, some studies also have reported deficiency of Trp (Gevi et al., 2016) and high levels of kynurenine (Murakami et al., 2019) in ASD individuals.

Autistic males exhibit reduced levels and synthesis of serotonin in frontal cortex and thalamus, which might explain the language production and sensory integration symptoms (Chugani et al., 1997; Pinares-Garcia et al., 2018). For this reason, it has been hypothesized that there is a correlation between severity of autistic symptoms and abnormal serotonin levels (Adams & Holloway, 2004). So, it is important to investigate the dysregulation of neurotransmitter synthesis in the CNS and peripheral organs to understand the molecular basis of ASD (Chen et al., 2021).

Acute or chronic manipulation of Trp levels, by depletion or supplementation, has been used to modulate peripheral and central serotonin levels (Jenkins et al., 2016). Undernutrition or over-restriction of Trp decreases brain serotonin levels and consequently causes behavioral changes such as loss of appetite, anxiety, depression, hyperactivity and behavioral impulsivity (Bell et al., 2001). A depletion of Trp, and consequently serotonin synthesis, results in a significant behavioral aggravation in ASD individuals, such as increased irritability and mild depression. (Adams et al., 2011; Hoshino et al., 1986; Kałużna-Czaplińska et al., 2014; Kałuzna-Czaplinska et al., 2010; McDougle et al., 1996; Naushad et al., 2013). Further, Delgado et al. (1991, 1994) proved that a depletion of peripheral Trp stores leads to a reduction in central serotonin levels and can induce depressive symptoms in susceptible individuals. Therefore, the concept of the acute Trp depletion method was thus created based in ingestion of diets low in Trp or by consuming others LNAA. In this way the TRP:LNAA ratio decreases and TRP transport by the BBB is also reduced (Jenkins et al., 2016; Roth et al., 2021). This methodology is crucial to understand the effect of lowered Trp levels and therefore the role of serotonin in the regulation of mood, anxiety and in cognition, including memory, learning and decision making, in both healthy controls and individuals with psychiatric disorders (Browne et al., 2012; Gibson, 2018).

On the other hand, supplementation with Trp has been largely used in research to increase the TRP:LNAA ratio and so facilitate the uptake of Trp into the brain and consequently increase production and release of serotonin (Pais et al., 2023). This method has demonstrated benefits for both cognitive and emotional functions and to reduce the cortisol response to stress (Gibson, 2018). Several authors indicated that daily dietary Trp supplementation could be an approach to treat/improve neuropsychiatric disorders such as stress, mood and anxiety disorders, or even ASD, ADHD, Alzheimer's disease and Parkinson's disease (Beretich, 2009; Kałużna-Czaplińska et al., 2019; Zahar et al., 2023). Lieberman et al. (2016) also proposed the intake of a Trp-supplemented diet as a potential treatment for depression and sleep disorders. Their study revealed that Trp ingestion is inversely correlated to self-reported depression level and positively associated with sleep duration. However, attention should be paid to the use of excessive doses that may have negative consequences. It can activate TDO and the oxidation of Trp, or it can also lead to the inhibition of TPH which consequently decreases brain levels of serotonin and reduces mood (Pais et al., 2023).

Scientific studies indicate that the pathogenesis of ASD may begin in fetal life, where maternal nutritional status during pregnancy might increase the risk (Moussa et al., 2016). Although, due to the various factors that can influence maternal Trp metabolism in pregnancy, it is unclear how gestational Trp fluctuation influence the development of the neuroendocrine system and the composition of the gut microbiota in the offspring. These factors consist mainly of: diet (Trp enriched or depleted diet),

emotional mental states (depression, anxiety), state of health (diabetes, hypertension), drug administration, stress and social support (Huang et al., 2022).

Altered Trp metabolism is a major indicator of maternal prenatal stress. Keane et al (2021) reported significantly reduced Trp and kynurenine plasmatic concentrations in severe pregnancy anxiety and depression (Keane et al., 2021). Prenatal stress has also been shown to decrease the gut microbial abundances that are responsible for metabolizing Trp in the mother and offspring, which alters fetal exposure both to Trp and its metabolites, impacting neurogenesis and microbial development in the fetus (Galley et al., 2021). Placental Trp can be metabolized into serotonin, degraded to kynurenine, or otherwise transferred to the fetus. The embryo receives Trp and its metabolites which regulate development of neurogenesis and neuroendocrine, and also the gut microbiota composition and diversity of the fetus. MGB axis development and its activity are regulated through maternal-fetal transmission of nutrients and microbes, and also by environmental factors, such as disease and medication background and dietary habits. Thus, during gestation, the factors as mentioned earlier can cause fluctuations in placental Trp, consequently altering all the interactions just mentioned (Huang et al., 2022).

Through the transmission from mother to child, the maternal Trp circuit is fundamental for MGB, HPA and hypothalamic-pituitary-gonadal axis development in the offspring, impacting long-term physical, physiological and behavioral outcomes (Hornef & Penders, 2017). It has been reported in individuals with mental disorders and emotional problems that there exists an abnormal metabolism of Trp during prenatal and/or early postnatal development which disrupts the serotonin signaling pathway and impacts neural maturation and synaptogenesis (Baumgarten & Göthert, 2000).

Breast milk is the principal source of Trp for newborns. Trp provided by breast milk is characterized by high antioxidant and anti-inflammatory properties that mainly contribute to the normal development of the CNS and GI tract in newborns, particularly in preterm infants (Nayak et al., 2019; Tsopmo et al., 2009).

It is important to keep Trp levels adequate by controlling dietary Trp and use of appropriate medication therapy throughout gestation and also during breastfeeding to avoid psychosocial disorders, emotional problems and mental illness (Huang et al., 2022).

# CHAPTER 2 | **AIMS OF THE STUDY**

Although Trp has been shown to play a role in the microbiome and serotonin production, its role in the MGB system, particularly during embryonic development, is still unclear.

Several studies have indicated that the pathogenesis of ASD may have an early onset in fetal life, where the nutritional status of mothers during pregnancy may increase risk and severity. Thus, prenatal diet is essential for the trajectory of brain development and may influence the risk of developing ASD.

Therefore, it is important to understand the effect of the prenatal Trp diet on shaping the microbiota, brain function and behavior, and the onset of core symptoms of ASD.

By conducting a longitudinal study, we aim to explore the following objectives:

1 – Investigating the influence of maternal Trp-rich diet on the neonatal development of the offspring, with a particular focus on physical, cognitive and pro-social skills and early communication;

2 – Understand the influence of maternal Trp-rich diet on juvenile offspring behavior with a particular focus on anxiety, communication and social interactions, repetitive behavior and memory/learning;

3 – Studying if Trp-rich diet during pregnancy influence maternal behavior;

4 - Exploring if prenatal Trp diet plays a role in serotonin levels in the offspring brain, focus on hypothalamus.

# CHAPTER 3 | **MATERIALS AND METHODS**

## 3.1. ANIMALS

Eighty-five animals (30 pups from 4 C57BL/6J *wild-type* (WT) dams and 44 pups from 7 *Nf1*$^{+/-}$ dams) were used in the experiments. *Nf1*$^{+/-}$ mice were generated by crossing *Nf1*$^{+/-}$ animals with mice with C57BL/6J background at least 10 times to keep the genetic background constant across experiments (Silva et al., 1997). To experimental groups, C57BL/6J WT females and *Nf1*$^{+/-}$ females were mated with males with 129T2/SvEmsJ background. The maintenance of the same background between experimental groups aims to eliminate/reduce the genetic effects of the background on the behavior and/or physiology of the animal.

This study was conducted following the subsequent timetable (Figure 6).

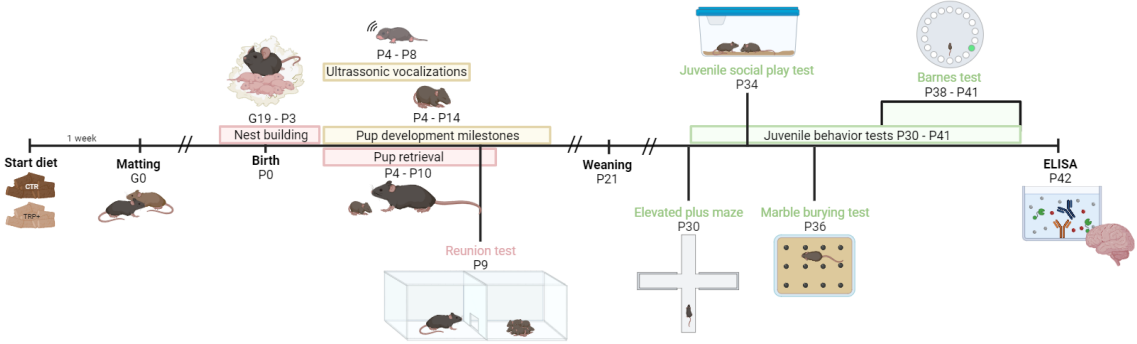



**Figure 6 | Schematic representation of the timetable**
One week before matting, the females were fed a control diet or a diet supplemented with tryptophan (Trp) (described below). After the birth of the litter, pup development tests are performed including recording ultrasonic vocalizations (USVs). Behavioral and maternal care tests (nest building test, pup retrieval and reunion test) are also conducted in the last days of gestation and in the early-life period. Later, at juvenile age of the litter, behavioral tests are performed such as: elevated plus maze for anxiety-like behavior; juvenile social play test for social interaction; marble burying test to analyze repetitive behavior and the last one, Barnes for learning and memory. At the end, the mice are sacrificed and the amounts of Trp, serotonin and kynurenine in the hypothalamus are analyzed by ELISA.

One week before matting to weaning, dams will be divided into four experimental groups: WT dams feeding with control diet (0.70% Trp, CTR), WT dams feeding with Trp-rich diet (1.50% Trp, TRP+), *Nf1*$^{+/-}$ dams feeding with CTR diet and *Nf1*$^{+/-}$ dams feeding with TRP+ , formulated by Mucedola (Milão, Italy) with the following composition described in Table 1 and based in previous work (Browne et al., 2012).

**Table 1 | Description of diet composition**
Tryptophan was the only amino acid that modified between the diets (0.7% in CTR diet and 1.5% in TRP+ diet).

| Diet components | % |
|---|---|
| Sucrose | 3.68 |
| Crude oil | 3.00 |
| Mineral mix | 2.01 |
| Glycine | 0.87 |
| Lysine | 0.97 |
| Histidine | 0.50 |
| Methionine | 0.47 |
| Phenylalanine | 0.89 |
| Leucine | 1.57 |
| Isoleucine | 0.84 |
| Threonine | 0.72 |
| Valine | 0.96 |
| Tyrosine | 0.65 |
| Arginine | 1.09 |
| Tryptophan | 0.70 / 1.50 |

Since our main objective is the study of offspring, they are identified in 6 different experimental groups (Figure 7):

- WT pups from WT dam with CTR diet;
- WT pups from WT dam with TRP+ diet;
- WT pups from *Nf1*$^{+/-}$ dam with CTR diet;
- WT pups from *Nf1*$^{+/-}$ dam with TRP+ diet;
- *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with CTR diet;
- *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with TRP+ diet.

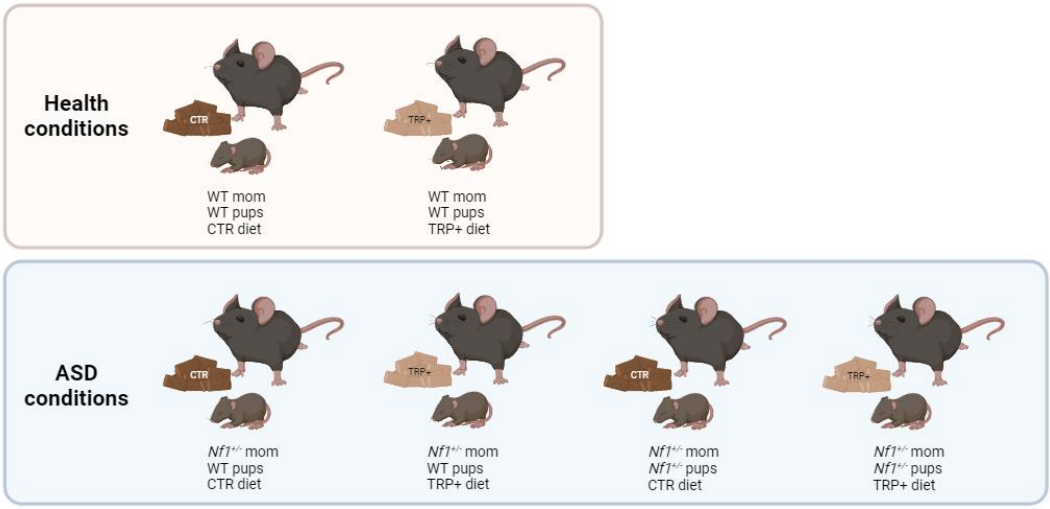



**Figure 7 | Schematic representation of experimental groups**
In total six experimental groups were established for this study, 2 in health conditions: *wild-type* (WT) pups from WT dam with control diet (CTR) diet and WT pups from WT dam with Trp-rich (TRP+) diet; and

4 in autism spectrum disorder (ASD) conditions: WT pups from *Nf1*$^{+/-}$ dam with CTR diet, WT pups from *Nf1*$^{+/-}$ dam with TRP+ diet, *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with CTR diet and *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with TRP+ diet.

At postnatal day (P)3, pups were identified with permanent tattoos on the toes, and tails tips were collected for posterior genotyping. All animals were measured on milestone days (P4 to P14) and weighed every 2 days until the end of the protocol (P42). At the end of the study, a brain index was calculated based on body and brain weight to normalized brain weight (Equation 1) (Santos et al., 2023):

$$Brain\ index = \frac{brain\ weight}{body\ weight} \quad \text{(eq 1)}$$

All pups were housed together with the dam until P21 (weaning), at which point each litter was segregated by sex. All animals were maintained in a housing room with a 12 h light/12h dark cycle, at 21 ± 2 °C, in animal facilities at ICNAS, University of Coimbra. The experiments were carried out in accordance with the European Union Council Directive (2010/63/EU), National Regulations and ORBEA board of the ICNAS and conducted under the authority of the Project License (8/2021).

## 3.2. BEHAVIORAL TESTS

### 3.2.1. Pup developmental milestones

Developmental milestone tests were performed on P4, 6, 8, 10, 12 and 14, in the same order, in a quiet room during the light period (8h00 – 13h00).

3.2.1.1. Surface righting reflex

The surface righting reflex measures the motor ability of a pup to be able to turn on its paws from a back position. The pup was positioned on its back on a plane surface, held for 5 seconds by the operator and then released. The amount of time required to return to a four-limbed position after release was noted, with a cut-off time of 30 seconds (Figure 8 A1, A2) (Feather-Schussler & Ferguson, 2016).

3.2.1.2. Negative geotaxis reflex

The negative geotaxis test is important for assessing motor coordination in young mice. The pup was positioned downward on a 35° inclined ramp, covered with a plastic net to allow traction. The time taken to turn and direct the front paws to the top was recorded, with a cut-off time of 30 seconds. If the puppy either fell or went down the ramp, it was allowed a maximum of 2 additional attempts, after which the time limit was recorded (Figure 8 B1, B2) (Feather-Schussler & Ferguson, 2016).

3.2.1.3. Nest seeking

A rectangular plastic field (25cm x 10cm) was divided into 3 compartments by drawing a goal line on each side. Home bedding material and fresh bedding material in a similar amount was placed in the left and right compartment respectively. In the first trial, the pup was placed in the central compartment, at a 90° angle to the bedding compartments, and was allowed to explore the area. The time taken to cross the goal line of the compartment with home bedding material with both forepaws was registered, with a cut-off time of 120 seconds. After 30 seconds of finishing the first trial, a second one is performed however this time the pup was positioned facing opposite side of the arena to discard possible head turning preferences. It's important to notice that, throughout the experiment for all pups, the pup's position in the arena was alternated between the two trials. If the pup crossed the compartment with fresh bedding material, cut-off time was scored. The final score was calculated by averaging the scored attributed in each trial (Figure 8 C1,C2) (VanRyzin et al., 2016).

3.2.1.4. Cliff aversion

Cliff aversion tests reflexes, strength and coordination. The pup was positioned with the nose and toes of the forepaws hanging over the border of an elevated (10 cm) planar platform above the table. The time required to turn the face and forepaws

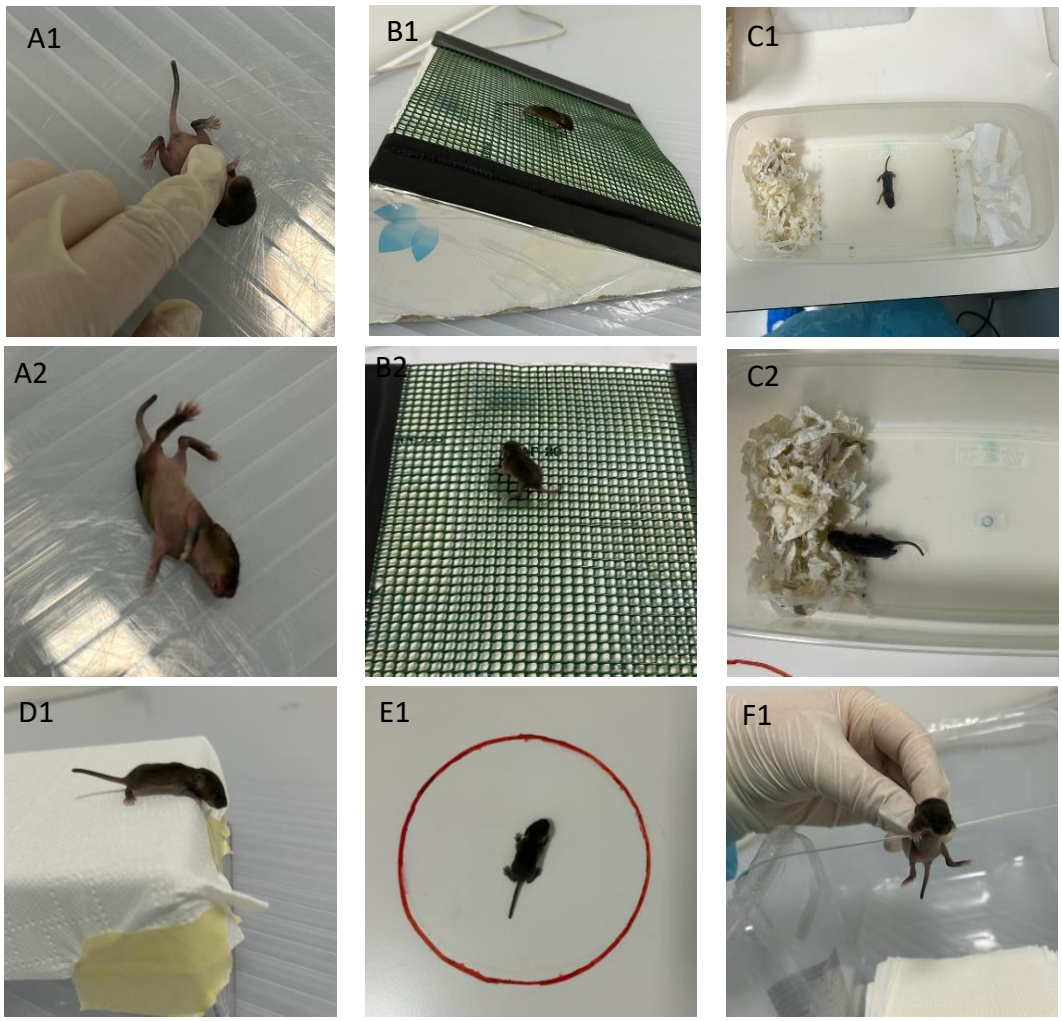
away from the border was recorded, with a cut-off time of 10 seconds. If the puppy fell from the platform, it was allowed a maximum of 2 additional attempts, after which the time limit was recorded (Figure 8 D1,D2) (Feather-Schussler & Ferguson, 2016).

3.2.1.5. Locomotion

This test, as the name suggests, tests the pup's ability to locomote. The pup was positioned in the center of a circle with a diameter of 13 cm. The time taken to cross the border of the circle with the two front paws, with a cut-off time of 30 seconds was recorded, with a cut-off time of 30 seconds (Figure 8 E1,E2) (VanRyzin et al., 2016).

3.2.1.6. Forelimb grasp

This test determines the strength of the posterior limbs. The pup was suspended by its forepaws on a horizontal wire that was 10 cm above a padded surface. The time between the operator releasing the pup and falling into the padded area was recorded with a cut-off time of 10 seconds. If the puppy is not capable of holding to wire or immediately falls after being released, it was given a maximum of 2 additional attempts (Figure 8 F1,F2) (VanRyzin et al., 2016).



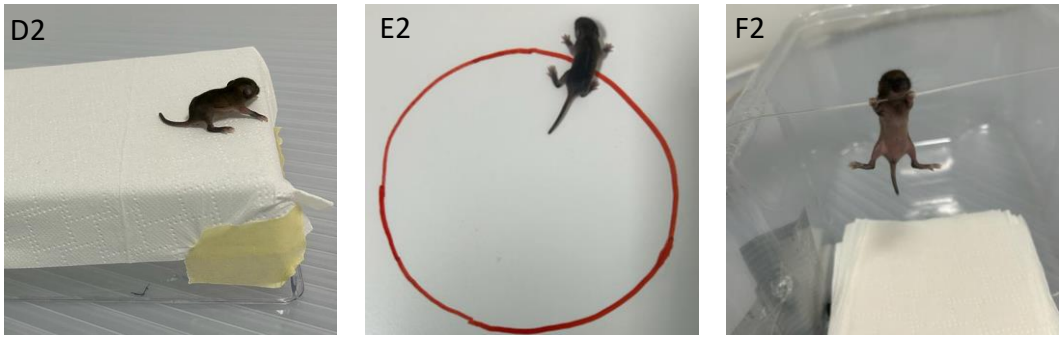



**Figure 8 | Developmental milestones tests**
Representation images of A. Surface righting reflex; B. Negative geotaxis reflex; C. Nest seeking; D. Cliff aversion; E. Locomotion; F. Forelimb grasp tests.

3.2.1.7. Auditory startle

A loud noise with a latex glove was produced near the pup's head. The pup was observed to see whether it reacted to the noise by shaking or quickly turning its head.

3.2.1.8. Eye opening

Each pup was observed to check that their eyes were open.

3.2.1.9. Pup ultrasonic vocalizations recording and analysis

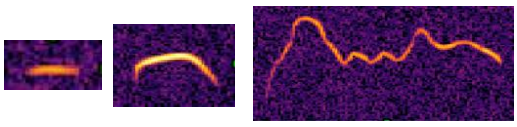
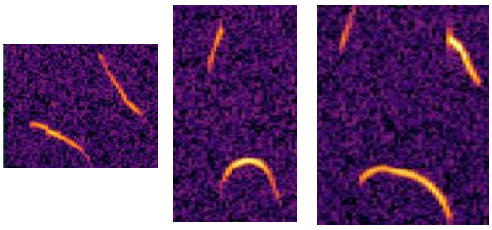
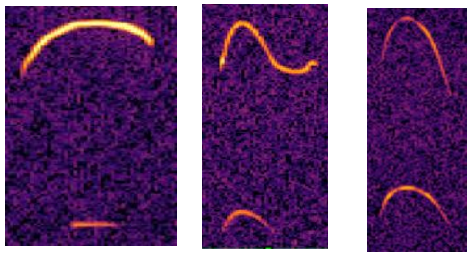
An anechoic chamber (55 cm x 50 cm x 70 cm) was set up with sheets of thick acrylic (1.5 cm) and completely covered with absorbent foam inside in order to block external sound (Figure 9A). The USVs of the pups were recorded on P4, 6 and 8, after the developmental milestone tests, also during the light period (8h00 – 13h00). The order in which the ultrasonic vocalizations (USVs) of each pup were recorded is the same order that the puppies were tested at developmental milestones previously. The pup was placed in a plastic container with tissue paper to maintain body temperature, which was then placed inside the chamber (Figure 9B). USVs recordings are recorded once the camera door closes and finish 5 minutes later, then the pup is placed back to its origin box. An ultrasound recording system is used with an Avisoft CM16/CMPA condenser microphone placed above the bottom of the test container, an UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany).

After the recording of the USVs, sonograms were generated, with FFT-length 512 points, 16-bit format, sampling frequency 250 kHz, time resolution 1 ms, frequency resolution 488 Hz, overlap 50%. USVs were further analyzed using MATLAB toolbox DeepSqueak version 3.0.4., which allowed the extraction of individual mouse USVs calls by applying the Mouse Call_Network_V2 neural network, with a chunk length analysis of 6 seconds, overlap of 0.1 seconds, high frequency cut-off of 125kHz and no score threshold.

**Figure 9 | Ultrasonic vocalizations recording system**
A. Anechoic chamber fully covered with absorbing foam to block external sound. B. Example of a recording of a mouse pup inside the box.

Several parameters were extracted such as: total number of USVs, total time of vocalization, latency (time to first call), call length, mean inter-call (silent time between vocalizations), mean power, tonality and principal, low and high frequency. Additionally, each vocalization was classified and separated into 3 different categories, single, multi-syllabic and stacked according to their complexity as summarized on Table 2 (Young et al., 2010):

**Table 2 | Description of each ultrasonic vocalizations category and respective examples**

| Call category | Description | Example |
|---|---|---|
| Single | A single waveform present in the sonogram, with no frequency shifts and no overlapping waveforms in time. |  |
| Multi-syllabic | Two or more waveforms that immediately follow each other with a frequency shift, with no time gap between them and no overlapping waveforms in time. |  |
| Stacked | Two or more waveforms which overlap in time |  |

### 3.2.2. Juvenile behavior tests

All the juvenile behavior tests were conducted between P30 and P41 during light period (8h00 – 13h00), in a quiet room. The animals were placed in the test room for 1 hour for acclimatization before each behavioral test.

#### 3.2.2.1. Elevated plus maze

The elevated plus maze (EPM) is used to evaluate anxiety-like behavior in mice. This test consists of four elevated arms connected by a central platform, forming a cross. Two opposite arms are closed (walled) and the other two opposite arms are open (Figure 10A). At P30, the mice were placed in the center of apparatus and allowed to freely explore the maze for 5 min (Figure 10B) (Walf & Frye, 2007). Light exposure was measured before test in different points of the maze to ensure it was between 80 and 100 lux. The test was video recorded with Microsoft LifeCam HD-3000 for later analysis.

Additionally, an anxiety index was calculated using the following equation 2 (Cohen et al., 2013; Tabbai et al., 2019):

$$Anxiety\ index = 1 - \left[\frac{\left(\frac{time\ in\ open\ arms\ (seconds)}{test\ duration\ (seconds)}\right) + \left(\frac{number\ of\ entries\ in\ open\ arms}{total\ numer\ of\ entries}\right)}{2}\right] \quad \text{(eq 2)}$$

This index varies between the values 0 and 1 and the closer to 1, the more anxious the mice are.

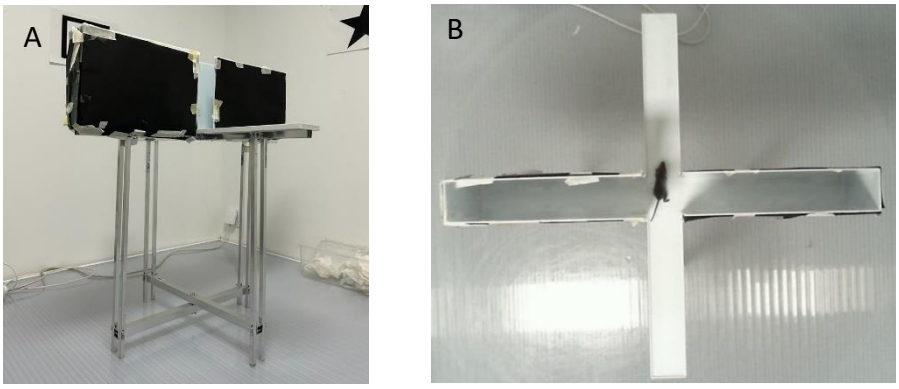



**Figure 10 | Elevated plus maze test**
A. Representation of configuration of the elevated plus maze test; B. Overhead view of an animal during the elevated plus maze

#### 3.2.2.2. Juvenile social play test

To stimulate social behavior, 24h before the test, the animals were isolated in new clean cages with fresh bedding. At P34, two animals of the same sex and genotype were placed in a box (35 cm x 35 cm) in the anechoic chamber (Figure 11). The animals were allowed to interact freely for 30 min and their behavior and USVs production was

recorded for later analysis (Ferreira et al., 2022). At the end of the experiment, both animals were returned to their home cage. Actions considered as social behaviors included affiliative interactions (allogrooming, group sitting, push under), investigative interactions (sniffing, following, mutual circle), play solicitation (approach, crawl, push past), and agonistic interactions (threat, aggression, defense, flight, submission). The number and duration of each social interaction was registered. In addition, the number of USVs in a social and non-social context was also analyzed. Also, a USVs social index was calculated based on the number of social and non-social USVs:

$$USVs\ Social\ index = \frac{number\ of\ USV\ social - number\ of\ USV\ non-social}{number\ of\ USV\ social + number\ of\ USV\ non-social} \quad \text{(eq 3)}$$

This index can range from -1 to 1, whereby positive values indicate better social communication.

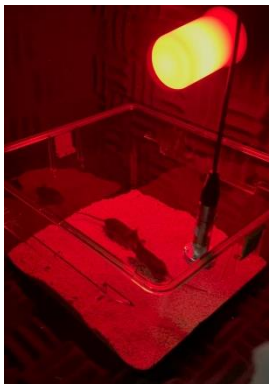


**Figure 11 | Juvenile social play test**
Representation of the juvenile social test inside the anechoic chamber for ultrasonic vocalizations (USVs) and video recording.

3.2.2.3. Marble burying test

A standard mouse cage was walled with acrylic sheet and filled with an 8 cm layer of sawdust. Twelve marbles were placed equidistantly on top of the sawdust in a 3x4 arrangement. At P36, the animal was placed on the cage and allowed to explore it freely for 20 minutes. The number of marbles buried every 5 minutes of the experiment were recorded (Figure 12 A, B) (Angoa-Pérez et al., 2013).

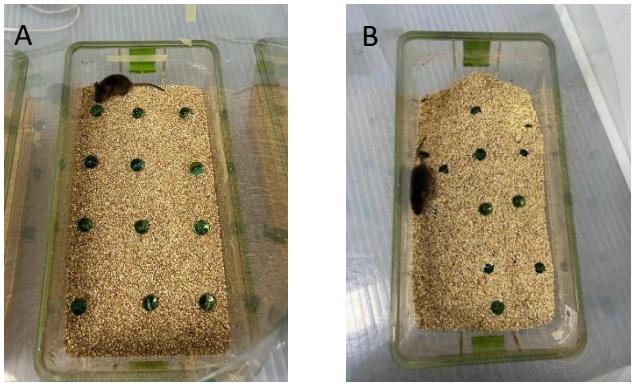


**Figure 12 | Marble burying test**
Representation of marble burying test. Standard mouse cage filled with sawdust with twelve marbles placed equidistantly on top. Example of an animal during test at A. begin time; B. end time

3.2.2.4. Barnes test

Finally, at P38 to P41, Barnes test was performed which consists of two parts: 3 days of learning and the fourth day to assess memory. The Barnes maze consist in an elevated circular platform with an escape hole, which leads to a small chamber attached underneath the platform. There are 19 other imitation holes circled around the maze look like the escape hole but do not lead to an escape chamber in order to distract from the real hole (Figure 13A). Because mice have natural preference for dark environments (Gawel et al., 2019), a bright light was used to stimulates the animals to complete the task so they will tend to search the target hole and hide.

On day one, the mouse was placed for 30 seconds inside the opaque cylinder in the middle of the platform to an adaptation period. After that, the cylinder was removed (Figure 13B) and the mouse was guided by hand to the escape hole. The main objective is to familiarize the animal with the maze and to reduce anxiety in a way that not interferes with the animal behavior. The first trial started 15 minutes later, in which the mouse was placed again in the starting cylinder for 30 seconds and then released to explore freely in order to find the escape hole (Figure 13C) for three minutes. If the animal enters the target (Figure 13D) before the end of the cut-off time (3 min), the test end immediately and was then returned to its original housing cage. If not, the animal is guided to the target and after that, the cylinder is put on the top of it for 30 seconds.

After 3 days of training period with four trial each with 15 minutes between, with the same procedures as describe above, in 4$^{th}$ day, a probe trial was performed. The mouse once again was placed on the maze however, this time, the escape hole was covered, to mimic all the other holes in order to access memory of target localization (Figure 13E) (Attar et al., 2013). After 90 seconds of freely exploring, the mouse was placed in its original housing cage.

The training and probe days were video recorded for later analysis with a video camera (Microsoft LifeCam HD-3000). The escape latency (time to enters the target), time to find target, time in target zone, exploring errors, total distance and search strategies was analyzed.

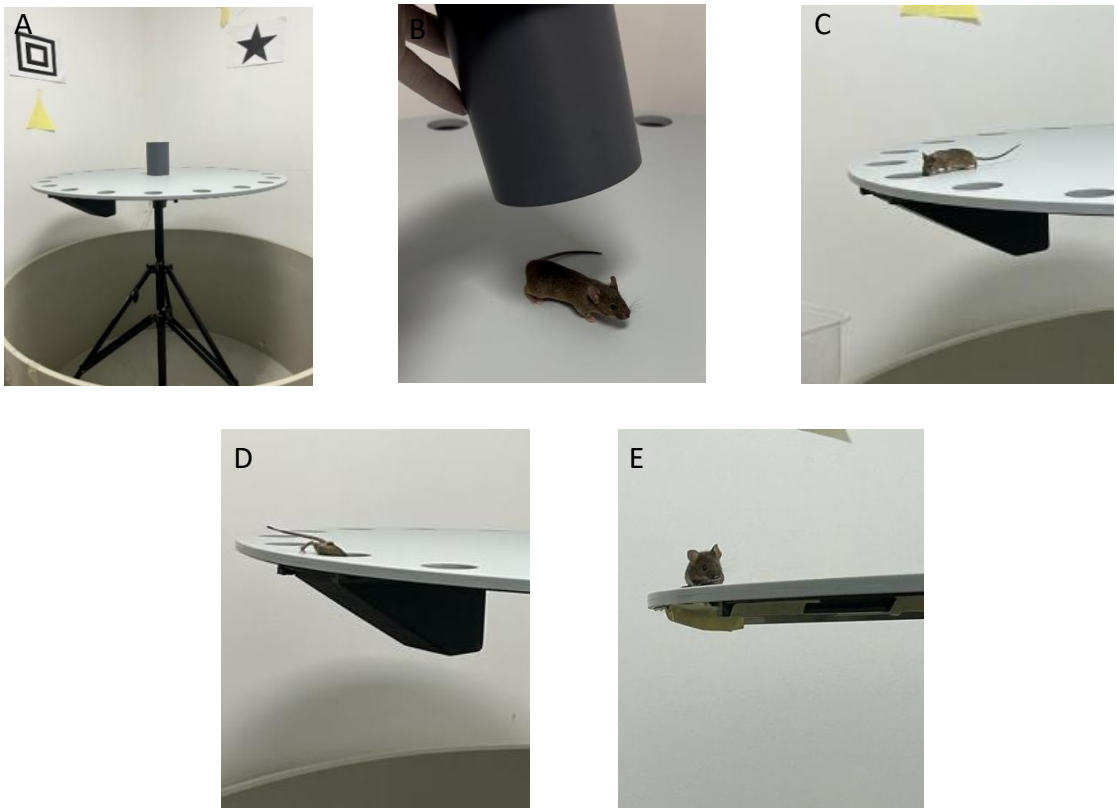



**Figure 13 | Barnes test**
A. Representation of setup of the Barnes test; B. Mouse being released from the start chamber; C. checking the target; D. entering the escape tunnel; E. probe day, the target has been covered.

### 3.2.3. Maternal behavior tests

Maternal behavior tests were performed during the light period (8h00 – 13h00), in a quiet room.

3.2.3.1 Nest building test

Each litter box was provided with a house-like shelter and nesting material. In the last 3 days of gestation until the first 3 days of the litter birth (G19 (Gestational day) to P3), photographs were taken of the nest and the nesting ability was scored in accordance to the following classification (Yun et al., 2019):

1 – paper dispersed, with no visible formed nest-like structure (Figure 14A);

2 – paper lightly scattered, with a nest-like structure (Figure 14B);

3 – nesting material forms a recognizable nest structure within house-like shelter, with only small amounts of scattered paper (Figure 14C);

4 – nesting material is well organized to form a full nest within house-like shelter (Figure 14D).

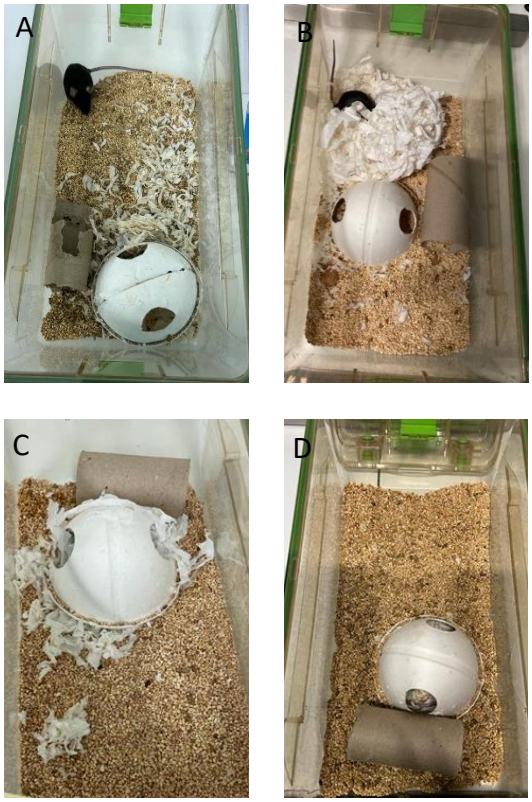



**Figure 14 | Nest building scores**
Representative images of nesting scores A. Score 1; B. Score 2; C. Score 3; D. Score 4.

3.2.3.2 Pup retrieval latency

Following the assessment of developmental milestones, at P4, 6, 8 and 10, each pup was returned to its home cage and placed in the farthest corner of the pup's nest. The time taken for the mother to collect the pup (Figure 15) and take it back to the nest was recorded (Wu et al., 2009).

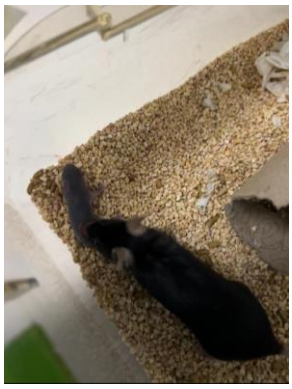


**Figure 15 | Pup retrieval**
Representation of the dam collecting the pup.

3.2.3.3 Reunion test

On P9 of litter, a transparent arena was prepared divided into two similar compartments of 20 cm x 42 cm each, with a removable transparent wall between them

(Panlab, Barcelona, Spain) (Figure 16A). The test was video recorded for later analysis with a video camera (Microsoft LifeCam HD-3000)

The test consisted in 3 phases: the first one, the habituation phase, where both dam and pups were placed on one side of the arena, with the partition closed, and allowed to freely explore for 5 min (Figure 16B). The second phase consisted of a separation phase, where the dam was placed for 3 min in the other compartment - the novel compartment - with the partition closed (Figure 16C). She is not allowed to have physical contact with the offspring but can see, listen and smell them. Finally, the reunion phase, the partition was removed and the dam was allowed to explore freely both compartments during 5 minutes (Figure 16D) (Guoynes & Marler, 2021).

An operator manually analyzed behavior such as, time spent exploring (investigating the environment) and grooming (licks its fur, grooms with forepaws and scratches a member) on either side of the arena, huddling the litter (any interaction with the pups, e.g. smell, touch or pick them) or on the partition next to the litter during the separation phase of the test.

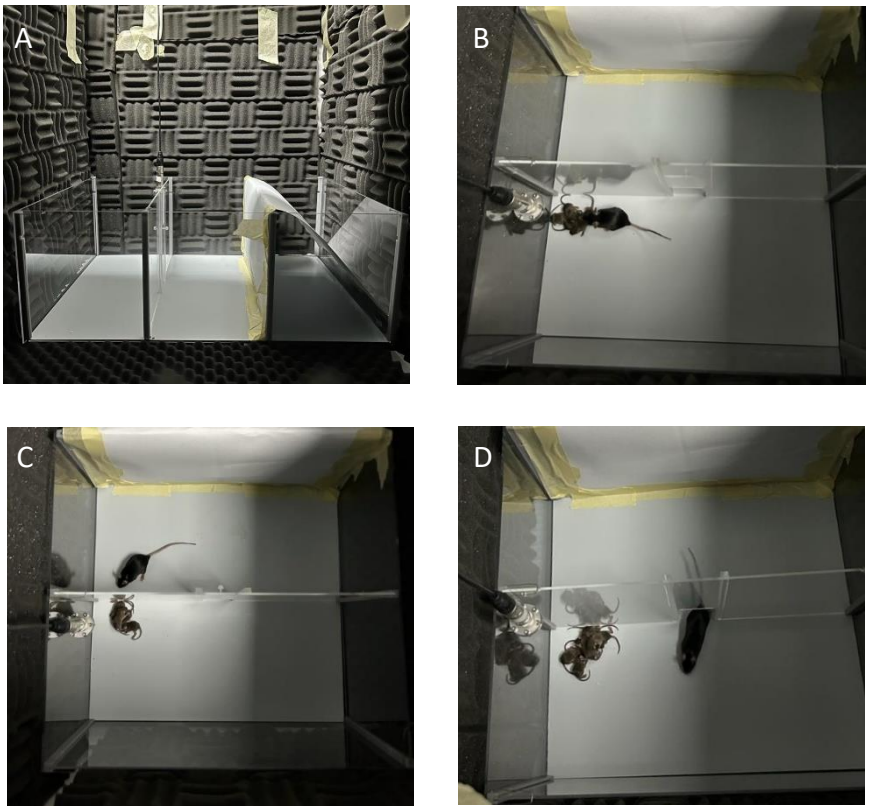



**Figure 16 | Reunion test**
A. Clean arena with two compartments divided with a transparent removable partition. Representation of: B. Habituation phase; C. Separation phase; D. Reunion phase.

### 3.3. ELISA ASSAY

Litters were sacrificed at P42, brain was removed and hypothalamus was dissected and stored at -80°C until further used. Hypothalamus was weighted and then the protein was quantified by the BCA method to obtain concentration of total protein. This method consists of homogenization the region of the brain in question in PBS with a protease inhibitor tablet (in a volume/weight ratio of 1:30) and centrifuged at 10000g during 5 minutes at 4°C. The supernatant is removed, quantified and stored for future use.

#### 3.3.1. Tryptophan ELISA

For Tryptophan ELISA procedure, manufacturer's instructions were followed (Tryptophan ELISA kit, Axxora, USA). Prepared standards and samples were incubated with tryptophan antiserum during overnight at 4°C. Next day, the wells were washed following incubation with enzyme Conjugate for 30 min at room temperature on a shaker. Then, samples were washed again and incubated with substrate under the same conditions as previously. To conclude, stop solution was added and the absorbance of samples was read at 450 nm.

#### 3.3.2. Serotonin ELISA

For serotonin ELISA procedure, manufacturer's instructions were followed (Serotonin ELISA kit, Enzo Biochem, USA). Samples and standards were incubated with serotonin conjugate and serotonin antibody for 2h at room temperature with shaking. Then, the wells were washed following incubation with substrate solution for 1 hour at room temperature with shaking. Finally, the stop solution was added and the optical density of the wells was measuring in a plate reader at 405 nm.

#### 3.3.3. Kynurenine ELISA

For Kynurenine ELISA procedure, manufacturer's instructions were followed (Kynurenine ELISA kit, Axxora, USA). Acylated standards and samples were incubated with Kynurenine antiserum during overnight at 4°C. Next day, the wells were washed following incubation with enzyme Conjugate for 30 min at room temperature on a shaker. Then, samples were washed again and incubated with substrate under the same conditions as previously. To conclude, stop solution was added and the absorbance of samples was read at 450 nm.

### 3.4. GENOTYPING

Tails tips were digested in a lysis solution [10mM Tris, 5mM EDTA, 200mM NaCl, 0.3% SDS, 2.5% proteinase K (Invitrogen, Waltham, MA, USA)], for 4 hours at 55°C with shaking. The resultant solution was centrifuged (1730R, Gyrozen, Gimpo, South Korea) at 12000 rpm for 15 minutes at 4°C. The supernatant was collected and the DNA was precipitated in two consecutive centrifugations at 4000 rpm for 15 min at 4°C with 100% ethanol and 75% ethanol (Sigma-Aldrich, St. Louis, MO, USA). The pellet was left to dry overnight and resuspended in TE buffer (10Mm Tris, 1mM EDTA, pH 8.0). The DNA chains were untangled at 70°C for 10 minutes. DNA was quantified (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and PCR was set up with Taq Master Mix 50% (Bioron Diagnostics GmbH, Römerberg, Germany), primers 5% (Metabion, Planegg, Germany) (NF1-P132, NF1-P133 and NF1-P134), PCR Water (Bioron Diagnostics GmbH, Römerberg, Germany) until the volume was complete and DNA (200ng/uL). PCR was performed (T100 Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA) with the following variations temperatures: 2 min 94°C; 34 cycles of 45 seconds 94°C, 1 min 56°C, 1 min 72°C; 10 min 72°C; infinite hold 4°C. Loading buffer 6x (Thermo Fisher Scientific, Waltham, MA, USA) was added to each PCR product and the resultant solutions were loaded onto a 3% agarose gel (NZYTech, Lisbon, Portugal) in TAE (40mM Tris, 5mM EDTA, 5.71% acetic acid, pH 8.0) with GreenSafe Premium (NZYTech, Lisbon, Portugal) for visualization of the bands. Electrophoresis was performed on a system with TAE at 110 V for 30 min (PowerPac, Bio-Rad Laboratories, Hercules, CA, USA). Results were observed with UV light.

### 3.5. STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA). Outliers were identified as all values outside the (mean ± standard deviation) interval, and excluded. Between the two groups of health conditions we used two-way or three-way analysis of variance (ANOVA), followed by Turkey or Sidak's multiple comparisons, as indicated in the figure legends. In the four groups of ASD conditions we used three-way analysis of variance (ANOVA), followed by Turkey or Sidak's multiple comparisons, as also indicated in the figure legends. Data were considered as statistically significant at $p<0.05$.

# CHAPTER 4 | **RESULTS**

This method of supplementation with Trp can be studied in two major fields of research (Nayak et al., 2019; Steenbergen et al., 2016). The first field is focused on healthy individuals with normal serotonin levels in the brain. In the second case the research is focused on individuals with neurodevelopmental disorders associated with an impairment in the serotoninergic system, such as ASD patients. Thus, this study will also be divided into the two domains.

## 4.1. EFFECT OF PRENATAL TRYPTOPHAN DIET IN HEALTH CONDITIONS

Since our main objective in this first part is to study the effect of the prenatal Trp diet in health conditions, these results are only regarding offspring that have been segregated by sex to form the following experimental groups:

- Female WT pups from WT dam with CTR diet (female CTR);
- Female WT pups from WT dam with TRP+ diet (female TRP+);
- Male WT pups from WT dam with CTR diet (male CTR);
- Male WT pups from WT dam with TRP+ diet (male TRP+).

### 4.1.1. Prenatal tryptophan suplemented diet affects body weight and length in health conditions

To investigates if the prenatal Trp diet affects the offspring's body weight, they were weighed every 2 days. Although no significant differences were found during the weaning period, after weaning (P21) several differences were observed. We found that overall males had a higher weight compared with females (Figure 17 and Table 3).

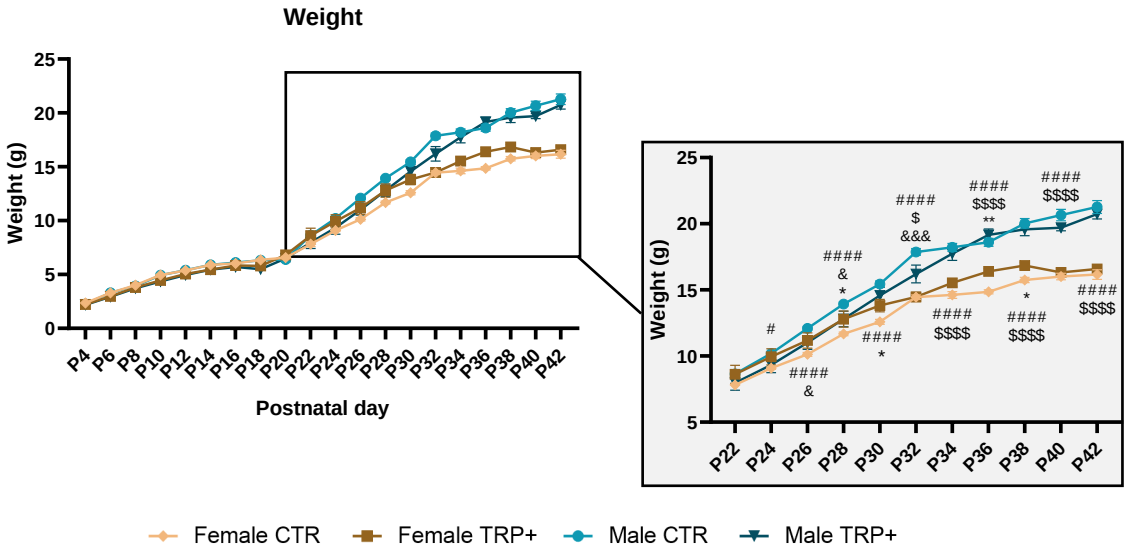


**Figure 17 | TRP+ diet led to a higher body weight of the offspring in health conditions**
Body weight was measured every 2 days until P42 and TRP+ led to a higher body weight of the offspring. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

**Table 3 | Significant differences in weight of offspring in health conditions**

| P24 | P value |
|---|---|
| Female CTR vs male CTR | **0.0394** |
| P26 | |
| Female CTR vs male CTR | **<0.0001** |
| Male CTR vs male TRP+ | **0.0461** |
| P28 | |
| Female CTR vs female TRP+ | **0.0379** |
| Female CTR vs male CTR | **<0.0001** |
| Male CTR vs male TRP+ | **0.0352** |
| P30 | |
| Female CTR vs female TRP+ | **0.0168** |
| Female CTR vs male CTR | **<0.0001** |
| P32 | |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **0.0002** |
| Male CTR vs male TRP+ | **0.0004** |
| P34 | |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **<0.0001** |
| P36 | |
| Female CTR vs female TRP+ | **0.0012** |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **<0.0001** |
| P38 | |
| Female CTR vs female TRP+ | **0.0410** |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **<0.0001** |
| P40 | |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **<0.0001** |
| P42 | |
| Female CTR vs male CTR | **<0.0001** |
| Female TRP+ vs male TRP+ | **<0.0001** |

The increment of weight between P4 and P20 (Figure 18A) and between P20 and P42 (Figure 18B) was also analyzed. Although in the breastfeeding period there was no

difference in weight augment between experimental groups, after weaning it was possible to observe differences between males and females of both diets (female CTR = 8.343 ± 0.347 vs male CTR = 12.62 ± 0.509, p<0.0001; female TRP+ = 6.323 ± 0.306 vs male TRP+ = 11.29 ± 0.433, p<0.0001), but also between females CTR and females TRP+ (female CTR = 8.343 ± 0.347 vs female TRP+ = 6.323 ± 0.306, p=0.0411). Overall, there was an effect of sex (F (1, 18) = 82.65, p<0.0001) and also of diet (F (1, 18) = 10.86, p=0.0040).

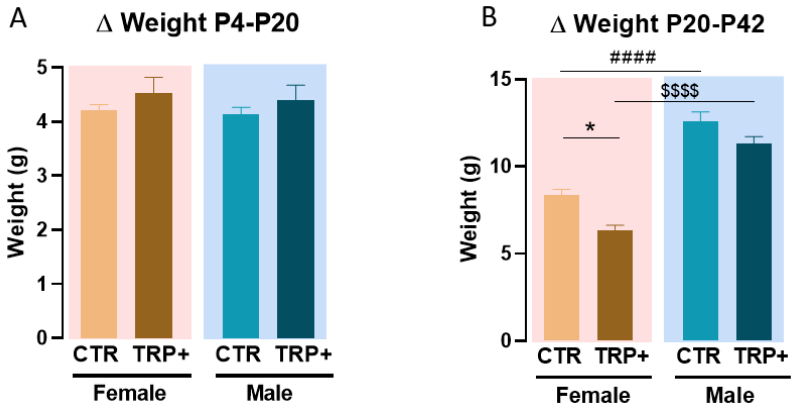



**Figure 18 | Females TRP+ showed less weight increase of offspring in health conditions**
Although there was no difference during the (A) breastfeeding period (P4-P20), (B) after weaning (P20-P42) females TRP+ showed a lower weight increment. Furthermore, after weaning, males showed a higher increase than females. A. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR diet) = 7; n (female TRP+ diet) = 8; n (male CTR diet) = 8; n (male TRP+ diet) = 7; and B. Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female CTR and female TRP+; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 8; n (male TRP+) = 3.

The offspring length was measured from P4 to P14, an important stage of initial and developmental growth and it was observed that there were differences between diets in both sexes (Figure 19 and Table 4).

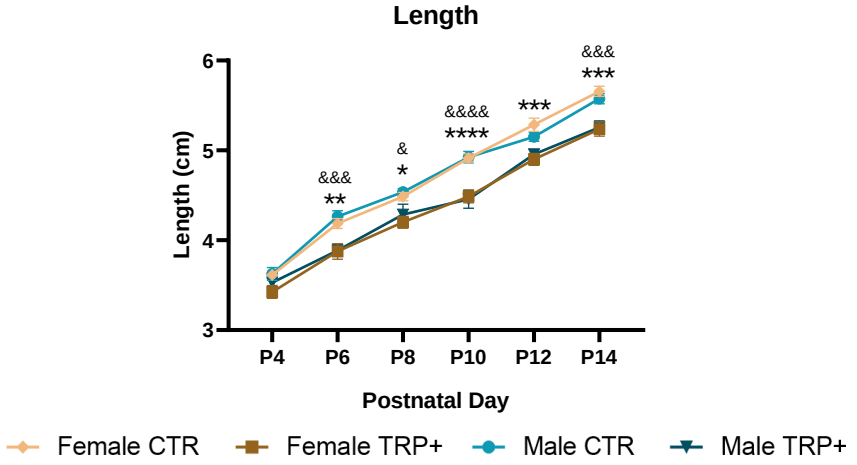



**Figure 19 | TRP+ diet resulted in lower body length of offspring in health conditions**
Length was measured on P4, 6, 8, 10, 12 and 14 and TRP+ diet led to shorter body length in both sexes.

Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female CTR and female TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

**Table 4 | Significant differences length of offspring in health conditions**

| P6 | P value |
|---|---|
| Female CTR diet vs female TRP+ | **0.0073** |
| Male CTR diet vs male TRP+ | **0.0007** |
| P8 | |
| Female CTR diet vs female TRP+ | **0.0164** |
| Male CTR diet vs male TRP+ | **0.0443** |
| P10 | |
| Female CTR diet vs female TRP+ | **<0.0001** |
| Male CTR diet vs male TRP+ | **<0.0001** |
| P12 | |
| Female CTR diet vs female TRP+ | **0.0005** |
| P14 | |
| Female CTR diet vs female TRP+ | **0.0001** |
| Male CTR diet vs male TRP+ | **0.0058** |

The increment of length (Figure 20) between this period was also analyzed and despite not observing differences between the experimental groups, there was diet effect (F (1, 26) = 8,087, p=0.0086).

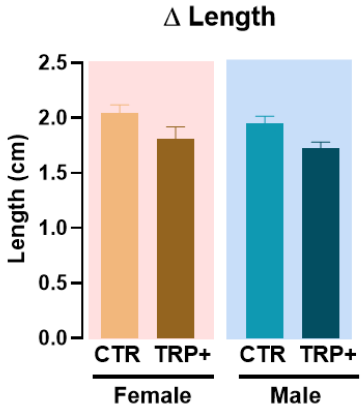



**Figure 20| TRP+ diet did not influence length increase of offspring in health conditions**
TRP+ diet did not influence length growth between P4 to P14. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

In addition, when the animals were sacrificed, the brain was extracted and weighed. To normalized brain weight, a brain index (Figure 21) was analyzed showing that females had a higher index than males in both diets (female CTR = 0.02543 ± 0.000595 vs male CTR = 0.01986 ± 0.000181, p<0.0001; female TRP+ = 0.02463 ± 0.000882 vs male TRP+ = 0.02057 ± 0.00047, p=0.0054). Overall, there was an effect of sex (F (1, 15) = 57.36, p<0.0001).

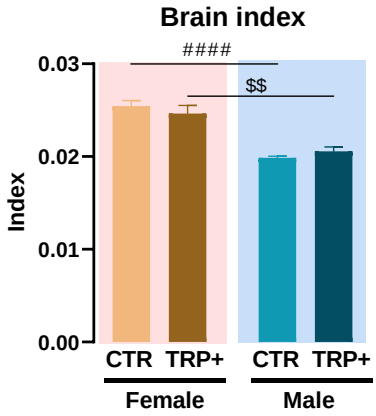



**Figure 21 | TRP+ diet did not influence brain index of offspring in health conditions**
An brain index was measured based on brain and body weight and the TRP+ diet did not influence the brain index, however females had a higher index. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 5; n (male TRP+) = 3.

So, in this study, we concluded that the prenatal TRP+ diet had an influence on length in early-life and, on the other hand, increased body weight in juvenile age of offspring in health conditions. Note that the TRP+ diet did not influence the brain index of offspring.

### 4.1.2. Prenatal tryptophan suplemented diet affects development of motor, vestibular and communicative skills and of pro-social behavior in health conditions

Several developmental milestones were performed to assess the development of sensorimotor skills of different experimental offspring groups at infancy period (P4 to P14).

On surface righting reflex test (Figure 22), we found that sensorimotor early changes. Indeed, at P4, it was observed that males TRP+ had a better development comparing with females TRP+ (female TRP+ = 6.265 ± 1.603 vs male TRP+ = 1.3875 ± 0.139, p=0.0237) and males CTR (male CTR = 6.066 ± 0.3766 vs male TRP+ = 1.3875 ± 0.139, p=0.0384). Further, at P6, female TRP+ scored significantly worse than the females CTR (female CTR = 2,580 ± 0.340 vs female TRP+ = 6.639 ± 1.115, p=0.0365). Overall, there was a significant effect of age in this reflex development ($F(2, 69) = 9.722$, p=0.0002).

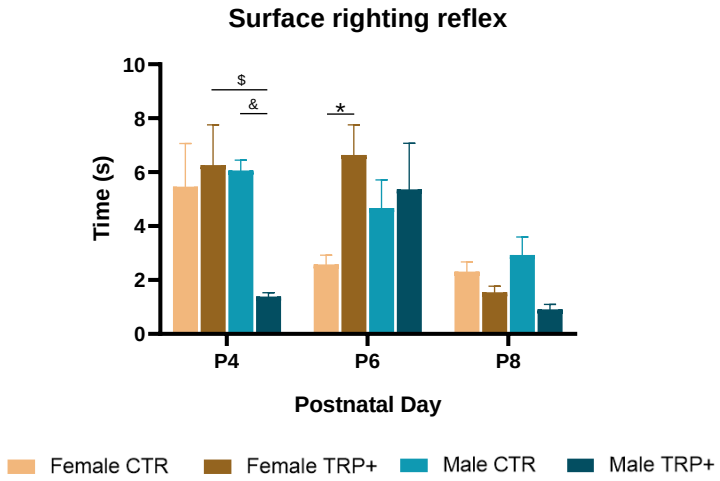



**Figure 22 | Females TRP+ had a delay in surface righting reflex of offspring in health conditions**
Female TRP+ showed a delay compared to male TRP+ and female CTR. On the other hand, male TRP+ showed an improvement over CTR+ males. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; P4: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 7; n (male TRP+) = 4; P6: n (female CTR) = 6; n (female TRP+) = 8; n (male CTR) = 7; n (male TRP+) = 7; P8: n (female CTR) = 7; n (female TRP+) = 7; n (male CTR) = 8; n (male TRP+) = 5.

On negative geotaxis test (Figure 23) there was no significant differences between experimental groups however there was an effect of age ($F(2, 76) = 11.41$, $p<0.0001$).

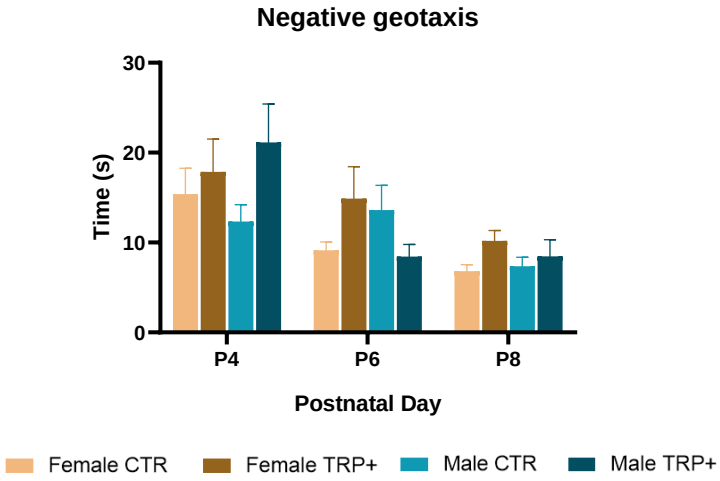



**Figure 23 | TRP+ diet did not influence the development in negative geotaxis test of offspring in health conditions**
TRP+ diet did not influence the development of negative geotaxis. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; P4: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P6: n (female CTR) = 6; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P8: n (female CTR) = 7; n (female TRP+) = 7; n (male CTR) = 8; n (male TRP+) = 7.

In nest seeking test (Figure 24) there were no significant differences between groups but we detected an effect of age in these results (F (2, 80) = 21.47, p<0.0001).

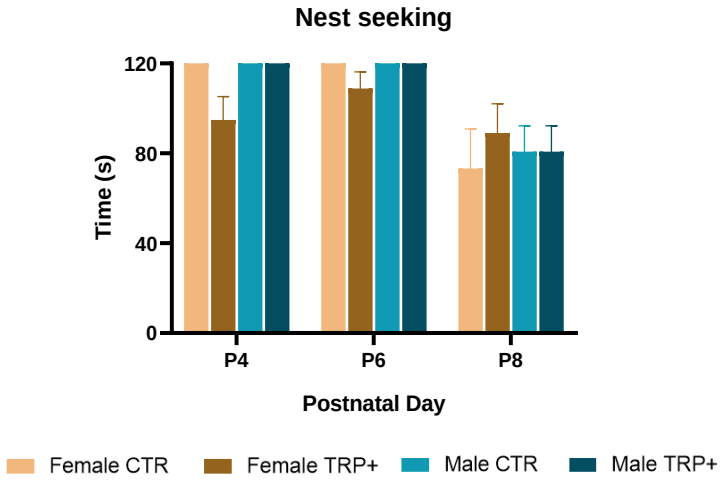



**Figure 24 | TRP+ diet did not influence the development in nest seeking test of offspring in health conditions**

TRP+ diet did not influence the development of nest seeking. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; P4: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 8; P6: n (female CTR) = 7; n (female TRP+) = 7; n (male CTR) = 8; n (male TRP+) = 8; P8: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 8.

On cliff aversion test (Figure 25), males TRP+ at P6 displayed a worse score in the cliff aversion test compared with males CTR (male CTR = 5.525 ± 0.774 vs male TRP+ = 8.464 ± 0.630, p=0.0276) and also with female TRP+ (female TRP+ = 5.523 ± 1.063 vs male TRP+ = 8.464 ± 0.630, p=0.0275). Also, there was a significantly difference in female between diets on P8 (female CTR = 2.817 ± 0.310 vs female TRP+ = 6.116 ± 0.865, p=0.0103) and P10 (female CTR = 2.169 ± 0.467 vs female TRP+ = 5.269 ± 1.150, p=0.0167), in both days females TRP+ showed a delay in the development of this reflex. Overall existed a significant effect in age (F (2, 78) = 13.58, p<0.0001) and diet (F (1, 78) = 23.84, p<0.0001).

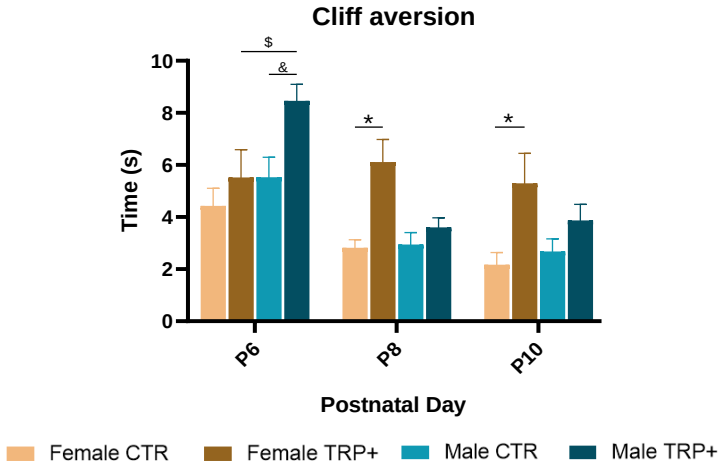


**Figure 25 | Females and males TRP+ showed a delay in development of the cliff aversion reflex of offspring in health conditions**

TRP+ diet showed an impairment in the development of the cliff aversion reflex in both sexes. Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female CTR and female TRP+; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; P6: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P8: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P10: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

Additionally, we observed that exist a significant different at P10 between sexes in CTR diet, males have developed faster locomotion (female CTR = 27.733 ± 1.564 vs male CTR = 17.273 ± 4.170, p=0.0193) (Figure 26). Regarding TRP+ diet, we observed that at P12, female TRP+ had a better development than female CTR diet (female CTR = 16.940 ± 4.143 vs female TRP+ = 4.860 ± 0.965, p=0.0031). Overall, there was a significant effect of age (F (2, 73) = 78.47 p<0.0001).

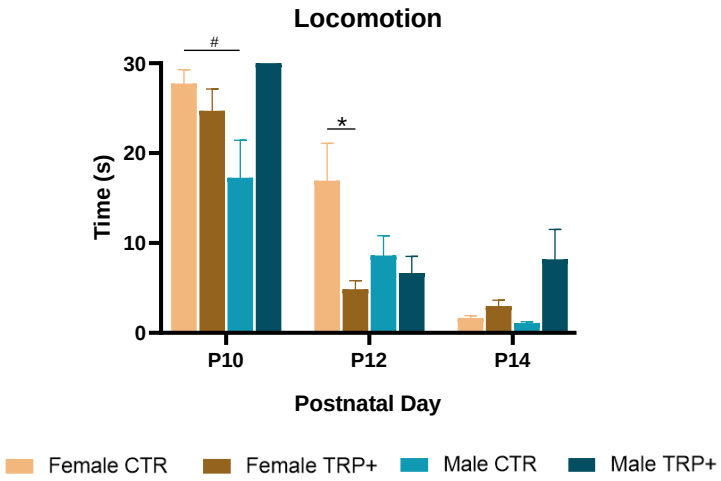



**Figure 26 | Females TRP+ showed an impairment in development of locomotion in offspring under health conditions**

TRP+ diet influenced the ability of locomotion in females. Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female CTR and female TRP+; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+. Data represented as mean ± SEM; P10: n (female CTR) = 6; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 5; P12: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P14: n (female CTR) = 6; n (female TRP+) = 8; n (male CTR) = 7; n (male TRP+) = 7.

Lastly, on forelimb grasp test (Figure 27), on P12 there was some significant differences, females CTR had a faster development comparing with females TRP+ (female CTR = 8.784 ± 0.079 vs female TRP+ = 4.211 ± 0.674, p=0.0021) and males CTR (females CTR = 8.784 ± 0.079 vs males CTR = 5.351 ± 0.747, p=0.0323). However, this differences seems to reverse on P14, where females TRP+ had a better score than the females CTR (female CTR = 3.907 ± 0.529 vs female TRP+ = 9.456 ± 0.222, p<0.0001), and also than males CTR (female CTR = 3.907 ± 0.529 vs male CTR = 7.086 ± 0.978, p=0.0381).

In the last day of development test, the females TRP+ scored better than the males (female TRP+ = 9.456 ± 0.222 vs male TRP+ = 6.163 ± 0.882).

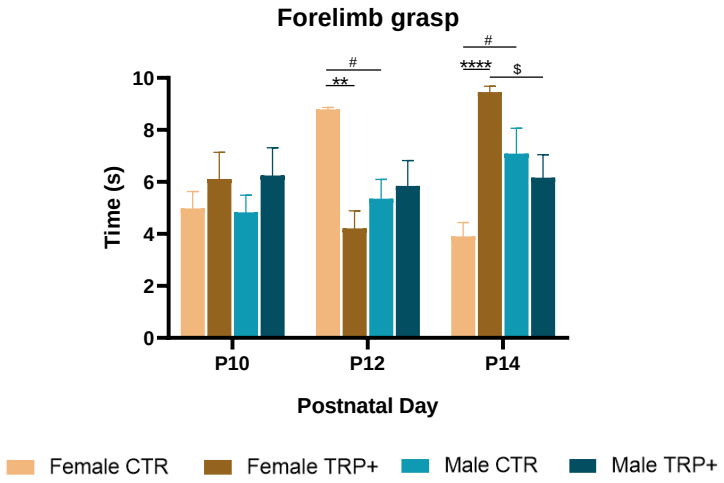



**Figure 27 | TRP+ diet delays the development of strength in forelimb grasp test in females of offspring in health conditions**

At P12 Females TRP+ had a worse score than the CTR females, however at P14 they improved their development considerably and therefore had a higher score in forelimb grasp. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+. Data represented as mean ± SEM; P10: n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P12: n (female CTR) = 5; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7; P14: n (female CTR) = 6; n (female TRP+) = 7; n (male CTR) = 8; n (male TRP+) = 7.

The TRP+ diet had a stronger impact on the development of females TRP+, with both positive and negative effects. This diet had a positive impact on these females in the forelimb test. On the other hand, the TRP+ diet had a negative impact on development in the cliff test in both sexes, which was the most affected developmental milestones.

To access communication skills, maternal separation-induced USVs were recorded following milestones on days P4, 6 and 8 and several parameters were analyzed.

In the total number (Figure 28A) and in total time of USVs (Figure 28B) no significant differences were observed between diets and sexes. However there was an overall effect of age ($F(2, 77) = 4.084$, $p=0.0206$), sex ($F(1, 77) = 9.468$, $p=0.0029$) and diet ($F(1, 77) = 11.61$, $p=0.0010$).

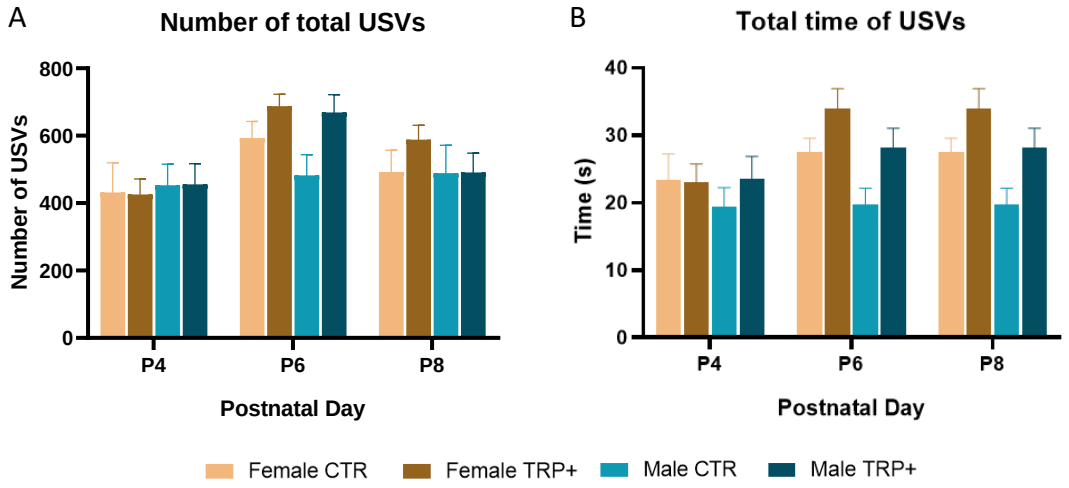
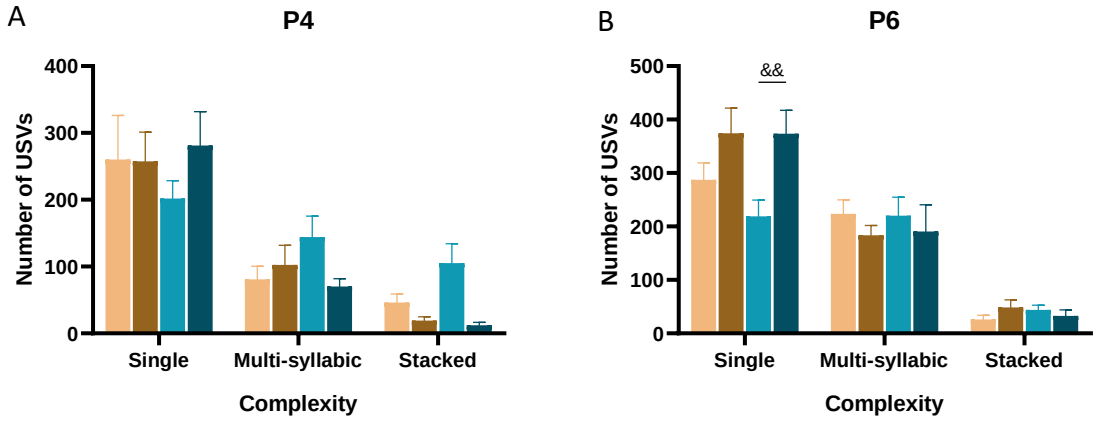



**Figure 28 | TRP+ diet did not influence differences in number and total time of USVs of offspring in health conditions**

TRP+ diet did not influence either the (A) number of total ultrasonic vocalizations (USVs) or the (B) total time of USVs. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

There was an effect overall of the type of vocalization in all ages (P4 (Figure 29A): $F(2, 73) = 37.14$, $p<0.0001$; P6 (Figure 29B): $F(2, 72) = 66.95$, $p<0.0001$; P8 (Figure 29C): $F(2, 74) = 24.38$, $P<0.0001$). Only on P6, males TRP+ produced significantly more single vocalizations when compared with males CTR (males CTR = 218.9 ± 30.38 vs male TRP+ = 373.4 ± 43.76, $p=0.0049$).



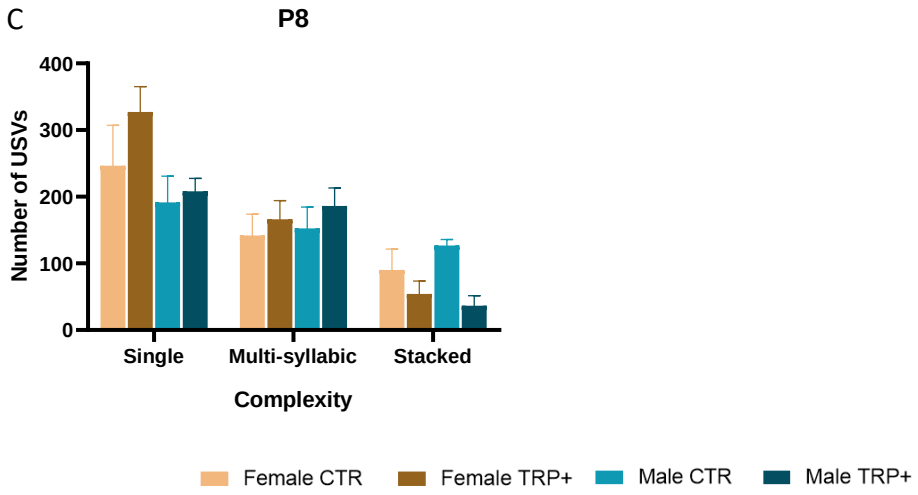



**Figure 29 | Males TRP+ produced significantly more single USVs on P6 of offspring in health conditions**

Despite at (A) P4 there was no significant difference, at (B) P6, males TRP+ produced more single vocalizations. However, this difference disappears at (C) P8. Two-way ANOVA followed by Turkey's multiple comparisons test; (A) No significant differences found between any of the experimental groups; (B) $p<0.05$; &: significant difference between male CTR and male TRP+; (C) No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

Latency time was also analyzed and it was found that at P4 males from mothers with TRP+ diet took longer to initiate vocalizations after separation from the mother, comparing with females on the same diet (female TRP+ = 0.614 ± 0.363 vs male TRP+ = 9.670 ± 5.110, p=0.0039) and males from mothers with CTR diet (male CTR = 0.325 ± 0.149 vs male TRP+= 9.670 ± 5.110, p=0.0009) (Figure 30A).

Accordingly, call length, time between calls (mean inter-call), and mean power showed alterations at P4. There was a difference in mean call length between males CTR and males TRP+, where the latter showed a longer mean call length (male CTR = 0.044 ± 0.002 vs male TRP+ = 0.054 ± 0.004, p= 0.0135) (Figure 30B). There was a significant sex effect ($F(1, 72) = 13.36$, p=0.0005) and also a diet effect ($F(1, 72) = 20.79$, p<0.0001).

Indeed, there was a significant difference at P4 where females TRP+ had a longer interval between calls) than females CTR (female CTR = 0.483 ± 0.046 vs female TRP+ = 0.717 ± 0.095, p= 0.0142) (Figure 30C). In particular, the females TRP+ vocalized with significantly increased power than females CTR on P4 (female CTR = -89.200 ± 1.265 vs female TRP+ = -93.82 ± 0.854, p= 0.0253) (Figure 30D).

The tonality of the USVs (Figure 30E) was also analyzed and at P8 there was a significant difference between sexes in the TRP+ diet, where males presented a lower tonality. Overall, there was an effect of sex ($F(1, 75) = 18.96$, p<0.0001) and also of diet ($F(1, 75) = 10.18$, p=0.0021).

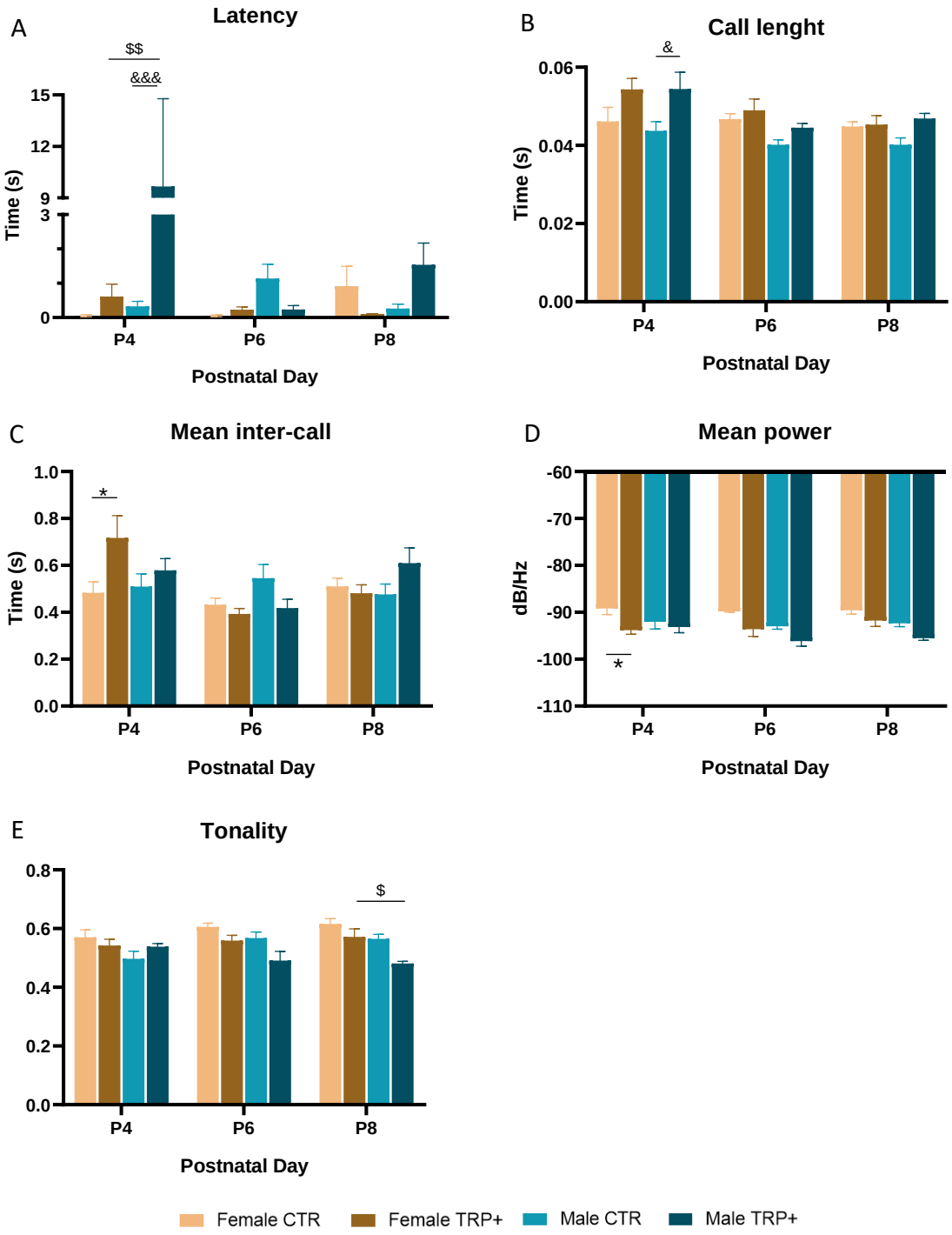



**Figure 30 | TRP+ diet increased the latency, mean inter-call, call length and power of USVs on P4 of offspring in health conditions**

At P4, males TRP+ showed longer (A) latency time and (B) call duration. On the same day, females TRP+ had a higher (C) mean inter-call and (D) power of USVs. At P8, differences were observed between the sexes on the TRP+ diet, where males TRP+ had a lower (E) tonality of USVs. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

Finally, the principal (Figure 31A), low (Figure 31B) and high frequencies (Figure 31C) were analyzed but there were no significant differences observed. However, there was a effect of age on the principal frequency (F (2, 78) = 7.666, p=0.0009) and in the low frequency, there was an effect of sex (F (1, 76) = 5.822, p=0.0182).

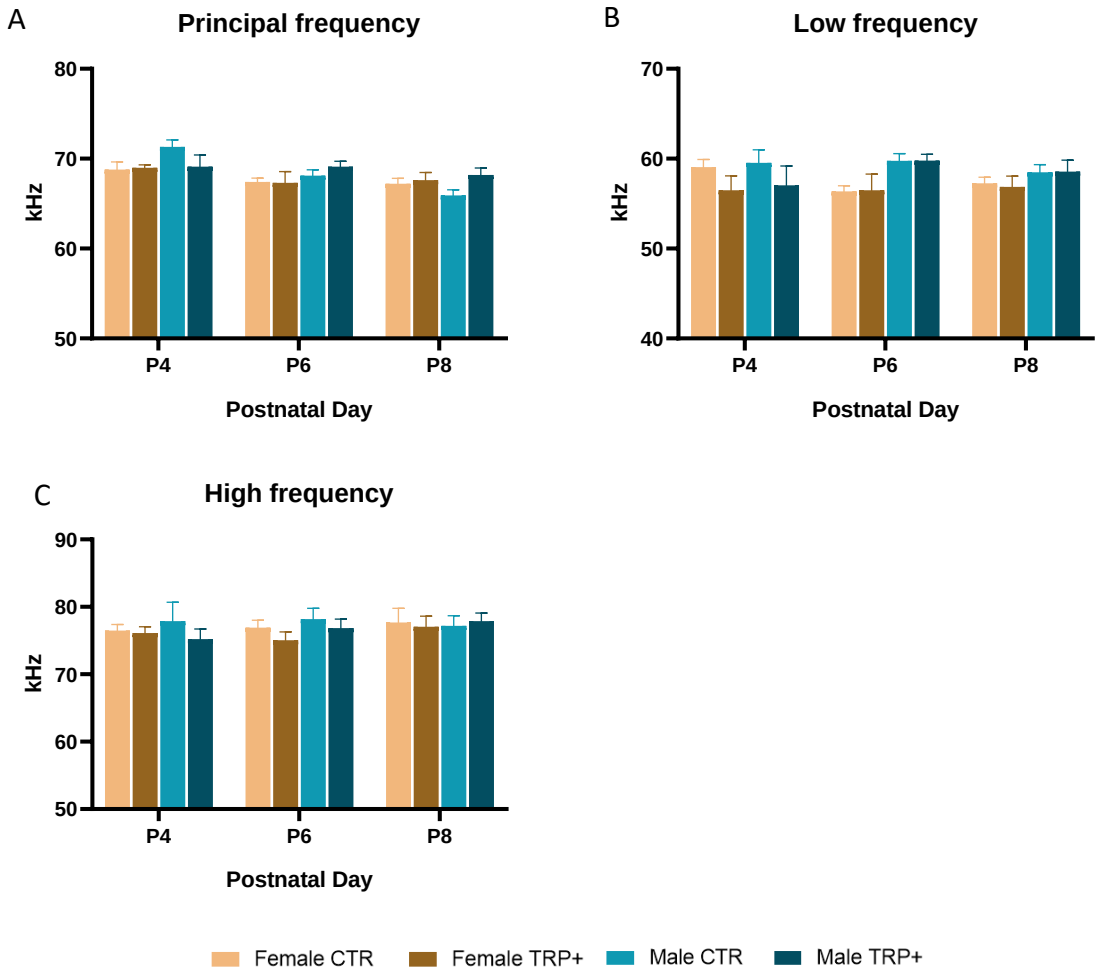



**Figure 31 | TRP+ diet did not influence principal, low and high frequency of USVs of offspring in health conditions**

TRP+ diet did not influence either (A) principal or (B) low or (C) high frequency of USVs. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

Thus, by analyzing the characteristics of the USVs, it can be seen that the males TRP+ were the group most affected. Although the TRP+ diet did not influence the number and time of USVs, it did provide an improvement in USVs, such as latency time, mean inter-call, call length and power, being the first characteristic on which the diet had the most impact.

Physical landmarks were registered for open eyes (Figure 32A) and auditory startle response (Figure 32B) during milestones and we observed no significant differences in both.

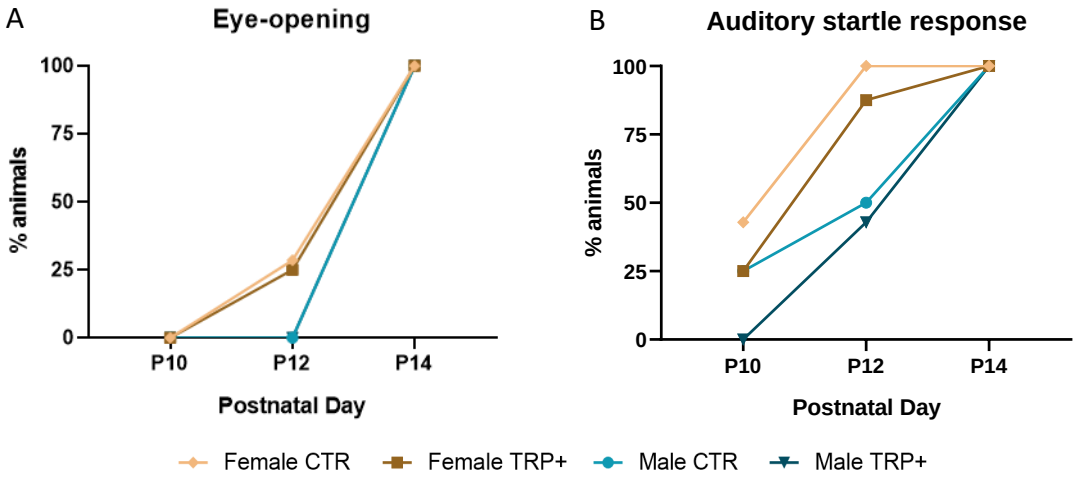


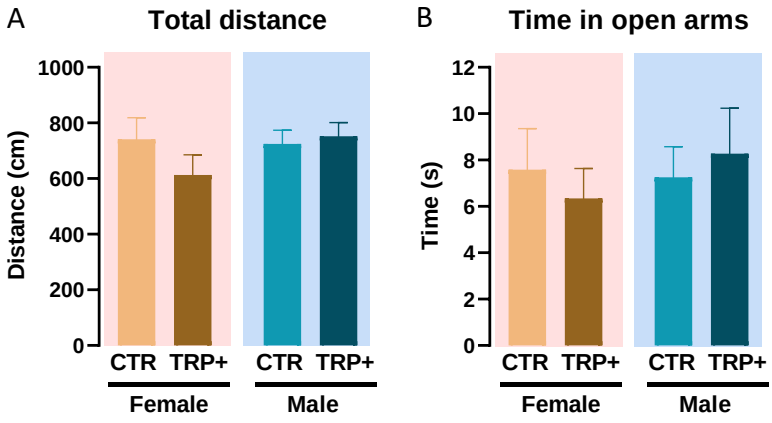

**Figure 32 | TRP+ diet did not influence eye-opening day and auditory startle response of offspring in health conditions**

TRP+ diet did not influence (A) eye-opening and (B) auditory startle response. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

### 4.1.3. Prenatal tryptophan suplemented diet increased number of open arms entries in males in health conditions

At juvenile age (P30 to P41), several tests were performed, the first being the EPM to assess anxiety-like behavior.

No difference between experimental groups were detected in total distance (Figure 33A), time (Figure 33B) and distance (Figure 33C) in open arms. However, males TRP+ showed a higher number of entries in the open arms comparing with males CTR (male CTR = 2.500 ± 0.598 vs male TRP+ = 5.000 ± 0.963, p=0.0275) and females TRP+ (female TRP+ = 2.500 ± 0.223 vs male TRP+ = 5.000 ± 0.963, p=0.0427) (Figure 33D). An anxiety index (Figure 33E) was also calculated and no significant differences were found.



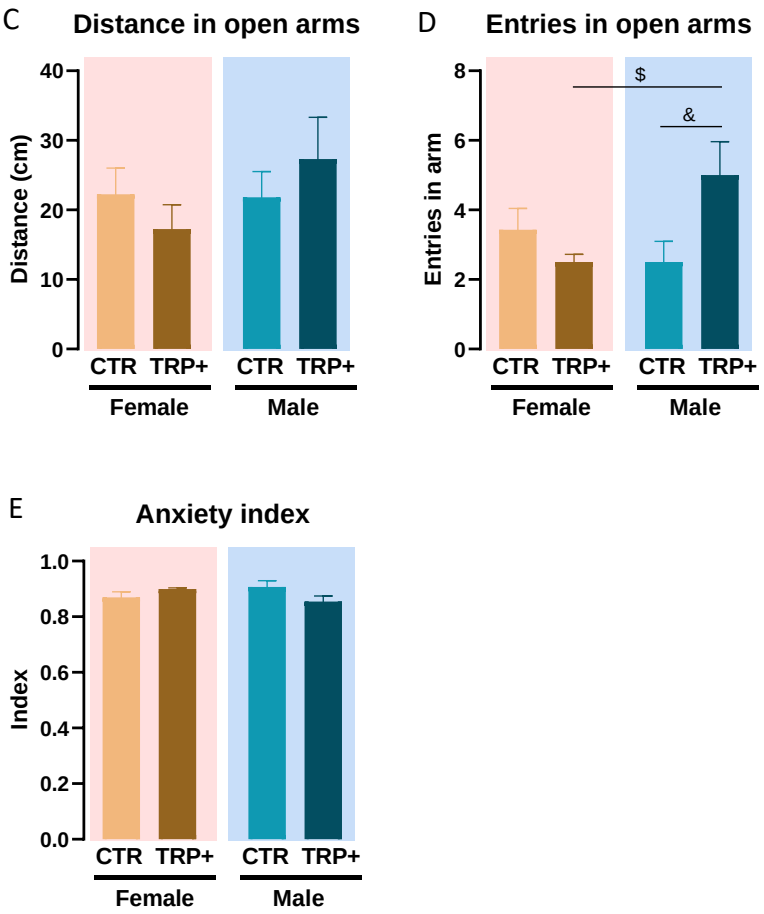



**Figure 33 | Males TRP+ showed a higher number of entries in the open arms of offspring in health conditions**

TRP+ diet did not influence (A) total distance, (B) time and (C) distance in open arms. However, males TRP+ showed a higher (D) number of entries in the open arms. Also, TRP+ diet did not influence (E) anxiety index. Two-way ANOVA followed by Turkey's multiple comparisons test; (A) No significant differences found between any of the experimental groups; (B) No significant differences found between any of the experimental groups; (C) No significant differences found between any of the experimental groups; (D) $p<0.05$; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+; (E) No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

TRP+ diet only showed significant differences in number of entries in open arms in males. No significant differences were observed in the other parameters, including the anxiety index, and therefore the TRP+ diet did not influence anxiety behavior.

### 4.1.4. Prenatal tryptophan suplemented diet does not affect social interaction in health conditions

To evaluate social interaction, a juvenile social play test was performed and the number (Figure 34A) and time of interactions (Figure 34B) were analyzed.

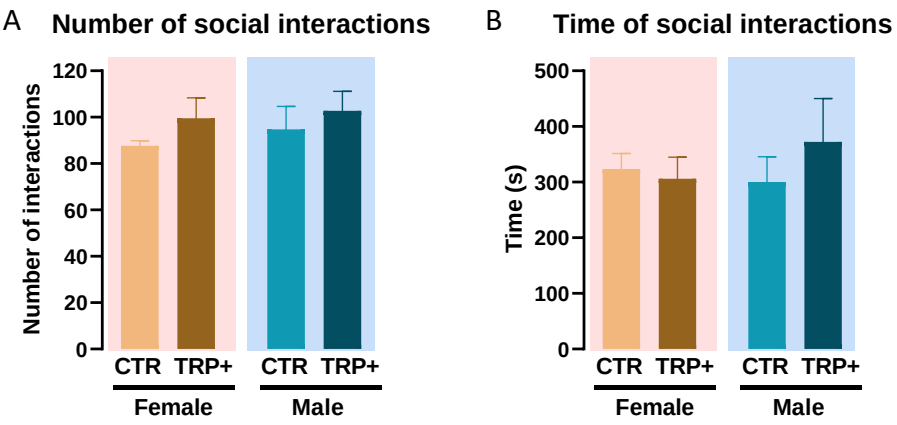



**Figure 34 | TRP+ diet did not influence number and time of social interactions of offspring in health conditions**

TRP+ diet did not influence (A) number of social interactions and (B) time of social interactions. Two-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 3; n (female TRP+) = 4; n (male CTR) = 4; n (male TRP+) = 3.

Moreover, the number of USVs in a social (Figure 35A) and non-social context (Figure 35B) and a respective USVs social index (Figure 35C) was also analyzed.

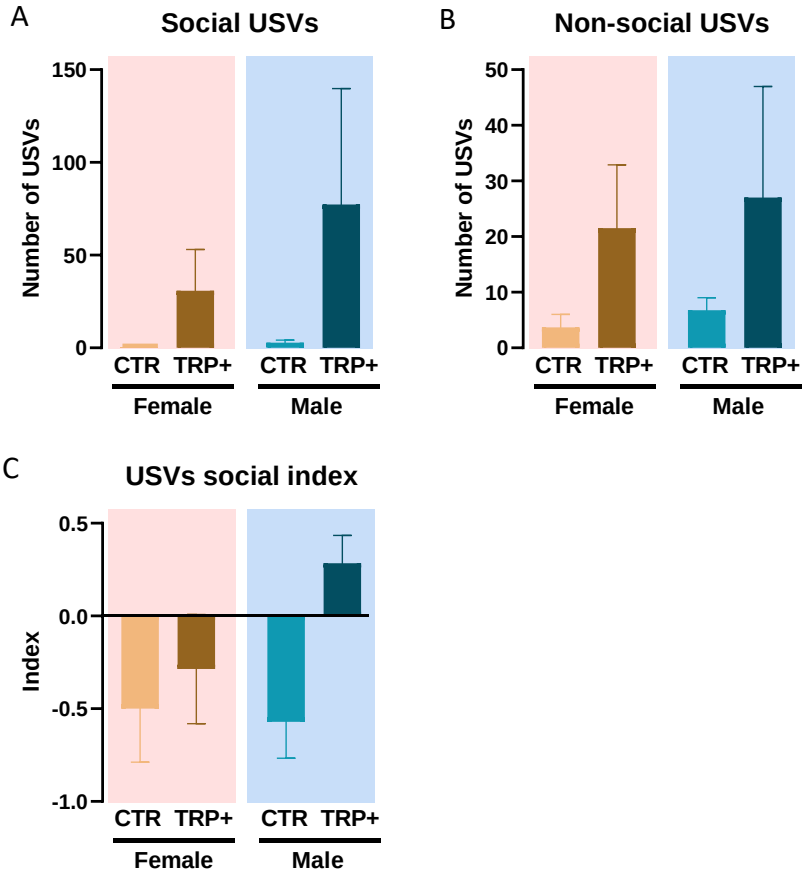


**Figure 35 | TRP+ diet did not influence number of USVs in a social and non-social context and the respective index of offspring in health conditions**

TRP+ diet did not influence (A) number of USVs in a social context, (B) number of USVs in a non-social context and (C) USVs social index. Two-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 3; n (female TRP+) = 4; n (male CTR) = 4; n (male TRP+) = 3.

### 4.1.5. Prenatal tryptophan suplemented diet increases repetitive behavior in males in health conditions

The marble test (Figure 36) was performed to analyze the effect of Trp diet on repetitive behavior, for which the number of marbles buried every 5 min was counted (Figure 25). Overall, there was an effect in time ($F(3, 102) = 11.37$, $p<0.0001$) and in diet ($F(1, 102) = 21.42$, $p<0.0001$). In addition, there was also an effect of diet and sex ($F(1, 102) = 18.00$, $p<0.0001$). Males TRP+ showed a higher number of buried marbles compared to females on the same diet from 10 minutes until the last time point (20 minutes) of the test (time point 10': female TRP+ = 0.429 ± 0.297 vs male TRP+ = 3.286 ± 0.918, p=0.0272; time point 15': female TRP+ = 1.375 ± 0.419 vs male TRP+ = 5.286 ± 1.322, p=0.0006;time point 20': female TRP+ = 1.750 ± 0.559 vs male TRP+ = 5.429 ± 1.110, p=0.0015). At 15 minutes there was also a significant difference between diets in males (male CTR = 2.375 ± 0.618 vs male TRP+ = 5.286 ± 1.322, p=0.0180).

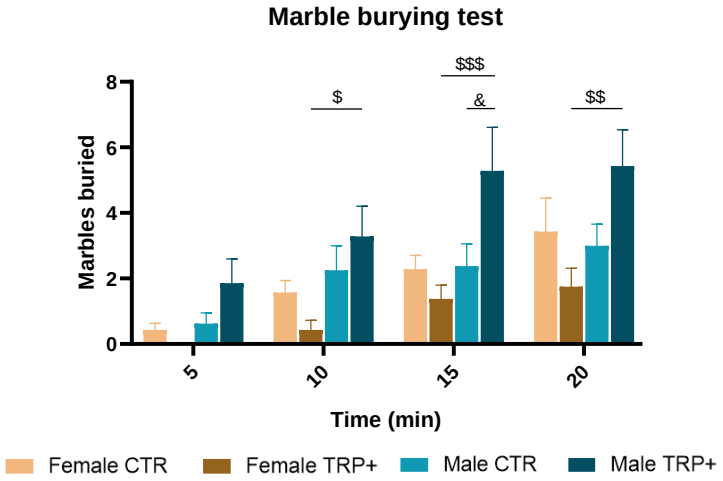



**Figure 36 | Males TRP+ showed an increased repetitive behavior of offspring in health conditions**

TRP+ diet influenced repetitive behavior in males. Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR diet and male TRP+. Data represented as mean ± SEM; time points 5' and 10': n (female CTR) = 7; n (female TRP+) = 7; n (male CTR) = 8; n (male TRP+) = 7; time points 15' and 20': n (female CTR) = 7; n (female TRP+) = 8; n (male CTR) = 8; n (male TRP+) = 7.

Thus, regarding the marble burying test, the diet only affected males TRP+ where, not only provided sexual dimorphism, but also increased repetitive behavior.

### 4.1.6. Prenatal tryptophan suplemented diet impairs learning and memory in males in health conditions

Finally, to assess learning and memory, Barnes test was performed. For this purpose, escape latency time (time taken for the mouse to enter the escape tube), time to find the target, total distance, time in the target zone, errors and search strategies were evaluated.

Regarding time of escape latency (Figure 37), on the first day of training sessions, males TRP+ took longer to enter the tunnel than males CTR (male CTR = 45.41 ± 9.969 vs male TRP+ = 120.70 ± 21.01, p=0.0491). No changes were detected in other days of test. It was found also an effect of diet (F (1, 48) = 11.19, p=0.0016).

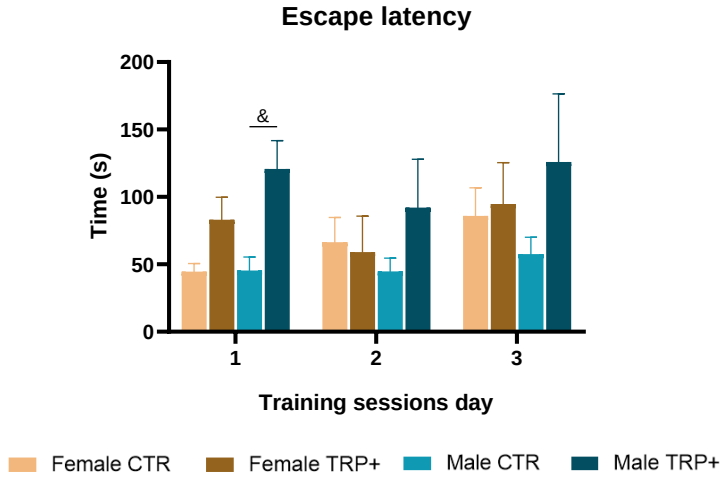



**Figure 37 |Males TRP+ had a longer escape latency time of offspring in health conditions**
TRP+ diet increased escape latency time in males on first day of training sessions. Two-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; n (female CTR) = 6; n (female TRP+) = 4; n (male CTR) = 7; n (male TRP+) = 3.

In the time to find the target (Figure 38A), there were diet- (F (1, 44) = 12.86, p=0.0008) and sex-dependent (F (1, 44) = 6,667, p=0.0132) effect. Furthermore, on day 1 of learning training, females TRP+ took longer to find the target comparing with female CTR (female CTR = 9.785 ± 0.497 vs female TRP+ = 43.900 ± 13.65, p= 0.0199). However, On day 2, females rescue this skills and were found that was males TRP+ that took longer to find the hole comparing with females TRP+ (female TRP+ = 8.908 ± 1.138 vs male TRP+ = 47.530 ± 19.56 p= 0.0340) and males CTR (male CTR = 14.480 ± 3.201 vs male TRP+ = 47.530 ± 19.56, p= 0.0312). On probe day (Figure 38B) no significant differences were observed in time to find target.

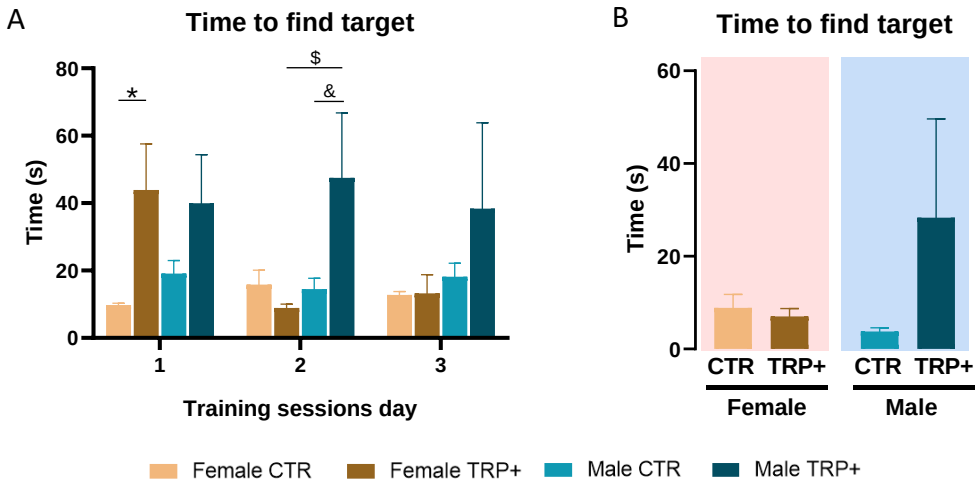



**Figure 38 | TRP+ diet increased time to find target in training sessions of offspring in health conditions**

TRP+ diet increased time to find target in both sex in (A) training sessions days however, it had no influence on (B) probe day. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; day 1: n (female CTR) = 5; n (female TRP+) = 4; n (male CTR) = 6; n (male TRP+) = 3; day 2: n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 7; n (male TRP+) = 3; day 3: n (female CTR) = 5; n (female TRP+) = 4; n (male CTR) = 7; n (male TRP+) = 3; probe day: n (female CTR) = 5; n (female TRP+) = 4; n (male CTR) = 6; n (male TRP+) = 3.

In time in the target zone no significant differences were observed between experimental groups (Figure 39 A, B). However, there was an day effect ($F(2, 50) = 24.04$, $p<0.0001$) and a diet x sex effect ($F(1, 49) = 8.057$, $p=0.0065$) in training sessions.

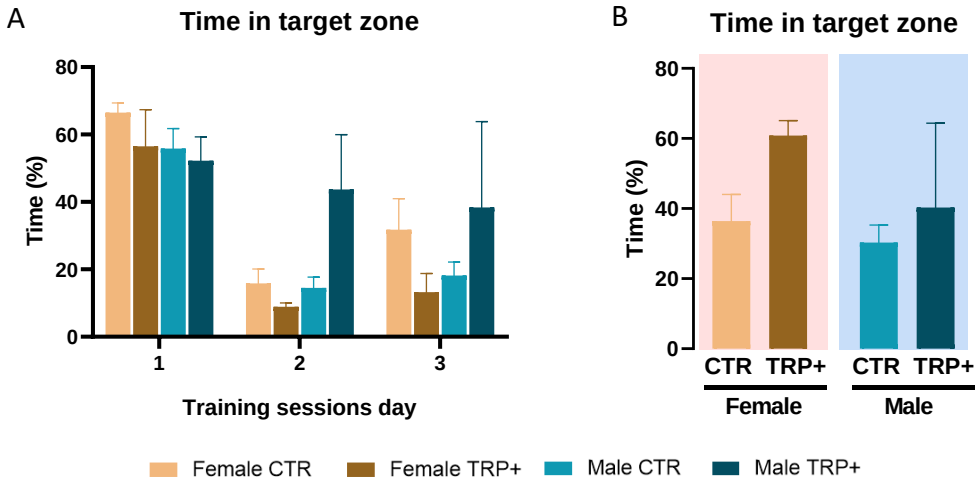



**Figure 39 | TRP+ diet did not influence time in target zone of offspring in health conditions**

TRP+ diet did not influence time in target zone in (A) training sessions day and (B) probe day in Barnes test. Two-way ANOVA followed by Sidak's multiple comparisons test. No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female CTR) = 6; n (female TRP+) = 4; n (male CTR) = 7; n (male TRP+) = 3.

On training sessions day (Figure 40A), there was an effect of sex in number of errors to explore (F (1, 49) = 7.725, p=0.0077). In addition, there was a diet x sex effect (F (1, 49) = 8.682, p=0.0049). Only on the second day of training it was possible observed alterations. In fact, males from mothers with TRP+ diet displayed more errors exploring for the target comparing with female from mothers with same diet (female TRP+ =2.677 ± 1.276 vs male TRP+ = 5.889 ± 2.04, p=0.0344). During test, no differences were observed between experimental groups (Figure 40B).

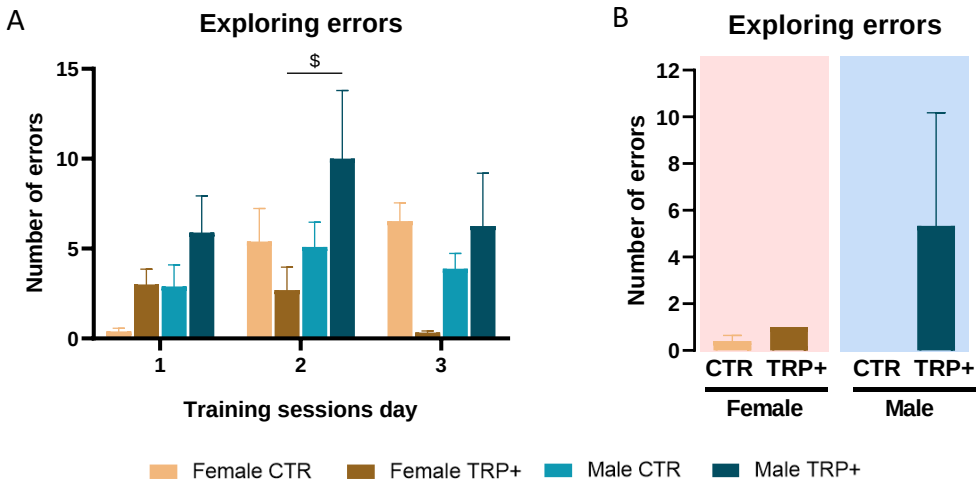



**Figure 40 | Males TRP+ committed more exploring errors in training sessions of offspring in health conditions**

TRP+ promotes a sexual dimorphism where males on this diet commit more exploring errors on day 2 of (A) training sessions. On (B) probe day, TRP+ diet did not influence the number of errors. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; $: significant difference between female TRP+ and male TRP+. Data represented as mean ± SEM; day 1: n (female CTR) = 5; n (female TRP+) = 4; n (male CTR) = 6; n (male TRP+) = 3; day 2: n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 8; n (male TRP+) = 3; day 3: n (female CTR) = 7; n (female TRP+) = 3; n (male CTR) = 8; n (male TRP+) = 3; probe day: n (female CTR) = 5; n (female TRP+) = 3; n (male CTR) = 7; n (male TRP+) = 3.

The distance traveled during training (Figure 41A) and test (Figure 41B) sessions was also evaluated. We observed that males TRP+ had higher activity comparing with female TRP+ and male CTR (female TRP+ = 129.80 ± 25.49 vs male TRP+ = 328.10 ± 31.29, p=0.0227; male CTR = 164.60 ± 36.34 vs male TRP+ = 328.10 ± 31.29, p=0.0385). Overall, there was a sex-dependent (F (1, 53) = 8.662, p=0.0048) effect. In addition, there were also diet and sex effect (F (1, 53) = 4.722, p=0.0343).

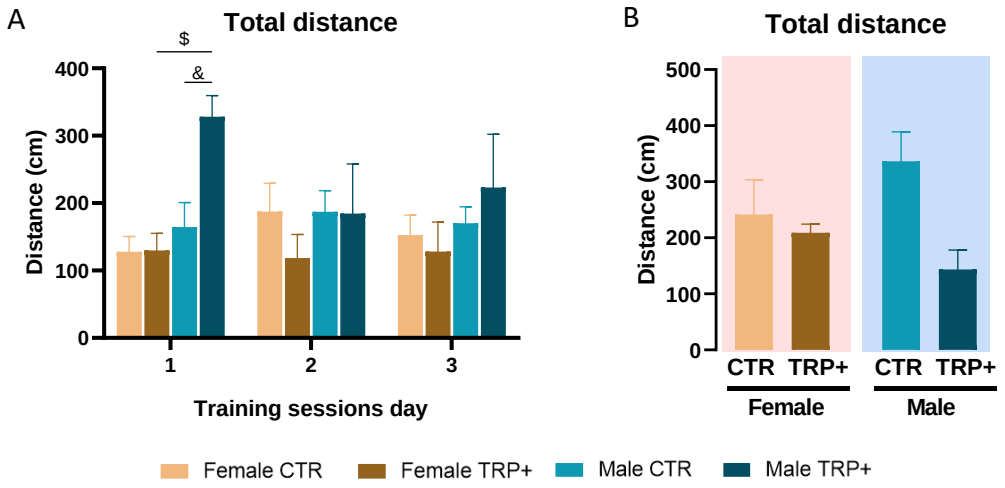



**Figure 41 | Males TRP+ traveled a higher total distance in training sessions of offspring in health conditions**

TRP+ diet increased the total distance traveled on (A) first day of training only in males. However, on (B) probe day, TRP+ diet did not influence the total distance in Barnes test. Two-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR diet and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 8; n (male TRP+) = 3.

The search strategies used by the animals to find the target were also evaluated, which can be divided into three categories: direct - the mouse moves directly to the target or to an adjacent hole; serial - the mouse goes to at least 2 holes until it reaches the target in a serial pattern; mixed - the mouse searches for the target randomly in a disorganized way (Sunyer et al., 2007).

On the first day (Figure 42A), it was detected that both sexes from mothers with CTR diet used more a directly approached to reach the target than those that from mothers with TRP+ diet (female CTR = 91.67 ± 8.325 vs female TRP+ = 58.33 ± 0.000, p=0.0273; male CTR = 58.34 ± 8.335 vs male TRP+ = 22.22 ± 0.000, p=0.0458). On the other hand, the animals TRP+ showed preference for serial strategy (female CTR = 4.165 ± 4.165 vs female TRP+ = 41.66 ± 0.000, p=0.0393; male CTR = 29.17 ± 4.165 vs male TRP+ = 66.67 ± 0.000, p=0.0392). In addition, it was observed a significant effect of diet ($F(2, 6) = 18.48$, $p= 0.0027$) and sex ($F(2, 6) = 25.15$, $p= 0.0012$) on strategy approaches.

On the other hand, at day 2 of training sessions (Figure 42B), there was no differences between experimental groups. On day 3 (Figure 42C), although, there was no differences in strategy approaches between experimental groups, there were differences between the sexes from mothers with TRP+ diet. Indeed, females predominate used direct strategy (female TRP+ = 87.50 ± 0.000 vs male TRP+ = 65.63 ± 9.375, p=0.0263) comparing with males. On test day (Figure 42D), it was clearly that males TRP+ had preference for serial strategy to reach the target than males CTR (male CTR diet = 0.00 ± 0.000 vs male TRP+ = 33.33 ± 0.000, p=0.0452).

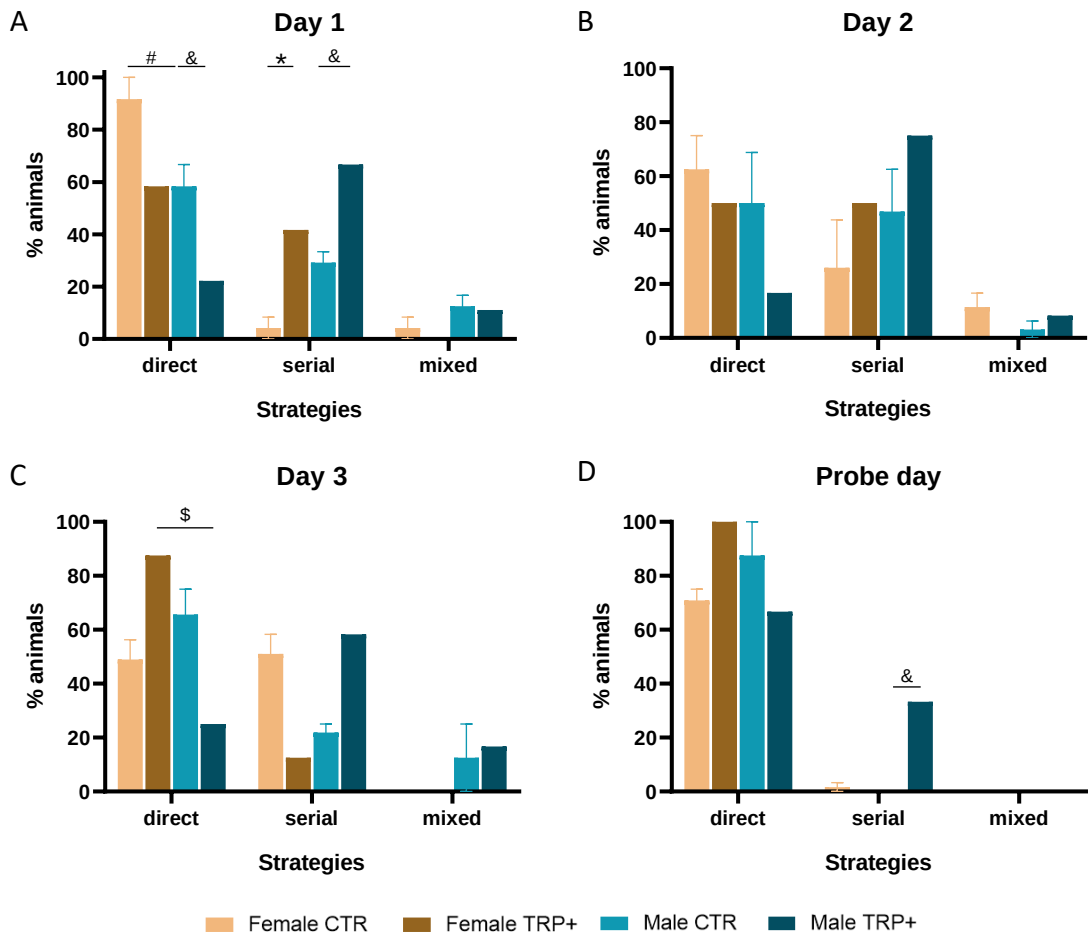



**Figure 42 | Males TRP+ showed learning and memory impairment in search strategy of offspring in health conditions**

TRP+ males searched more serially and less directly than control males on (A) day 1. Female TRP+ males also searched mostly with a serial strategy. However, on days (B) 2 and (C) 3 the diet did not influence the search strategy. On (D) probe day, TRP+ males searched much more in a serial strategy. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female CTR and female TRP+; #: significant difference between female CTR and male CTR; $: significant difference between female TRP+ and male TRP+; &: significant difference between male CTR and male TRP+. Data represented as mean ± SEM; n (female CTR) = 7; n (female TRP+) = 4; n (male CTR) = 8; n (male TRP+) = 3.

Taking into account the results of the Barnes test, it was possible to conclude that males TRP+ showed deficits in learning, performing worse in almost all parameters. In addition, on the probe day, males TRP+ presented a more serial target search strategy while the other groups mainly went for direct search and therefore also had a memory impairment.

## 4.2. EFFECT OF PRENATAL TRYPTOPHAN DIET IN ASD CONDITION

Since our main objective in the second part is to study the effect of the prenatal Trp diet in ASD condition, these results are only regarding offspring that have been segregated by sex to form the following 8 experimental groups:

- Female WT pups from *Nf1*$^{+/-}$ dam with CTR diet (female WT CTR);
- Female *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with CTR diet (female *Nf1*$^{+/-}$ CTR);
- Female WT pups from *Nf1*$^{+/-}$ dam with TRP+ diet (female WT TRP+);
- Female *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with TRP+ diet (female *Nf1*$^{+/-}$ TRP+);
- Male WT pups from *Nf1*$^{+/-}$ dam with CTR diet (male WT CTR);
- Male *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with CTR diet (male *Nf1*$^{+/-}$ CTR);
- Male WT pups from *Nf1*$^{+/-}$ dam with TRP+ diet (male WT TRP+);
- Male *Nf1*$^{+/-}$ pups from *Nf1*$^{+/-}$ dam with TRP+ diet (male *Nf1*$^{+/-}$ TRP+).

### 4.2.1. Prenatal tryptophan suplemented diet affect body weight but not between genotype in ASD condition

To investigate if the prenatal Trp diet affects the offspring's body weight, they were weighed every 2 days. Although no significant differences were found during the weaning period, after weaning (P21) there were several differences observed between sex in CTR and TRP+ diets (males had higher weight) and also between diets either in males or females, mostly in males, where the TRP+ diet offspring had a lower weight in both genotypes. However, no differences in weight were observed between genotypes (Figure 43 and Table 5).

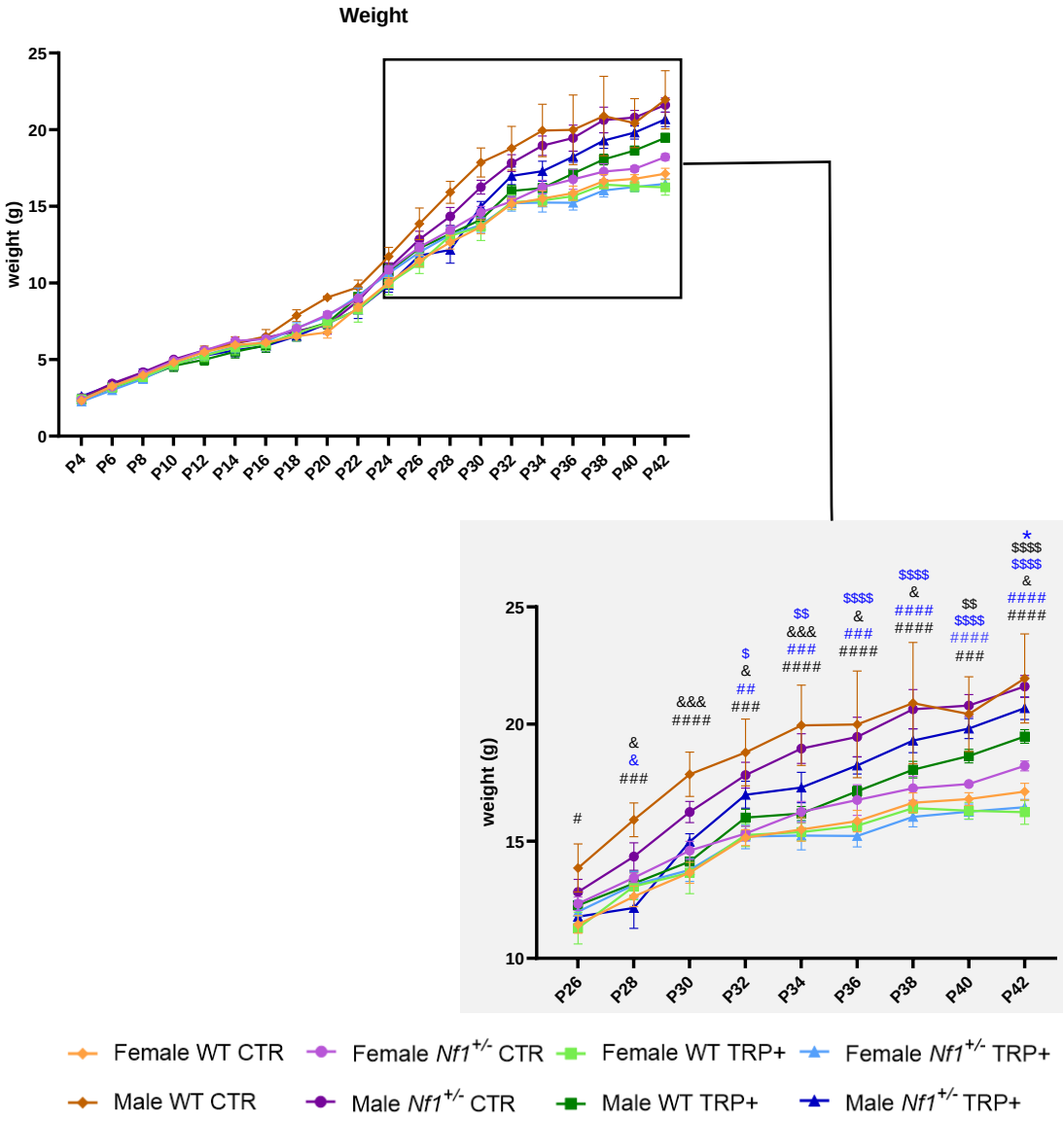



**Figure 43 | TRP+ diet led to a lower body weight in both genotype of offspring in ASD condition**
Body weight was measured every 2 days until P42 and TRP+ led to a lower body weight of the WT and *Nf1*$^{+/-}$ offspring. Two-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female WT CTR and female WT TRP+; #: significant difference between female WT CTR and male WT CTR; #: significant difference between female *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ CTR; $: significant difference between female WT TRP+ and male WT TRP+; $: significant difference between female *Nf1*$^{+/-}$ TRP+ and male *Nf1*$^{+/-}$ TRP+; &: significant difference between male WT CTR and male WT TRP+; &: significant difference between male *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

**Table 5 | Significant differences in weight of offspring in ASD condition**

| P26 | P value |
|---|---|
| Female WT CTR vs male WT CTR | **0.0442** |
| P28 | |
| Female WT CTR vs male WT CTR | **0.0009** |
| Male WT CTR vs male WT TRP+ | **0.0185** |
| Male *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ TRP+ | **0.0113** |
| P30 | |
| Female WT CTR vs male WT CTR | **<0.0001** |
| Male WT CTR vs male WT TRP+ | **0.0001** |
| P32 | |
| Female WT CTR vs male WT CTR | **0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **0.0019** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **0.0431** |
| Male WT CTR vs male WT TRP+ | **0.0138** |
| P34 | |
| Female WT CTR vs male WT CTR | **<0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **0.0005** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **0.0100** |
| Male WT CTR vs male WT TRP+ | **0.0001** |
| P36 | |
| Female WT CTR vs male WT CTR | **<0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **0.0005** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **<0.0001** |
| Male WT CTR vs male WT TRP+ | **0.0101** |
| P38 | |
| Female WT CTR vs male WT CTR | **<0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **<0.0001** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **<0.0001** |
| Male WT CTR vs male WT TRP+ | **0.0110** |
| P40 | |
| Female WT CTR vs male WT CTR | **0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **<0.0001** |
| Female WT TRP+ vs male WT TRP+ | **0.0027** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **<0.0001** |
| P42 | |
| Female WT CTR vs male WT CTR | **<0.0001** |
| Female *Nf1*$^{+/-}$ CTR vs female *Nf1*$^{+/-}$ TRP+ | **0.0498** |
| Female *Nf1*$^{+/-}$ CTR vs male *Nf1*$^{+/-}$ CTR | **<0.0001** |
| Female WT TRP+ vs male WT TRP+ | **<0.0001** |
| Female *Nf1*$^{+/-}$ TRP+ vs male *Nf1*$^{+/-}$ TRP+ | **<0.0001** |
| Male WT CTR vs male WT TRP+ | **0.0453** |

The difference in weight between P4 to P20 and also from P20 to P42 was also analyzed.

During infancy (Figure 44A), differences in increment of weight were observed between females WT CTR and males WT CTR (female WT CTR = 4.461 ± 0.393 vs male WT CTR = 6.640 ± 0.020, p=0.0357). It was observed a sex x genotype effect in increment weight of offspring (F (1, 34) = 7.695, p=0.0089).

After weaning and following juvenile age (Figure 44B), sex differences were observed in all experimental groups (female WT CTR = 8.904 ± 0.087 vs male WT CTR = 12.24 ± 1.435, p=0.0013; female *Nf1*$^{+/-}$ CTR = 9.236 ± 0.272 vs male *Nf1*$^{+/-}$ CTR = 12.78 ± 0.310, p<0.0001; female WT TRP+ = 8.054 ± 0.653 vs male WT TRP+ = 10.370 ± 0.531, p=0.0032; female *Nf1*$^{+/-}$ TRP+ = 7.503 ± 0.275 vs male *Nf1*$^{+/-}$ TRP+ = 12.42 ± 0.658, p<0.0001). In addition, it was observed that females *Nf1*$^{+/-}$ TRP+ had a higher weight gain than females *Nf1*$^{+/-}$ CTR (female *Nf1*$^{+/-}$ CTR = 9.236 ± 0.272 vs female *Nf1*$^{+/-}$ TRP+ = 7.503 ± 0.275, p=0.0354). In males TRP+, *Nf1*$^{+/-}$ had a higher weight gain comparing with their littermate WT (male WT TRP+ = 10.370 ± 0.531 vs male *Nf1*$^{+/-}$ TRP+ = 12.420 ± 0.658, p=0.0129). Overall, there were sex- (F (1, 34) = 105.4, p<0.0001) and diet-dependent (F (1, 34) = 12.26, p=0.0013) effect.

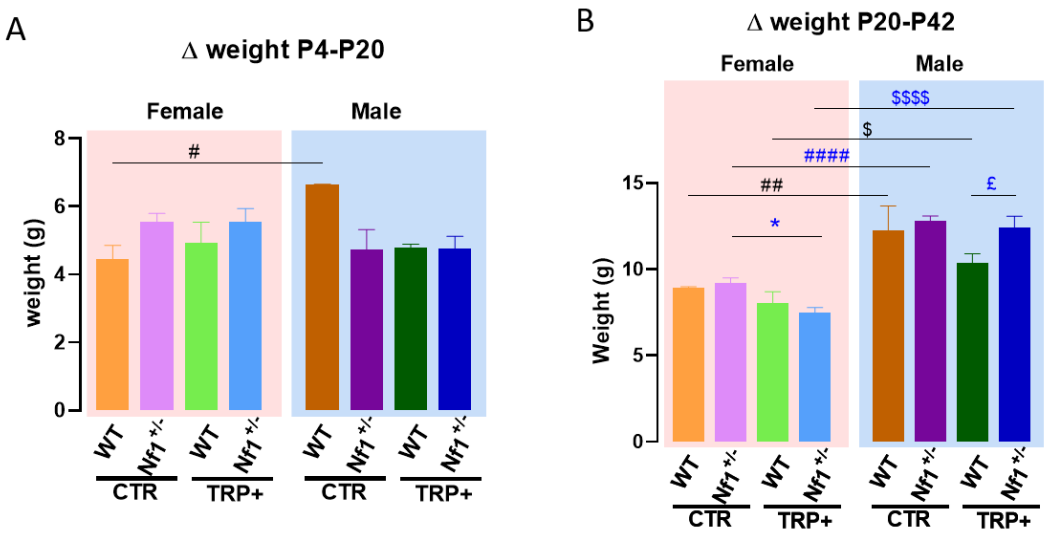



**Figure 44 | Females *Nf1*$^{+/-}$ TRP+ showed less weight increase of offspring in ASD condition**
During (A) breastfeeding period (P4-P20) males WT CTR showed a higher increase than females WT CTR. Moreover, (B) after weaning (P20-P42) females *Nf1*$^{+/-}$ TRP+ showed a lower weight increment and in all experimental groups, males showed a higher increase weight than respective females. Two-way ANOVA followed by Sidak's multiple comparisons test; Three-way ANOVA followed by Sidak's multiple comparisons test; p<0.05; #: significant difference between female WT CTR and male WT CTR. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 5; n (male *Nf1*$^{+/-}$ TRP+) = 5; and B. Three-way ANOVA followed by Sidak's multiple comparisons test; p<0.05; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; #: significant difference between female WT CTR and male WT CTR; #: significant difference between female *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ CTR; $: significant difference between female WT TRP+ and male WT TRP+; $: significant difference between female *Nf1*$^{+/-}$ TRP+ and male *Nf1*$^{+/-}$ TRP+; £: significant difference between male WT TRP+ and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Also, pup length was measured during infancy (Figure 45) and although no significant differences were observed between experimental groups, there was an effect of sex (F (1, 96) = 7.186, p=0.0086).

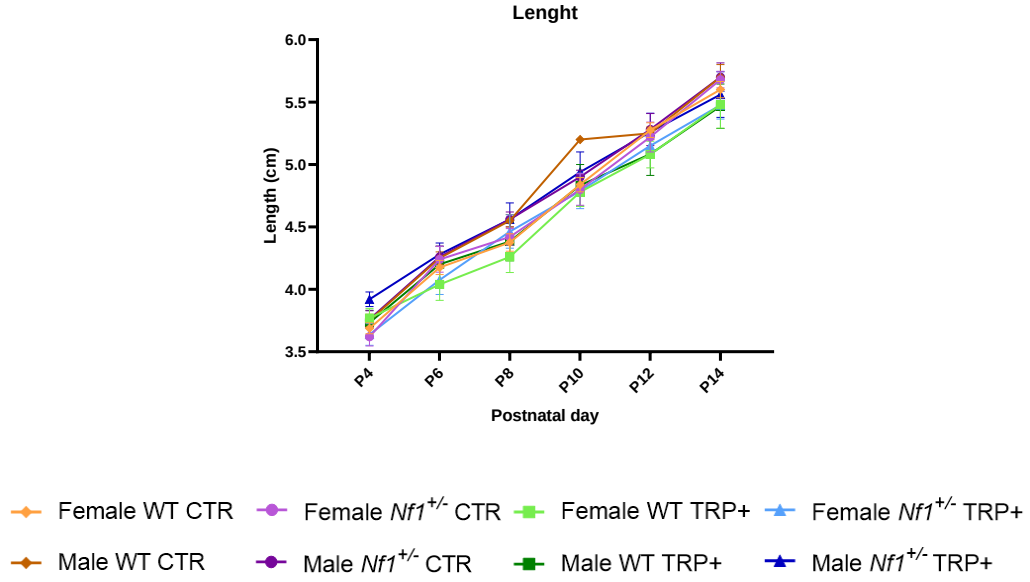



**Figure 45 | TRP+ diet did not influence length of offspring in ASD condition**
Length was measured on P4, 6, 8, 10, 12 and 14 and TRP+ diet did not influence the length. Two-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 8; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

The increment of length (Figure 46) was also calculated and although there were no significant differences between the experimental groups, there was an effect of the diet (F (1,32) = 9.119, p=0.0049).

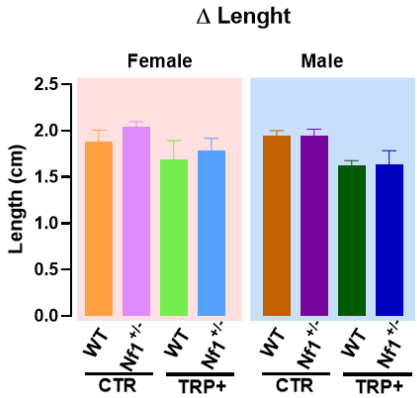



**Figure 46 | TRP+ diet did not influence length increase of offspring in ASD condition**
TRP+ diet did not influence length growth between P4 to P14. Three-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 6; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 4; n (female *Nf1*$^{+/-}$ TRP+) = 6; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 5; n (male *Nf1*$^{+/-}$ TRP+) = 5.

To determine and normalize the weight of brain on each experimental group, a brain index was calculated (Figure 47) and it found that female *Nf1*$^{+/-}$ TRP+ displayed a higher index than male *Nf1*$^{+/-}$ TRP+ (female *Nf1*$^{+/-}$ TRP+ = 0.02369 ± 0.000194 vs male *Nf1*$^{+/-}$ TRP+ = 0.02013 ± 0.000364, p= 0.0103). Also, an effect of sex in this variable was detected (F (1, 22) = 16.85, p=0.0005).

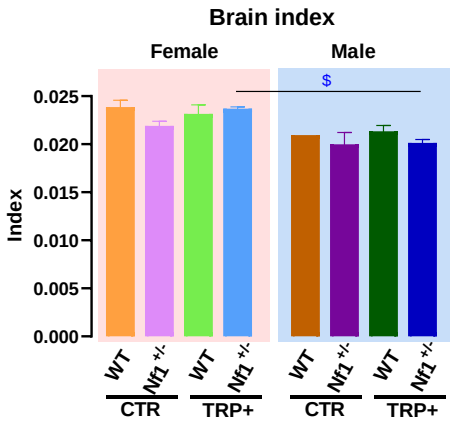



**Figure 47 | Females *Nf1*$^{+/-}$ TRP+ had a higher index than males *Nf1*$^{+/-}$ TRP+ of offspring in ASD condition**

An brain index was measured based on brain and body weight and the TRP+ diet did not influence the brain index, however females *Nf1*$^{+/-}$ TRP+ had a higher index than males *Nf1*$^{+/-}$ TRP+. Three-way ANOVA followed by Sidak's multiple comparisons test; p<0.05; $: significant difference between female *Nf1*$^{+/-}$ TRP+ and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 2; n (female *Nf1*$^{+/-}$ TRP+) = 5; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 3.

Thus, in this study, we concluded that the prenatal TRP+ diet in ASD condition did not influence neither weight nor length, and their respective increments in early-life. Meanwhile, TRP+ diet only had influence on body weight in juvenile age of offspring. Note that, this diet promoted a dimorphism in *Nf1*$^{+/-}$offspring in brain index.

### 4.2.2. Prenatal tryptophan suplemented diet improves motor development performance in females WT and influences communication skills in both sexes in ASD condition

Between P4 to P14, developmental milestones tests described above were conducted to evaluate the development of the sensorimotor abilities of the different groups of experimental.

On surface righting reflex test (Figure 48), it was observed at P6 female WT TRP+ diet developed this reflex faster than females WT CTR diet (female WT CTR = 2.817 ± 0.310 vs female WT TRP+ = 2.945 ± 0.461, p=0.0413). Overall, both sexes had an effect of age (female (F (2,59) = 13.66, p<0.0001); male: (F (2, 40) = 3.535, p=0.0386). Particularly, in males there was an effect of genotype (F (1, 40) = 6.465, p=0.0150) and in females there was an effect of diet (F (1, 59) = 4.129, p=0.0467).

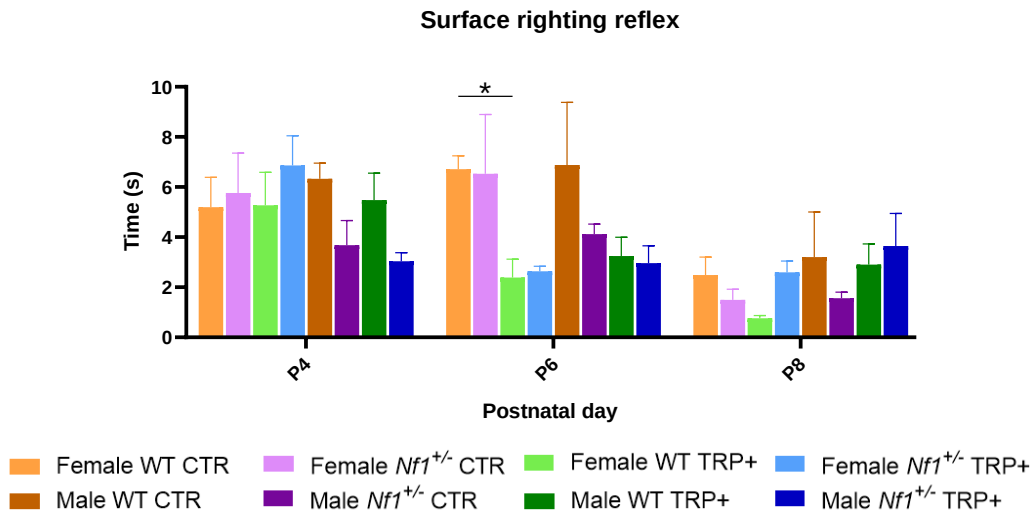



**Figure 48 | Females WT TRP+ developed faster in surface righting reflex of offspring in ASD condition**

Female WT TRP+ showed an improved in development in this reflex compared to Female WT CTR on P6. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female WT CTR and female WT TRP+; Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Moreover, in the negative geotaxis test (Figure 49) at P4 is observed a significantly difference between genotype in males CTR (male WT CTR = 21.45 ± 8.55 vs male *Nf1*$^{+/-}$ CTR = 7.49 ± 2.02, p=0.0425), where males *Nf1*$^{+/-}$ CTR developed faster. Also, there was an effect of genotype in males (F (1, 40) = 14.28, p=0.0005).

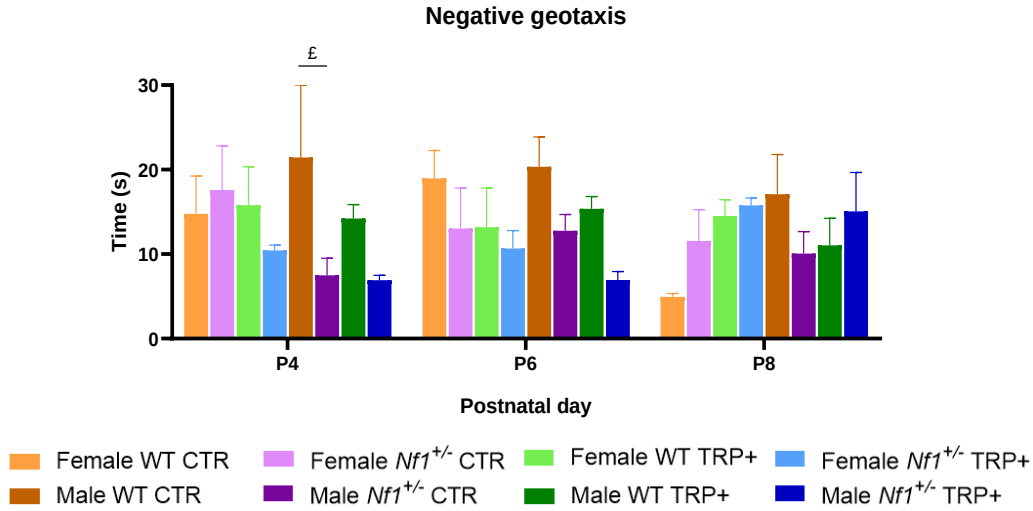



**Figure 49 | Males *Nf1*$^{+/-}$ CTR developed faster in negative geotaxis of offspring in ASD condition**

Males *Nf1*$^{+/-}$ CTR developed faster than male WT CTR in this reflex on P4. However, diet did not influence the development of negative geotaxis test. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; £: significant difference between male WT CTR and male Nf1$^{+/-}$ CTR. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

In addition, on nest seeking test (Figure 50), no differences were observed between experimental groups. There was an effect of age in both sexes (female: F (2, 63) = 23.83, p<0.0001; male: F (2, 42) = 9.111, p=0.0005).

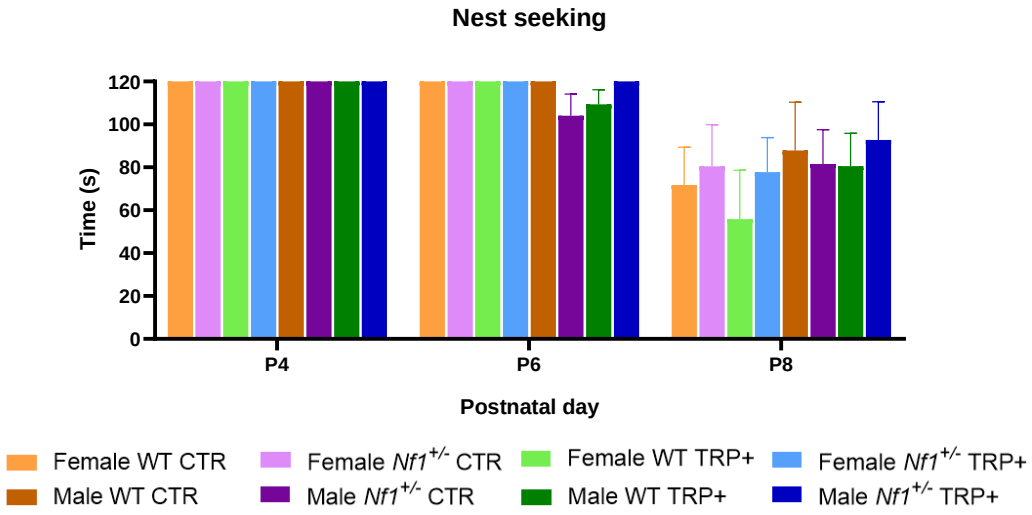



**Figure 50 | TRP+ diet did not influence the development in nest seeking of offspring in ASD condition**

TRP+ diet did not influence the development of nest seeking. Three-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Also, in cliff aversion test (Figure 51), there were no significant differences between the experimental groups.

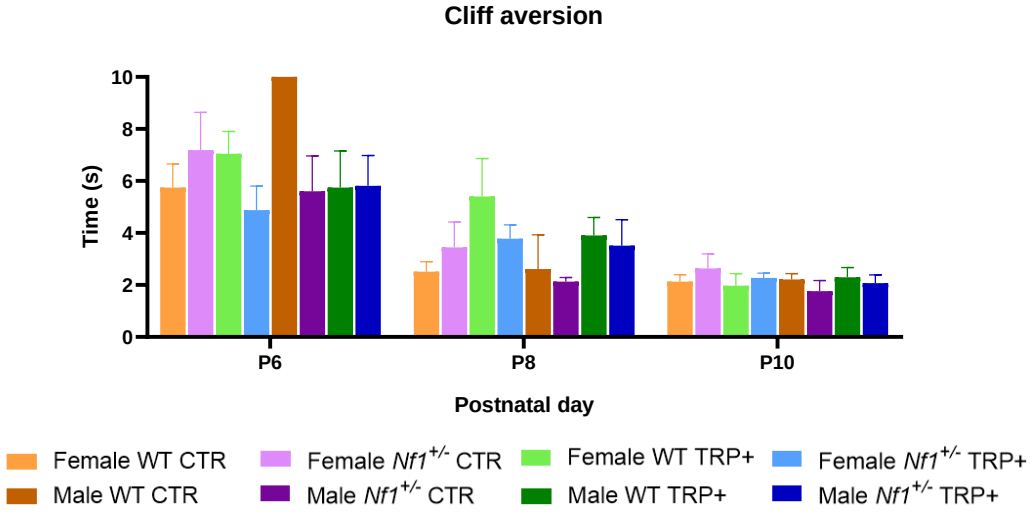



**Figure 51 | TRP+ diet did not influence the development in cliff aversion of offspring in ASD condition**

TRP+ diet did not influence the development of cliff aversion reflex. Three-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT

TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

On day 10, males *Nf1*$^{+/-}$ CTR showed a delay in development of locomotion (Figure 52) when compared to male WT CTR (male WT CTR = 3.62 ± 0.80 vs male Nf1$^{+/-}$ CTR = 30.00 ± 0.000), moreover, overall it is possible to observe an effect of genotype in males (F (1, 40) = 6.172, p=0.0173). In females, there was a diet effect (F (1, 57) = 7.271, p=0.0092). Furthermore, in both sexes was observed an age effect (females F (2, 57) = 16.49, p<0.0001; males: F (2, 40) = 7.617, p=0.0016).

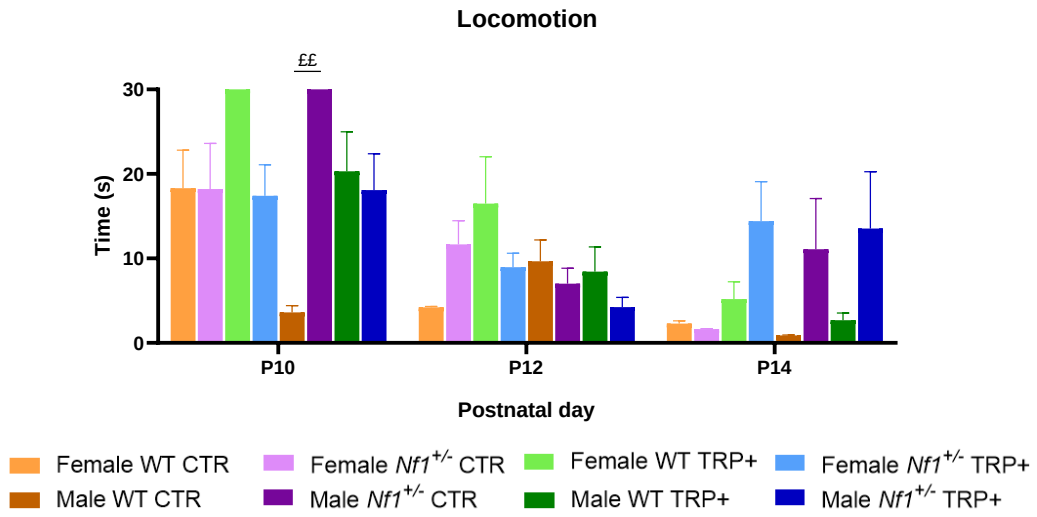



**Figure 52 | Males *Nf1*$^{+/-}$ CTR showed a delay in development of locomotion of offspring in ASD condition**

Males *Nf1*$^{+/-}$ CTR showed a delay compared than male WT CTR in this test on P10. However, diet did not influence the development of locomotion. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; £: significant difference between male WT CTR and male Nf1$^{+/-}$ CTR. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

In forelimb grasp test (Figure 53) an effect of diet on females was observed (F (1, 64) = 8.671, p=0.0045).

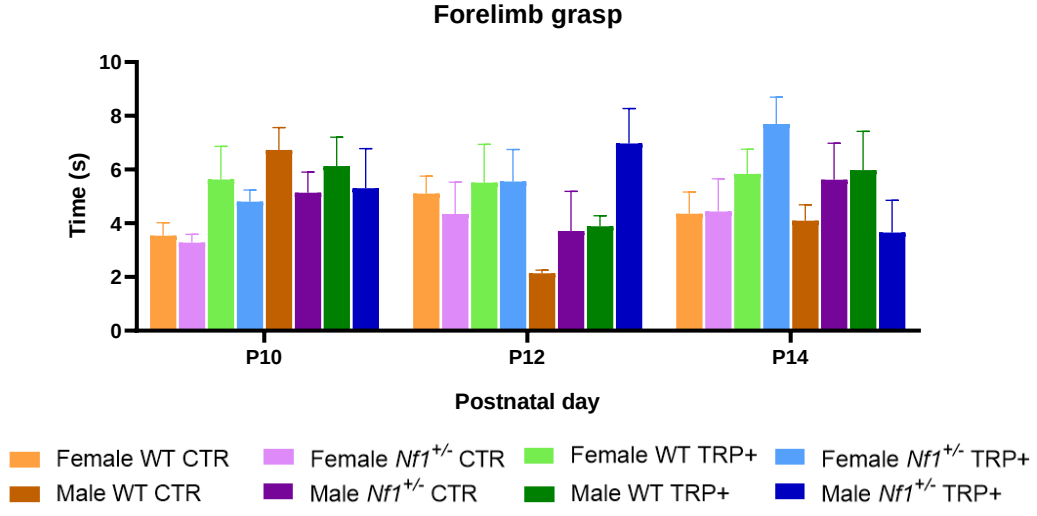


**Figure 53 | TRP+ diet did not influence the strength in forelimb grasp test of offspring in ASD condition**

TRP+ diet did not influence the development of forelimb grasp test. Three-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 8; n (female WT TRP+) = 8; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Thus, TRP+ diet only affected females WT in the surface righting reflex. In this test, TRP+ diet resulted in faster development in WT females.

To access communication skills, maternal separation-induced USVs were recorded following milestones on days P4, 6 and 8 and several parameters were analyzed.

On P8, it was possible to observe that WT females on CTR diet vocalized less often than *Nf1*$^{+/-}$ females on the same diet (female WT CTR = 287.90 ± 74.18 vs female *Nf1*$^{+/-}$ CTR = 545.00 ± 65.43, p=0.0326) (Figure 54A). It should be noted that there was an age effect in total number of USVs in both sexes (female: F (2, 63) = 6.856, p=0.0020; male: F (2, 40) = 4.054, p=0.0249).

Similar to the number, the total time of USVs (Figure 54B) also had an age effect (female: F (2, 62) = 8.784, p=0.0004; male: F (2, 40) = 7.938, p=0.0012). There was also an effect of diet in males (F (1, 40) = 9.198, p=0.0042).

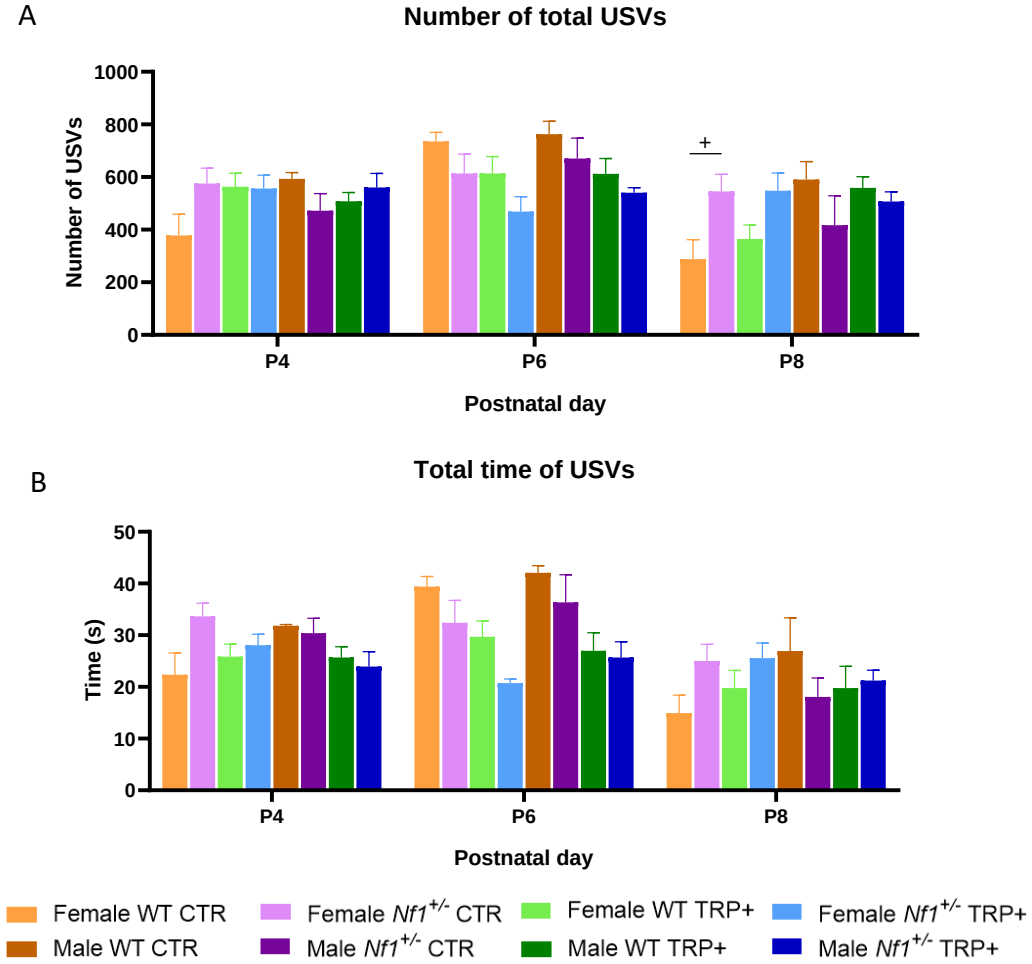
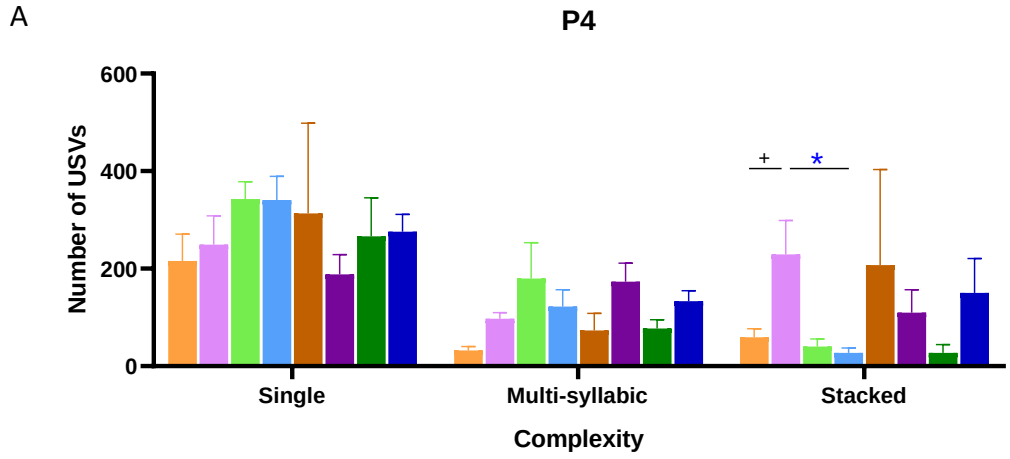


**Figure 54 | Females WT CTR vocalized less of offspring in ASD condition**
Females WT CTR had a lower (A) number of total USVs than females *Nf1*$^{+/-}$ CTR. Meanwhile, TRP+ diet did not influence the number of total USVs neither the (B) total time of USVs. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; +: significant difference between female WT CTR and female Nf1$^{+/-}$ CTR. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

The category of vocalizations was also evaluated. At P4 (Figure 55A) it was possible to observed that females *Nf1*$^{+/-}$ CTR produced significantly more stacked vocalizations than females WT CTR (female WT CTR = 59.29 ± 17.68 vs female *Nf1*$^{+/-}$ CTR = 229.00 ± 69.94, p=0.0365) and than females *Nf1*$^{+/-}$ TRP+ (female *Nf1*$^{+/-}$ CTR = 229.00 ± 69.94 vs female *Nf1*$^{+/-}$ TRP+ = 27.20 ± 10.17, p=0.0175). There was an effect of type of USVs in males (F (2, 41) = 6.942, p=0.0025) and females (F (2, 59) = 25.53, p<0.0001).

At P6 (Figure 55B) the difference between diets in *Nf1*$^{+/-}$ females in stacked vocalizations continued (female *Nf1*$^{+/-}$ CTR = 200.08 ± 43.03 vs female *Nf1*$^{+/-}$ TRP+ = 45.57 ± 13.43, p=0.0208), and then also a difference between diets in *Nf1*$^{+/-}$ males was observed (male *Nf1*$^{+/-}$ CTR = 267.80 ± 88.18 vs male *Nf1*$^{+/-}$ TRP+ = 83.50 ± 27.18, p=0.0268). The effect of type of USVs continued in both sexes (female: F (2, 63) = 17.85, p<0.0001; male: F (2, 41) = 11.95, p<0.0001).

At P8 (Figure 55C) there also was an type of USVs effect in females (F (2, 62) = 20.55, p<0.0001) and in males (F (2, 42) = 3.395, p=0.0430). Also, there was an type x diet effect in both sexes (female: F (2, 62) = 3.892, p=0.0256; male: F (2, 42) = 6.637, p=0.0031). There was an effect of genotype in females (F (1, 62) = 9.514, p=0.0030). Nevertheless, there was no difference between the experimental groups.



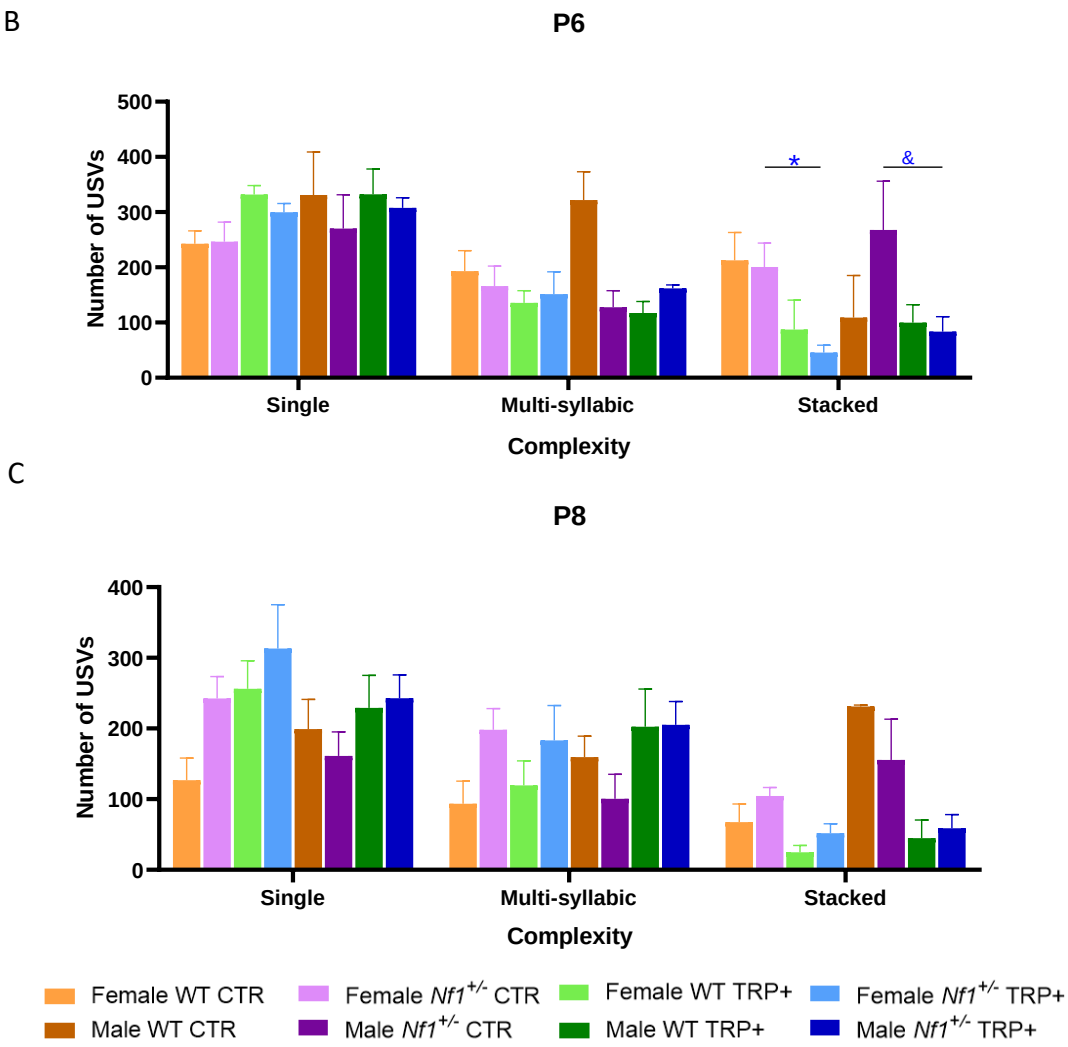



**Figure 55 | Pups *Nf1*$^{+/-}$ TRP+ produced significantly less stacked USVs of offspring in ASD condition**

At (A) P4 and (B) P6, females *Nf1*$^{+/-}$ TRP+ produced less stacked vocalizations than females *Nf1*$^{+/-}$ CTR. Also, at P6, males *Nf1*$^{+/-}$ TRP+ produced less stacked vocalizations than males *Nf1*$^{+/-}$CTR. However, at (C) P8, TRP+ diet did not influence the type of USVs. Three-way ANOVA followed by Turkey's multiple comparisons test; (A) $p<0.05$; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; +: significant difference between female WT CTR and female *Nf1*$^{+/-}$ CTR; (B) $p<0.05$; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; &: significant difference between male *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ TRP+; (C) no significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

The time to first call (latency) was also analyzed (Figure 55A). On the first day of vocalizations, males WT on both diets took much longer to start vocalizing than females WT on the same diets (female WT CTR = 0.1216 ± 0.0129 vs male WT CTR = 4.403 ± 4.272, p=0.0067; female WT TRP+ = 0.0775 ± 0.0158 vs male WT TRP+ = 20.600 ± 7.102, p=0.0030). Moreover, males WT CTR had a longer latency period than males *Nf1*$^{+/-}$ CTR (male WT CTR = 4.403 ± 4.272 vs male *Nf1*$^{+/-}$ CTR = 0.069 ± 0.026, p=0.0042. The same difference was also observed at P8 (male WT CTR = 3.533 ± 0.432 vs male *Nf1*$^{+/-}$ CTR =

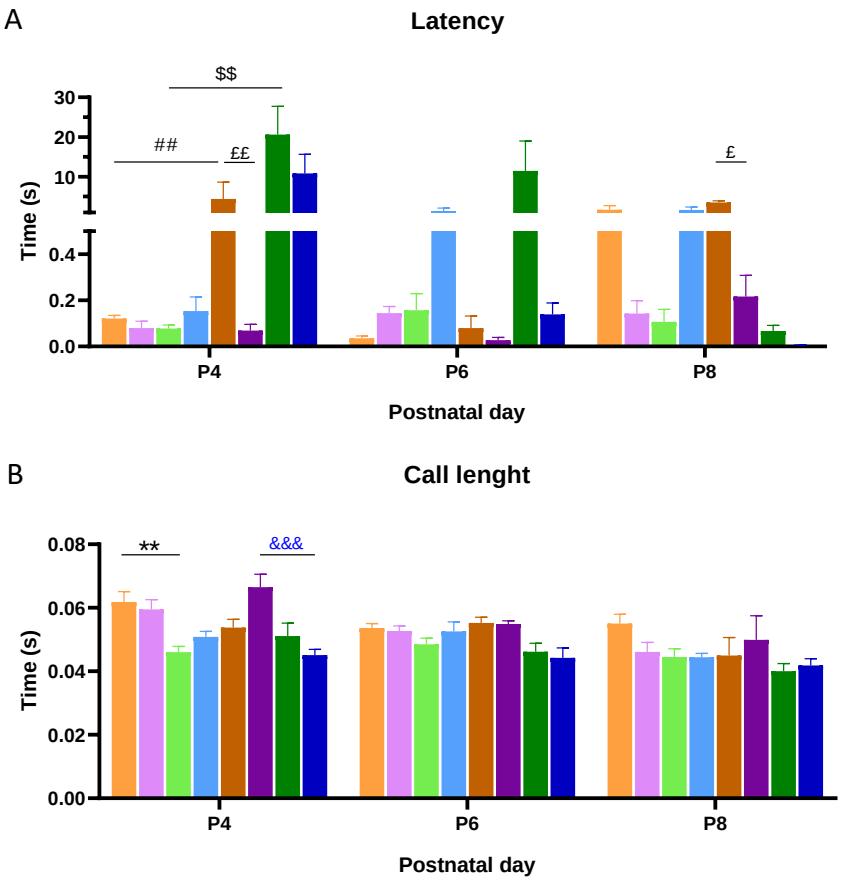
0.216 ± 0.092, p=0.0480). There was an effect between diet and genotype (F (1, 35) = 12.04, p=0.0014).

At P4, it was possible to observe a decrease in call length (Figure 55B) in females WT TRP+ compared to female WT CTR (WT CTR female = 0.06180 ± 0.0033 vs WT TRP+ female = 0.04602 ± 0.0018, p=0.0037). Also, males *Nf1*$^{+/-}$ TRP+ had a shorter call length than males *Nf1*$^{+/-}$ CTR (*Nf1*$^{+/-}$ CTR male =0.06648 ± 0.00408 vs *Nf1*$^{+/-}$ TRP+ male = 0.04513 ± 0.00179, p=0.0001). Overall, there was an effect of diet in males (F (1, 40) = 15.27, p=0.0004) and also, an effect of age in both sexes (females: F (2, 61) = 7.307, p=0.0014; males: F (2, 40) = 5.716, p=0.0066).

The time of interval between calls (mean inter-call) (Figure 55C) had an age effect in females (F (2, 60) = 4.903, p=0,0107). At P8, females WT CTR had longer time between calls than females *Nf1*$^{+/-}$ CTR (female WT CTR = 0.9704 ± 0.2399 vs female *Nf1*$^{+/-}$ CTR = 0.537 ± 0.0708, p=0.0341).

In addition, female WT CTR showed lower power (Figure 55D) than females WT TRP+ (female WT CTR = -83.69 ± 1.419 vs female WT TRP+ = -92.90 ± 1.665, p=0.0041) and than females *Nf1*$^{+/-}$ CTR (female WT CTR = -83.69 ± 1.419 vs female *Nf1*$^{+/-}$ CTR = -90.33 ± 0.932, p<0.0001). There was an effect of diet on females (F (1, 62) = 8,075, p=0.0061).

However, at P4, females WT CTR had a higher tonality (Figure 55E) than females WT TRP+ (female WT CTR =0.6578 ± 0.0179 vs female WT TRP+ = 0.5045 ± 0.0281, p<0.0001) and than females *Nf1*$^{+/-}$ CTR (female WT CTR = 0.6578 ± 0.0179 vs female *Nf1*$^{+/-}$ CTR = 0.5676 ± 0.0343, p=0.0311).



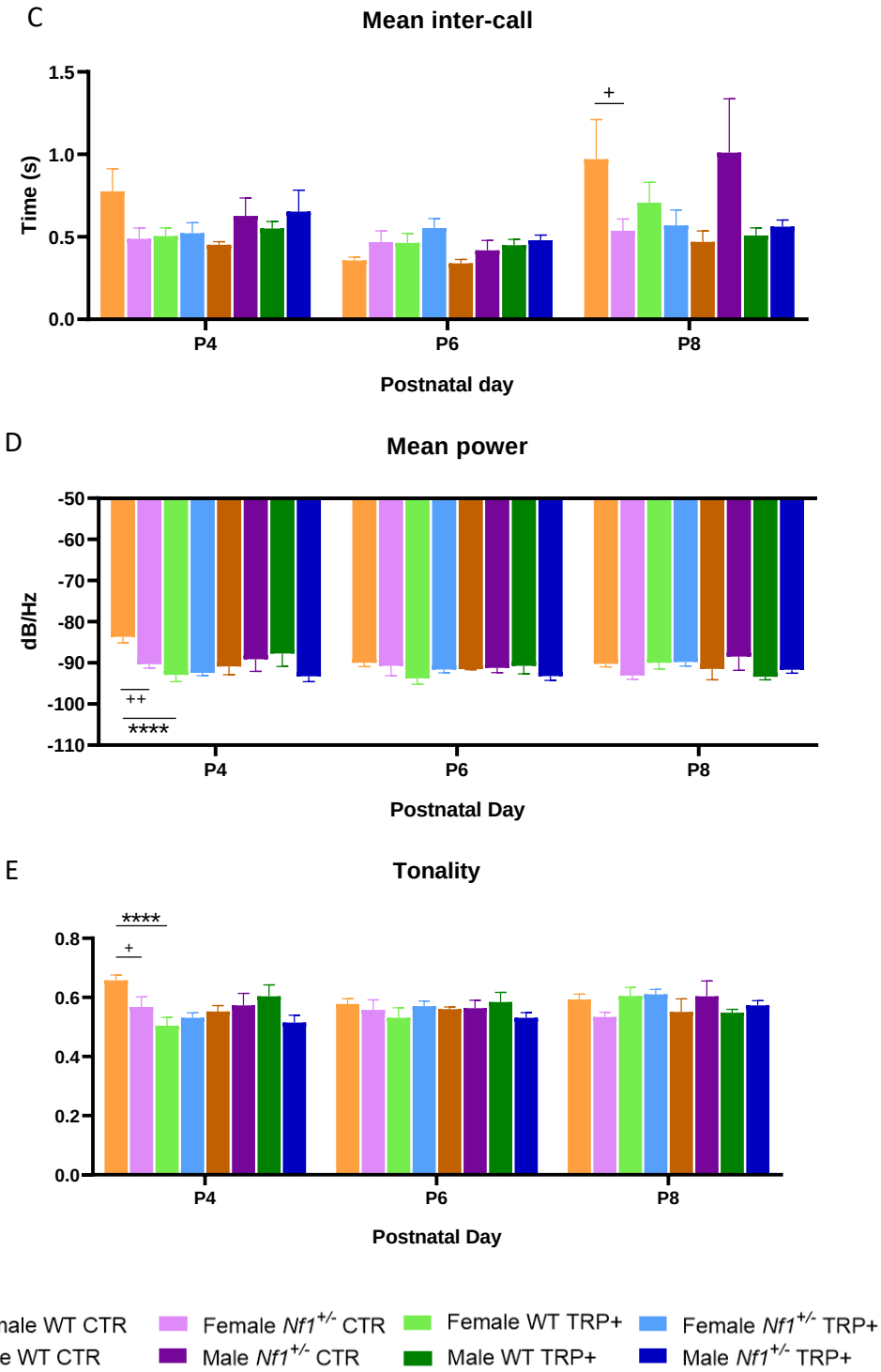



**Figure 56 | Females WT TRP+ showed shorter call length, higher power and lower tonality of USVs of offspring in ASD condition**

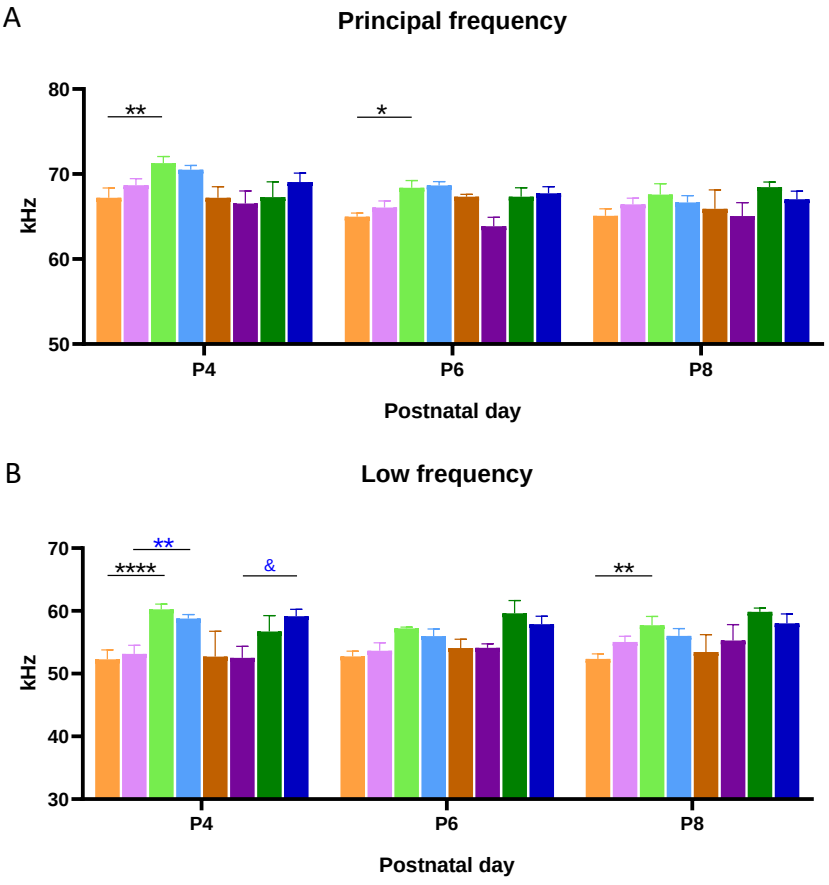
Mainly at P4, males WT CTR had a longer (A) latency than males *Nf1*$^{+/-}$ CTR and than females WT CTR. Also, males WT TRP+ had a longer latency time than females WT TRP+. At P4, females WT TRP+ had a shorter (B) call length than females WT CTR and males *Nf1*$^{+/-}$ TRP+ also had shorter call length than males *Nf1*$^{+/-}$ CTR. Regarding the (C) mean inter-call females WT CTR had longer time of interval between calls than females *Nf1*$^{+/-}$CTR at P8. Also at P4, females WT TRP+ showed higher (D) power than female WT CTR. In addition, females WT CTR had lower power than females *Nf1*$^{+/-}$CTR. Lastly, females WT TRP+ showed a lower (D) tonality than females WT CTR. Also, females *Nf1*$^{+/-}$CTR had lower tonality than females WT CTR.

Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; *: significant difference between female WT CTR and female WT TRP+; #: significant difference between female WT CTR and male WT CTR; $: significant difference between female WT TRP+ and male WT TRP+; &: significant difference between male *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ TRP+; +: significant difference between female WT CTR and female *Nf1*$^{+/-}$ CTR; £: significant difference between male WT CTR and male *Nf1*$^{+/-}$ CTR. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Furthermore, female WT TRP+ showed a higher principal frequency (Figure 57A) than female WT CTR at P4 and P6 (P4: female WT CTR = 67.21 ± 1.144 vs female WT TRP+ = 71.30 ± 0.762, p=0.0047; P6: female WT CTR = 65.10 ± 0.820 vs female WT TRP+ = 67.59 ± 1.281, p=0.0085). Overall, females had a diet - (F (1, 61) = 25.03, p<0.0001) and age-depended effect (F (2, 61) = 13.94, p<0.0001) in both diets in principal frequency.

Regarding low frequency (Figure 57B), there was an diet effect (female: F (1, 61) = 45.29, p<0.0001; male: F (1, 42) = 20.37, p<0.0001). On P4, females TRP+ with both genotypes and males *Nf1*$^{+/-}$ TRP+ had a higher low frequency than the respectives CTR (female WT CTR = 52.28 ± 1.518 vs female WT TRP+ = 60.24 ± 0.829, p<0.0001; female *Nf1*$^{+/-}$ CTR = 53.16 ± 1.365 vs female *Nf1*$^{+/-}$ TRP+ = 58.80 ± 0.630; male *Nf1*$^{+/-}$ CTR = 52.50 ± 1.850 vs male *Nf1*$^{+/-}$ TRP+ = 59.14 ± 1.102, p=0.0434). The difference between diets in WT females appeared also at P8 (female WT CTR = 52.36 ± 0.775 vs female WT TRP+ = 57.72 ± 1.381, p=0.0061).

Meanwhile, at high frequency (Figure 57C) no significant difference between groups nor any effect of variables was observed.



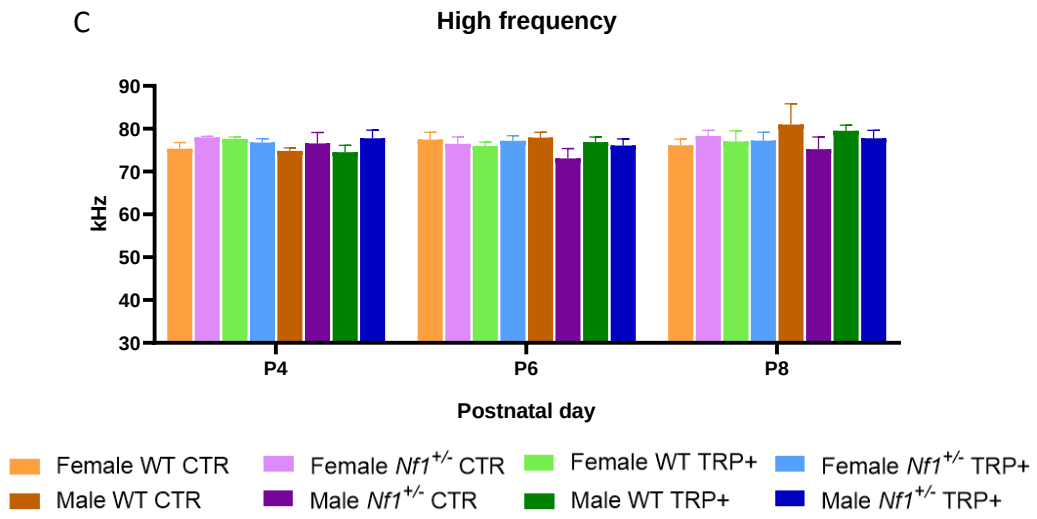



**Figure 57 | TRP+ diet increased principal and low but not the high frequency of USVs of offspring in ASD condition**

Females WT TRP+ showed a higher (A) principal and (B) low frequency. Also, females and males *Nf1*$^{+/-}$ TRP+ diet has increased low frequency. TRP+ diet did not influence (C) high frequency. Three-way ANOVA followed by Turkey's multiple comparisons test; $p<0.05$; *: significant difference between female WT CTR and female WT TRP+; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; &: significant difference between male *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

The TRP+ diet mainly affected the WT females in various parameters, showing lower call length, higher power, lower tonality and higher principal and low frequencies.

Physical landmarks were registered for open eyes (Figure 58A) and auditory startle response (Figure 58B) during milestones and we observed no significant differences in both.

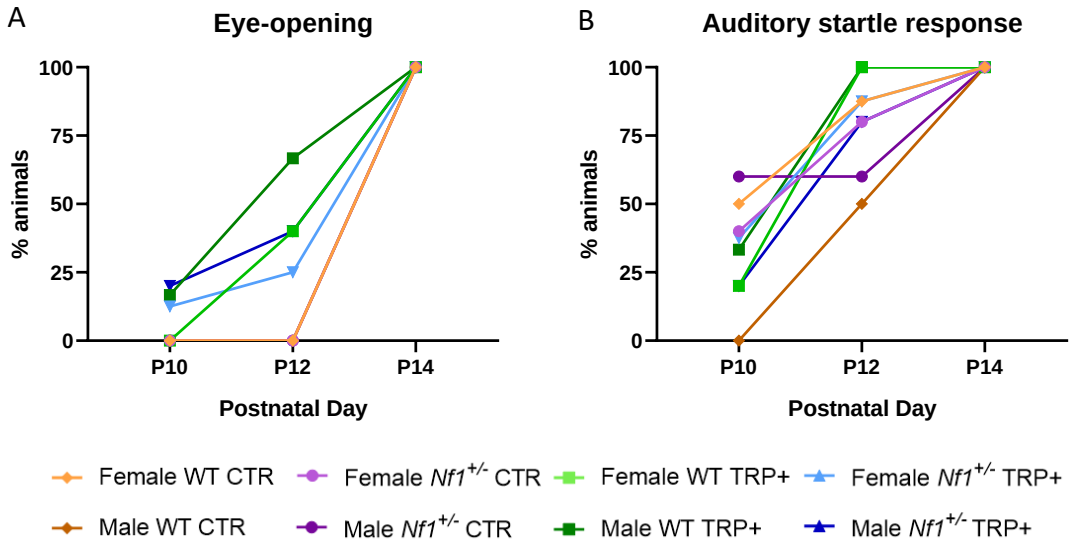
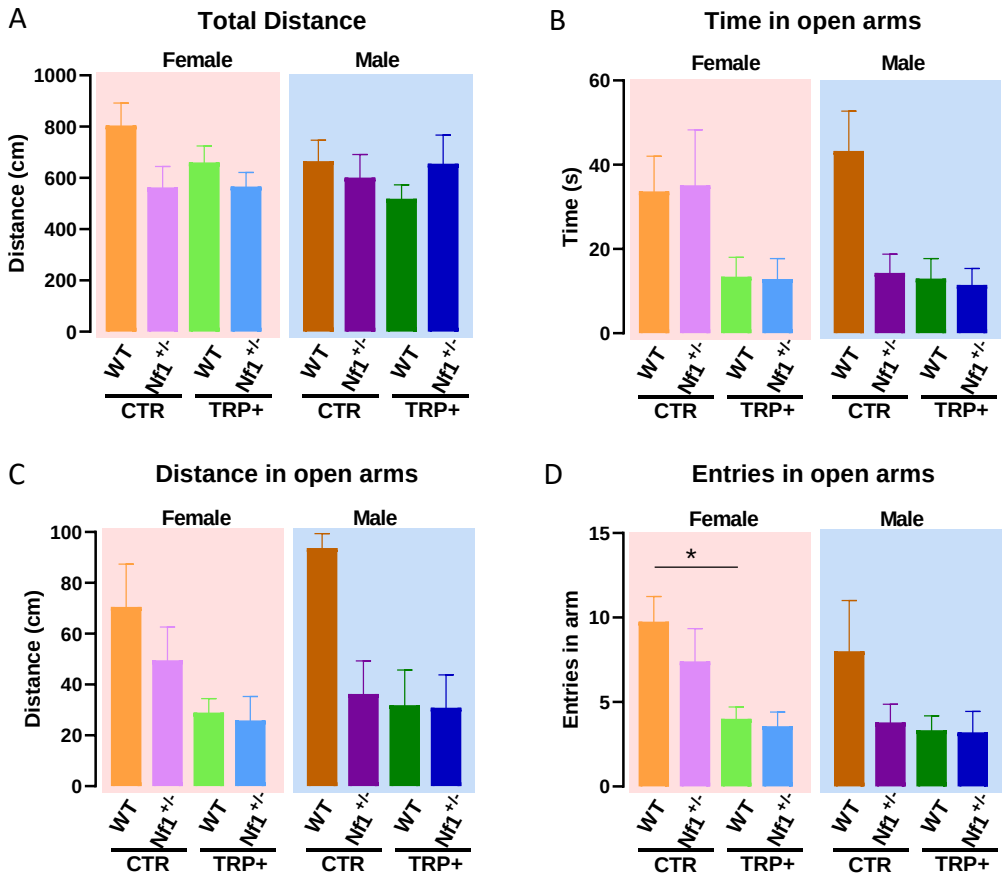


**Figure 58 | TRP+ diet did not influence eye-opening day and auditory startle response of offspring in ASD condition**

TRP+ diet did not influence (A) eye-opening and (B) auditory startle response. Two-way ANOVA followed by Turkey's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

### 4.2.3. Prenatal tryptophan suplemented diet decreased number of open arms entries in females in ASD condition

In the EPM test, the total distance (Figure 59A) traveled was measured and no differences were observed between the experimental groups. However, in the time (Figure 59B), distance (Figure 59C), entries in the open arms (Figure 59D) and in anxiety index (Figure 59E) there was an overall effect of diet (time in open arms: F (1, 34) = 11.04, p=0.0021; distance in open arms: F (1, 34) = 9.708, p=0.0037; entries in open arms: F (1, 35) = 13.34, p=0.0008); anxiety index: (F (1, 34) = 10.53, p=0.0026) , both in females and males. Furthermore, in the open arm entries there was a difference in diet specifically between WT females (female WT CTR = 9.750 ± 1.497 vs female WT TRP+ = 4.00 ± 0.707, p=0.0447), where females WT TRP+ showed a lower number of entries in open arms.



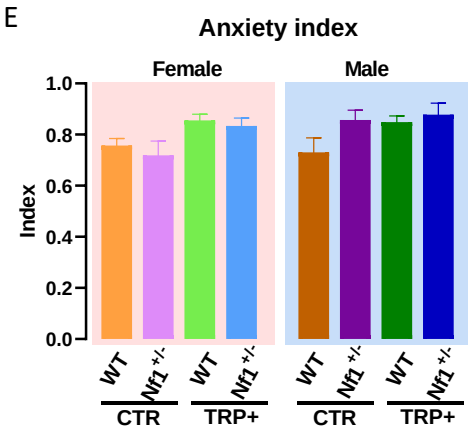



**Figure 59 | Females WT TRP+ showed a lower number of entries in the open arms of offspring in ASD condition**

TRP+ diet did not influence (A) total distance, (B) time and (C) distance in open arms. However, females WT TRP+ showed a lower (D) number of entries in the open arms. Also, TRP+ diet did not influence (E) anxiety index. Three-way ANOVA followed by Sidak's multiple comparisons test; (A) No significant differences found between any of the experimental groups; (B) No significant differences found between any of the experimental groups; (C) No significant differences found between any of the experimental groups; (D) $p<0.05$; *: significant difference between female WT CTR and female WT TRP+; (E) No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female *Nf1*$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

TRP+ diet only showed significant differences in number of entries in open arms in females WT. No significant differences were observed in the other parameters, including the anxiety index, and therefore the TRP+ diet does not influence anxiety behavior.

### 4.2.4. Prenatal tryptophan suplemented diet seems to improves the number and time of social interactions in *Nf1*$^{+/-}$ mice in ASD condition

To evaluate social interaction, a juvenile social play test was performed and the number (Figure 60A) and time of interactions (Figure 60B) were analyzed. As this test required two animals of the same sex and genotype, it was difficult to have enough pairs to have more concrete results that would give differences. Thus, no difference was observed between the experimental groups. However, was a general effect of the diet in number of social interactions was observed ($F(1, 8) = 7.742$, $p=0.0238$). The TRP+ diet in females *Nf1*$^{+/-}$ seems to have increased the number of interaction, on the other hand, TRP+ diet in males *Nf1*$^{+/-}$ seems to have increased the time of interactions. However, due to the low number as mentioned above, it was not statistically relevant but there seemed to be a trend.

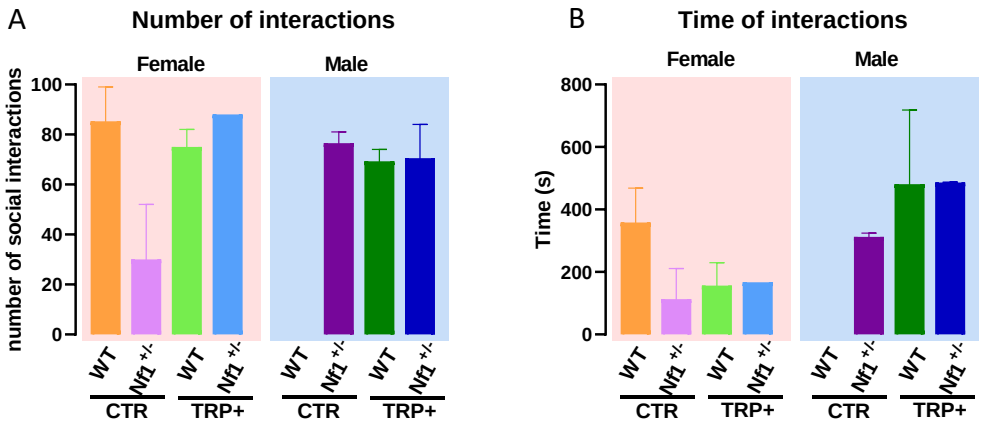



**Figure 60 | TRP+ diet did not influence number and time of social interactions of offspring in ASD condition**

TRP+ diet did not influence (A) number of social interactions and (B) time of social interactions. Three-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 3; n (female *Nf1*$^{+/-}$ CTR) = 2; n (female WT TRP+) = 2; n (female *Nf1*$^{+/-}$ TRP+) = 1; n (male WT CTR) = 0; n (male *Nf1*$^{+/-}$ CTR) = 2; n (male WT TRP+) = 3; n (male *Nf1*$^{+/-}$ TRP+) = 2

Moreover, the number of USVs in a social (Figure 61A) and non-social context (Figure 61B) and a respective USVs social index (Figure 61C) were also analyzed.

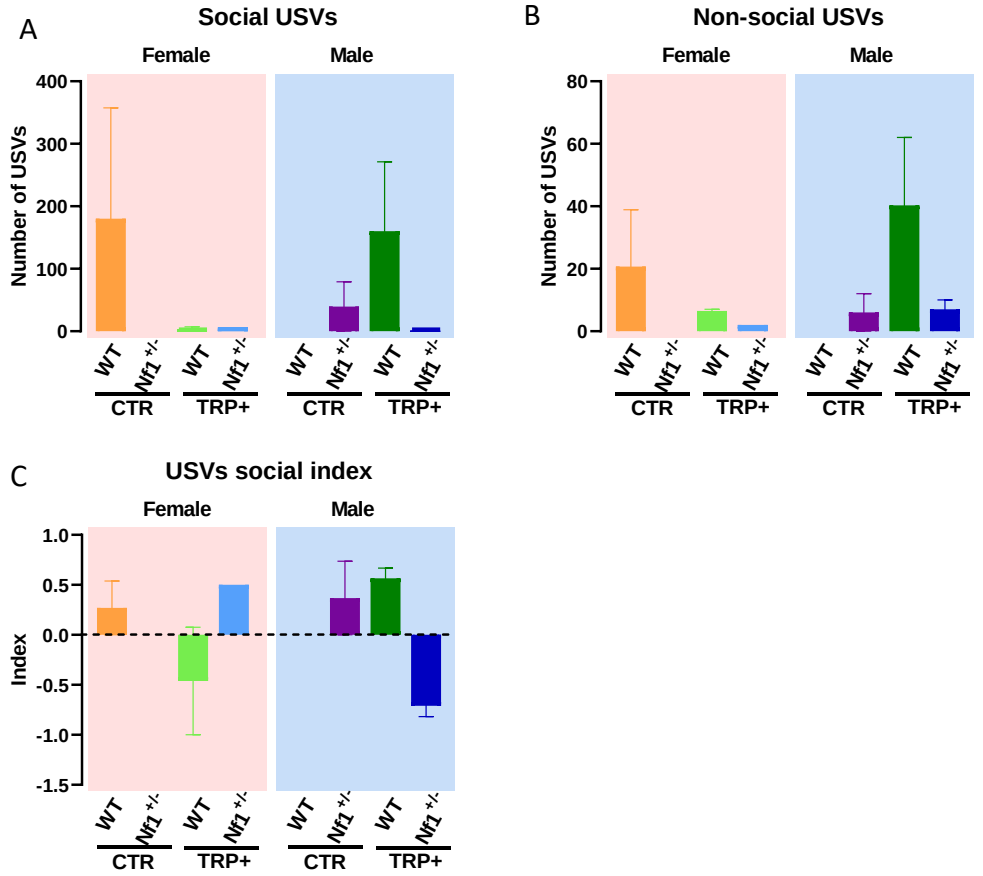


**Figure 61 | TRP+ diet did not influence number of USVs in a social and non-social context and the respective index of offspring in ASD condition**

TRP+ diet did not influence (A) number of USVs in a social context, (B) number of USVs in a non-social context and (C) USVs social index. Three-way ANOVA followed by Sidak's multiple comparisons test; No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (female WT CTR) = 3; n (female *Nf1*$^{+/-}$ CTR) = 2; n (female WT TRP+) = 2; n (female *Nf1*$^{+/-}$ TRP+) = 1; n (male WT CTR) = 0; n (male *Nf1*$^{+/-}$ CTR) = 2; n (male WT TRP+) = 3; n (male *Nf1*$^{+/-}$ TRP+) = 2.

### 4.2.5. Prenatal tryptophan suplemented diet decrease repetitive behavior in males *Nf1*$^{+/-}$ in ASD condition

In marble test it was possible to observed significant differences between diets in *Nf1*$^{+/-}$ males, where TRP+ diet decreases repetitive behavior (male *Nf1*$^{+/-}$ CTR = 4.200 ± 0.374 vs male *Nf1*$^{+/-}$ TRP+ = 1.200 ± 0.490, p=0.0453) (Figure 62). It was also possible to observe differences between females and males *Nf1*$^{+/-}$ with diet CTR, where males buried more marbles (female *Nf1*$^{+/-}$ CTR = 1.000 ± 0.632 vs male *Nf1*$^{+/-}$CTR = 4.200 ± 0.374, p=0.0162).

In both sex and in both diets there is an effect of time (female: F (3, 80) = 9.211, p<0.0001; male: F (3, 56) = 11.85, p<0.0001; diet CTR: F (3, 61) = 13.24, p<0.0001; diet TRP+: F (3, 75) = 7.940, p=0.0001). Also, in males, there was a diet effect (F (1, 56) = 8.567, p=0.0049) and a diet x genotype effect (F (1, 56) = 4.051, p=0.0490). Overall, there was an effect of sex in CTR diet F (1, 61) = 23.21, p=0.0001).

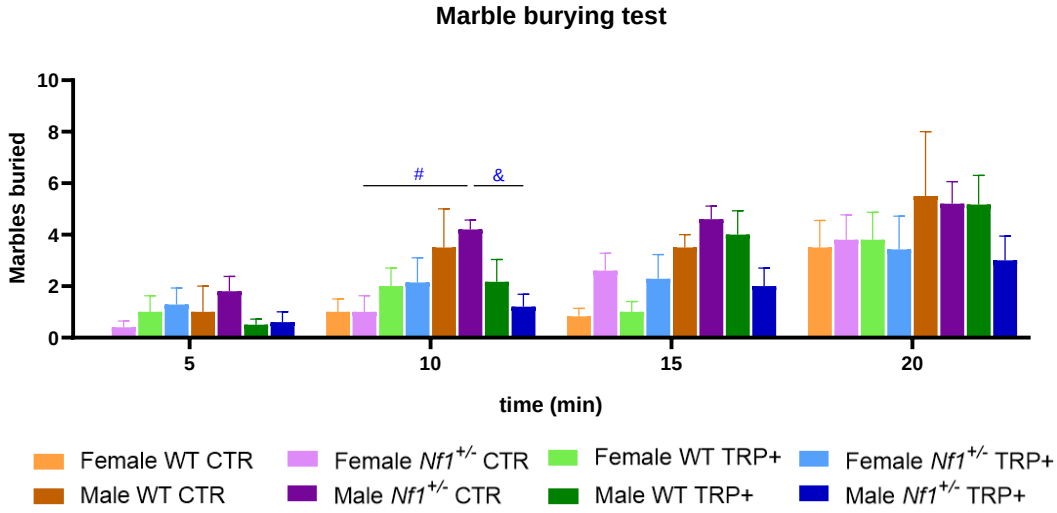



**Figure 62 | Males *Nf1*$^{+/-}$ TRP+ showed a decrease in repetitive behavior of offspring in ASD condition**

TRP+ diet influenced repetitive behavior in males *Nf1*$^{+/-}$ by decreasing the number of marbles burying. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; #: significant difference between female *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ CTR; &: significant difference between male *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 8; n (female Nf1$^{+/-}$ CTR) = 5; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 5; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

Thus, regarding the marble burying test, the diet only affected males *Nf1*$^{+/-}$ TRP+ where decreased repetitive behavior.

### 4.2.6. Prenatal tryptophan suplemented diet improves learning in ASD condition

The first parameter analyzed in the Barnes test was the escape latency time (Figure 63), in this aspect on the last day of training the females *Nf1*$^{+/-}$ TRP+ females took less time to enter the escape tunnel than the females WT with same diet (female WT TRP+ = 65.87 ± 14.29 vs female *Nf1*$^{+/-}$ TRP+ = 144.30 ± 11.93, p=0.0096).

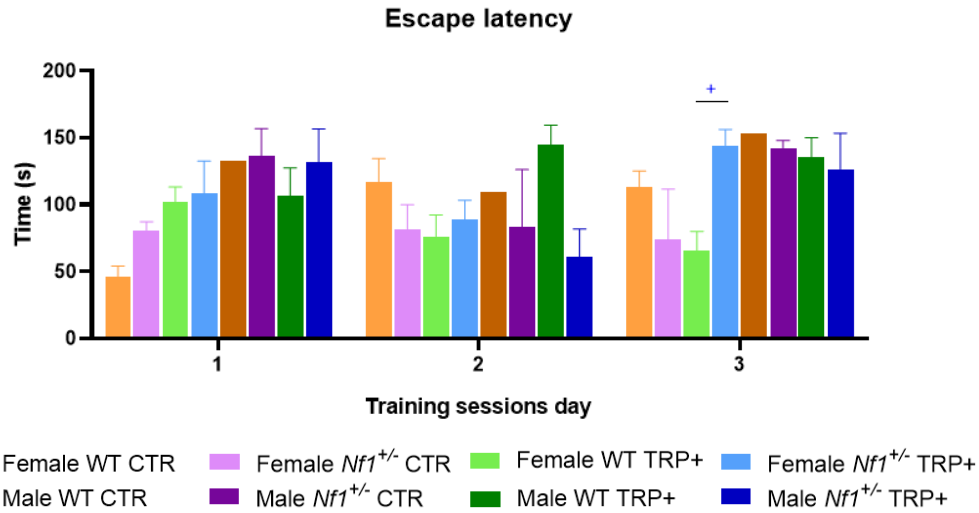



**Figure 63 |Females *Nf1*$^{+/-}$ TRP+ had a longer escape latency time of offspring in ASD condition**
TRP+ diet increased escape latency time in Females *Nf1*$^{+/-}$ on third day of training sessions. Three-way ANOVA followed by Turkey's multiple comparisons test; p<0.05; +: significant difference between female WT TRP+ and female *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 5; n (male *Nf1*$^{+/-}$ TRP+) = 3.

In time to find the target (Figure 64), on the first day of training TRP+ *Nf1*$^{+/-}$ males took much longer to find than *Nf1*$^{+/-}$ females on the same diet (female *Nf1*$^{+/-}$ TRP+ = 23.58 ± 4.64 vs male *Nf1*$^{+/-}$ TRP+. = 81.89 ± 17.96, p=0.0034). In addition, there was an effect of diet in both sexes (females: F (1, 52) = 6.601, p=0.0131; males: F (1, 26) = 4.956, p=0.0349). On the day of the probe, no differences were observed between the experimental groups.

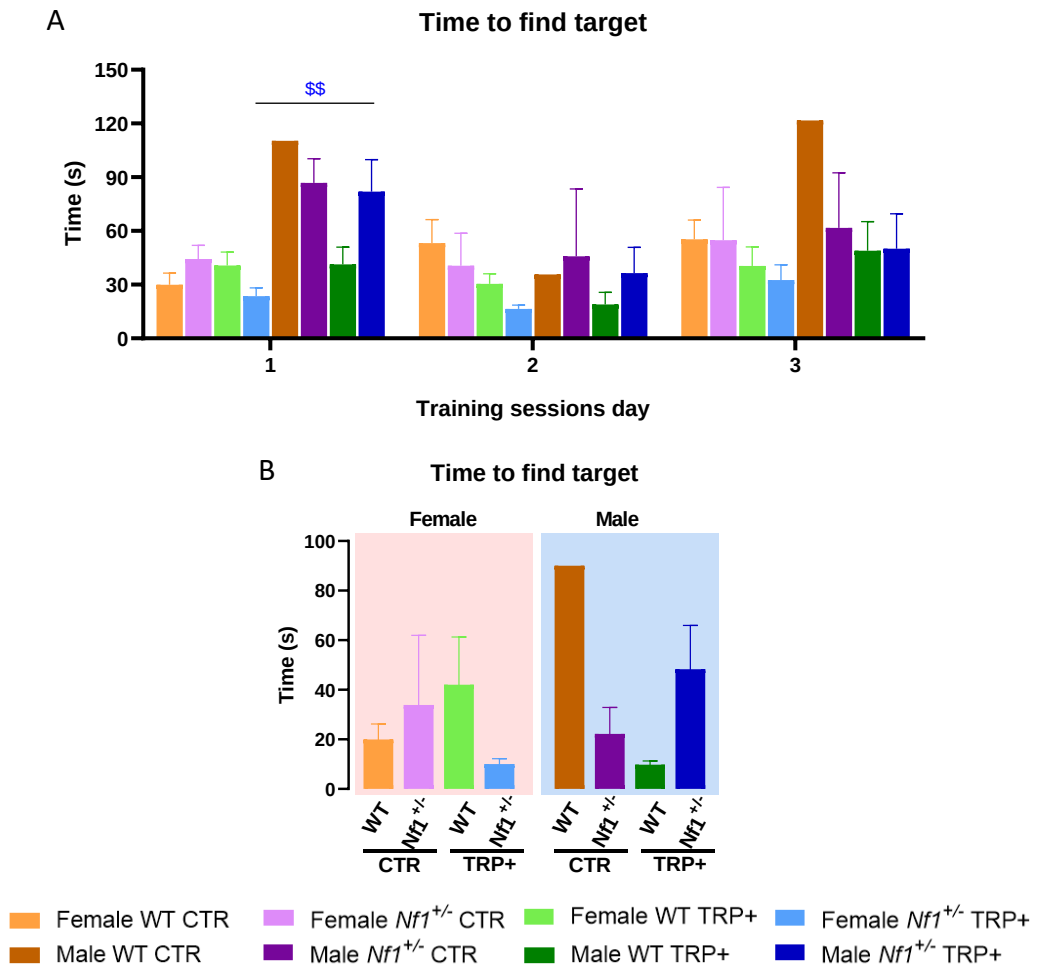



**Figure 64 |Females *Nf1*$^{+/-}$ TRP+ were faster to find target than males *Nf1*$^{+/-}$ TRP+ in training sessions of offspring in ASD condition**

TRP+ diet promote a dimorphism in *Nf1*$^{+/-}$ mice in day 1 of (A) training sessions days, where females were faster to find the target. However, diet had no influence in any experimental groups on (B) probe day. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; $: significant difference between female *Nf1*$^{+/-}$ TRP+ and male *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 5.

On the last day of training (Figure 65A) it was possible to observe differences in time in the target zone between females WT and *Nf1*$^{+/-}$ with CTR diet, with the last ones spent less time in the target zone (female WT CTR = 58.14 ± 4.82 vs female *Nf1*$^{+/-}$ CTR = 12.52 ± 1.91, p=0.0063). Females *Nf1*$^{+/-}$ TRP+ spent much more time in the target zone than females *Nf1*$^{+/-}$ CTR (female *Nf1*$^{+/-}$ CTR = 12.52 ± 1.91 vs female *Nf1*$^{+/-}$ TRP+ = 61.16 ± 5.34, p=0.0024). Overall, in training sessions there was a diet effect (F (1, 52) = 0.6392, p=0.0225) and a diet x genotype effect (F (2, 52) = 1.987, p=0.0009) only in females. On prove day (Figure 65B) no significant differences were observed between groups however there was a sex x genotype effect (F (1, 27) = 7.240, p=0.0121).

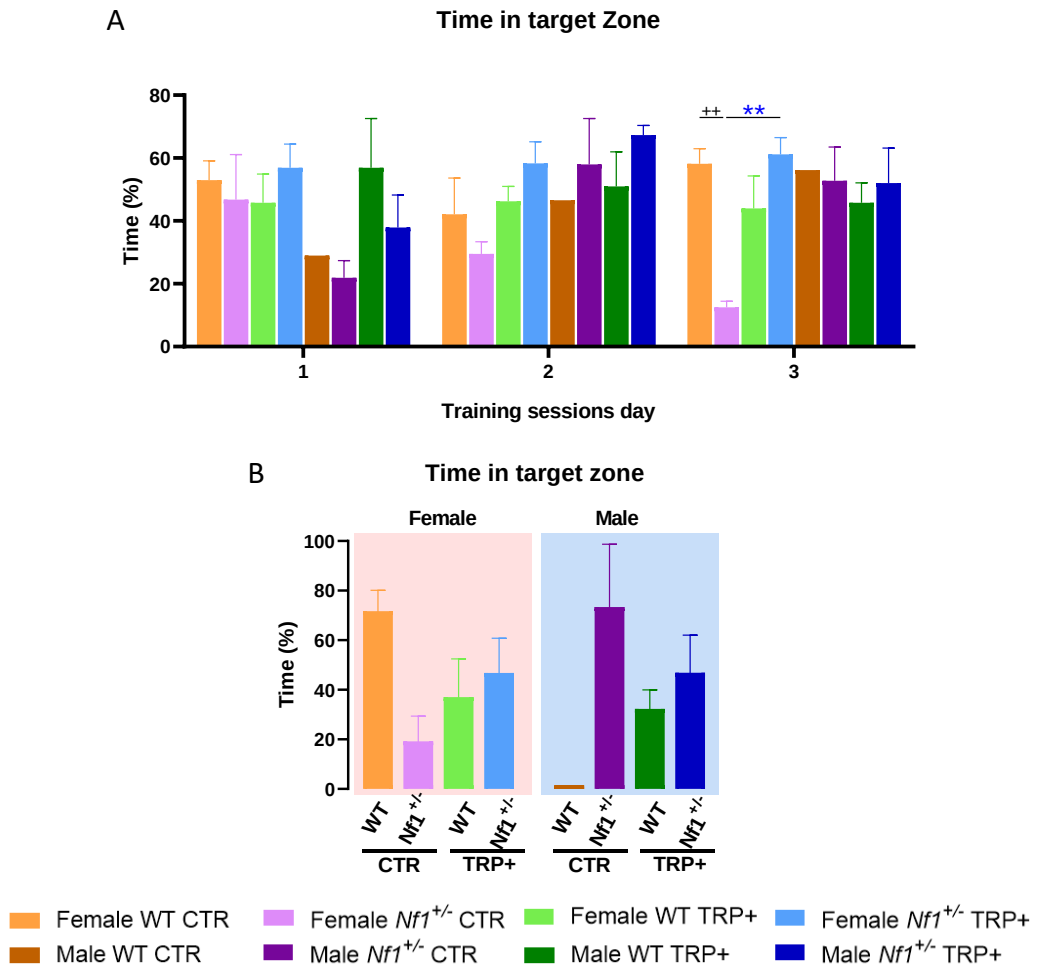



**Figure 65 |Females *Nf1*$^{+/-}$ TRP+ spent more time in target zone in training sessions of offspring in ASD condition**

The TRP+ diet improved learning capacity in females *Nf1*$^{+/-}$, who spent more time in the target zone than females *Nf1*$^{+/-}$ CTR in day 3 of (A) training sessions days. In addition, it was possible to observed a difference in genotype in females CTR, where females *Nf1*$^{+/-}$ showed an impairment in learning. On the other hand, TRP+ diet did not influence any experimental groups on (B) probe day. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; +: significant difference between female WT CTR and female *Nf1*$^{+/-}$ CTR. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 3.

Regarding on exploring errors, in the first day of training sessions (Figure 66A) it was possible to observe differences between females and males WT CTR, where males performed worse (female WT CTR = 6.762 ± 2.071 vs male WT CTR = 24.00 ± 0.00, p=0.0041). However, the TRP+ diet in WT males reverses the poor development of WT males CTR (male WT CTR = 24.00 ± 0.00 vs male WT TRP+ = 4.133 ± 1.759, p=0.0008). Besides that, males had an effect on days (F (2, 27) = 14.92, p<0.0001) and diet (F (1, 27) = 14.38, p=0.0008). Also, in females there was an effect of diet x genotype (F (1, 52) = 6.316, p=0.0151). On probe day (Figure 66B), males WT CTR committed more exploring errors than females WT CTR (female WT CTR = 0.00 ± 0.00 vs male WT CTR = 8.00 ± 0.00, p=0.0193).

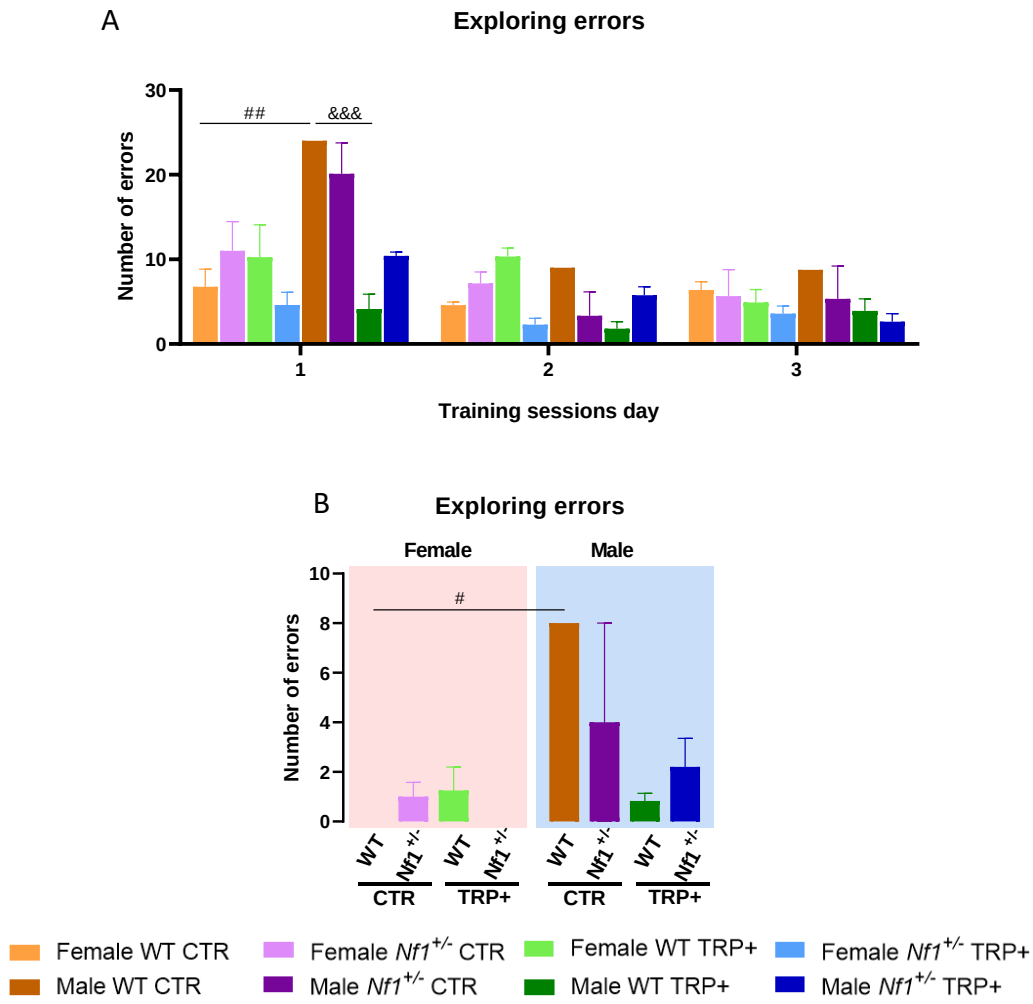



**Figure 66 |Males WT TRP+ committed fewer exploring errors in training sessions of offspring in ASD condition**

The TRP+ diet improved learning capacity in males WT, who committed less errors than males WT CTR in day 1 of (A) training sessions days. Moreover, it was possible to observe a sex difference in WT CTR mice, where males showed an impairment in learning. This difference was also seen on (B) probe day. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; #: significant difference between female WT CTR and male WT CTR; &: significant difference between male WT CTR and male WT TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 3.

On the first day of training there was a difference in total distance (Figure 67A) between sex in *Nf1*$^{+/-}$ CTR, males traveled more distances than the females (female *Nf1*$^{+/-}$ CTR = 207.30 ± 26.85 vs male *Nf1*$^{+/-}$ CTR = 372.10 ± 82.27, p=0.0197). Also in this day, females WT TRP+ traveled more total distance than females WT CTR (female WT CTR = 119.10 ± 20.55 vs female WT TRP+ = 220.30 ± 26.85, p=0.0246). On third day of training, these females WT TRP+ traveled the least distance compared to females *Nf1*$^{+/-}$ and males WT on the same diet (female WT TRP+ = 86.44 ± 9.23 vs female *Nf1*$^{+/-}$ TRP+ = 217.90 ± 29.62, p=0.0016; female WT TRP+ = 86.44 ± 9.23 vs male WT TRP+ = 256.20 ± 46.19, p= 0.0149). Besides these differences, there was a genotype effect in the females ($F(1, 48) = 6.058$, p=0.0175). On the probe day (Figure 67B) no significant differences were observed between any of the experimental groups.

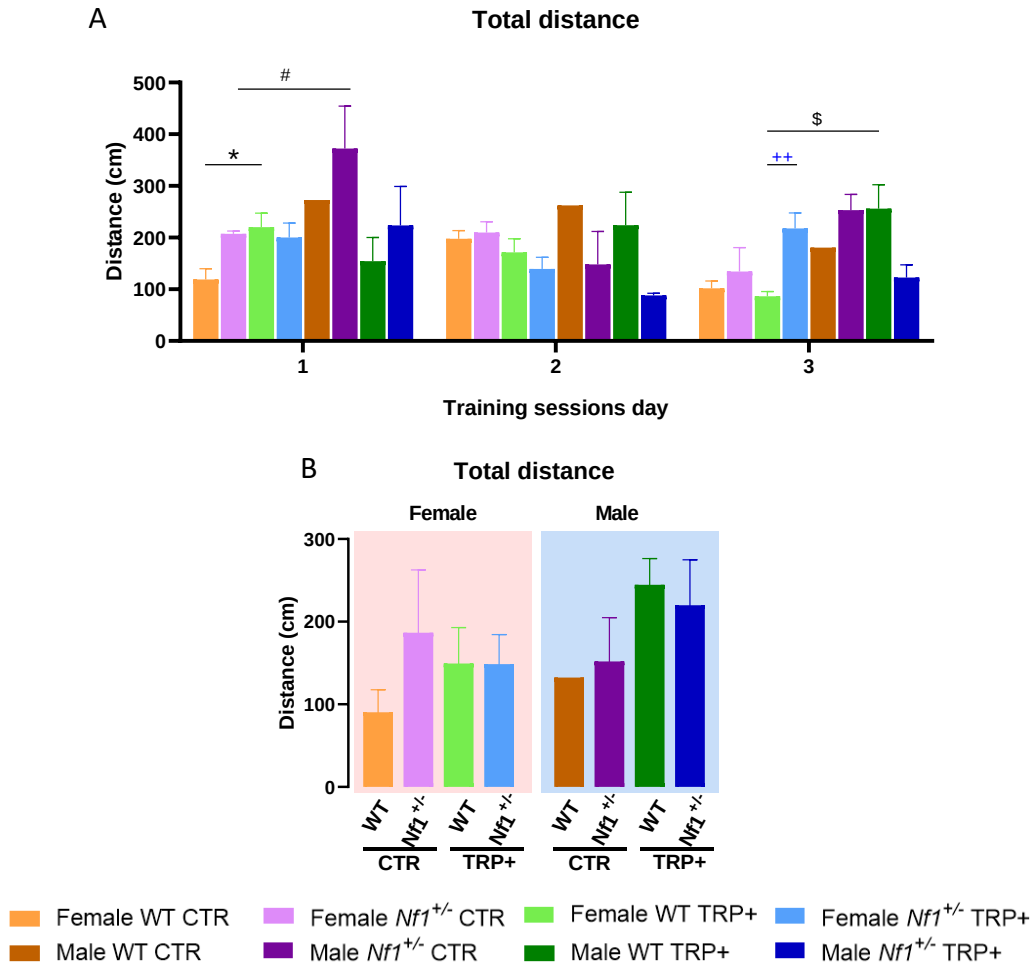
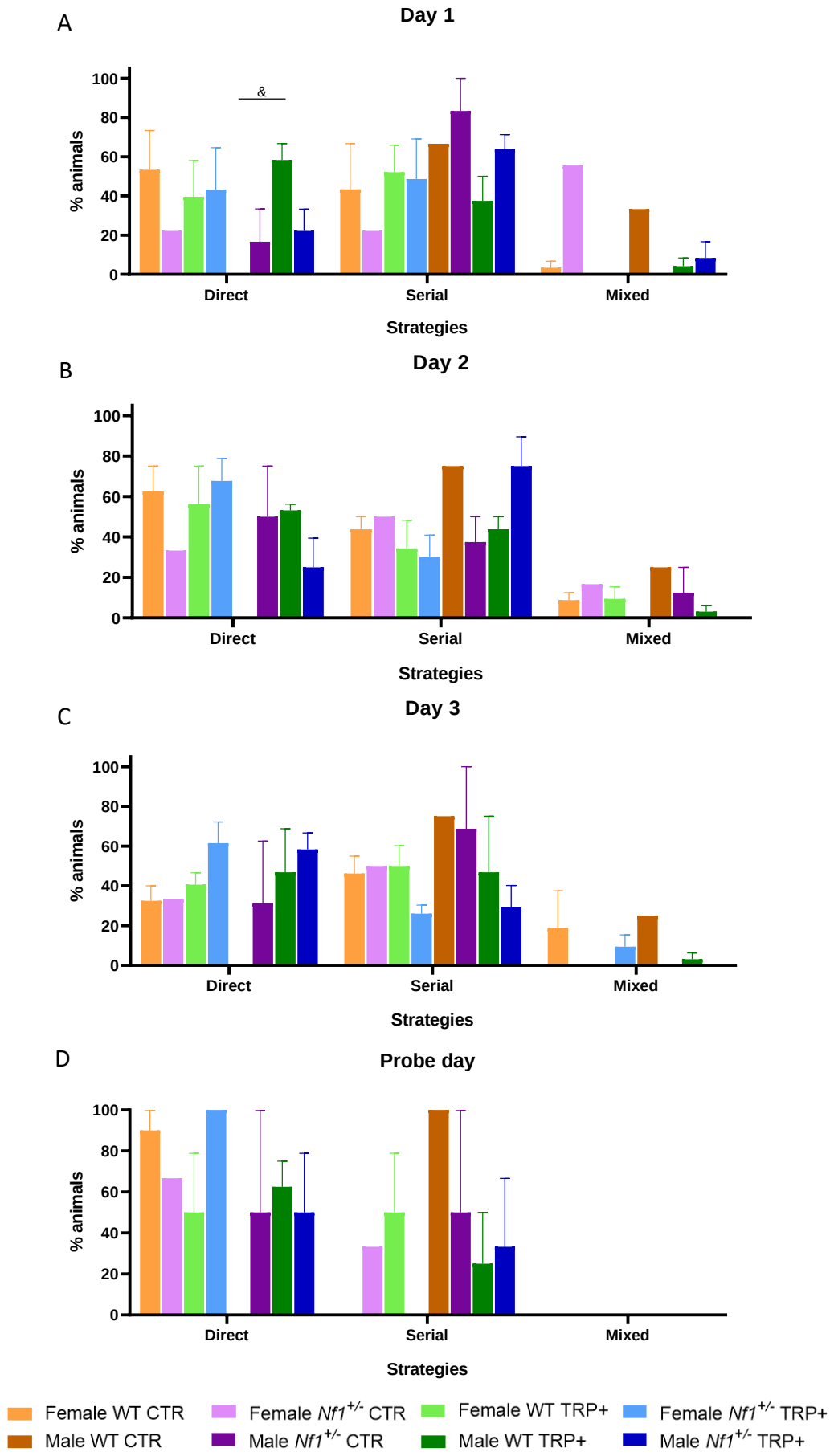



**Figure 67 |Female WT TRP+ traveled more distance in training sessions of offspring in ASD condition**

TRP+ diet increased total distance in females WT in day 1 of (A) training sessions days. Also, on this day, males *Nf1*$^{+/-}$CTR traveled more distance than females. An dimorphism sexual it was also possible to observed on day 3, where females WT TRP+ traveled less total distance than males WT TRP+. An genotype difference in TRP+ diet was observed in females, females *Nf1*$^{+/-}$TRP+ traveled more total distance. However, on (B) probe day, TRP+ diet did not influence any experimental groups. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between female WT CTR and female WT TRP+; #: significant difference between female WT CTR and male WT CTR; $: significant difference between female WT TRP+ and male WT TRP+; +: significant difference between female WT TRP+ and female *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 3.

Considering the 3 types of strategies before mentioned, on the first day (Figure 68A), more males WT TRP+ directly approached the target than males WT CTR (male WT CTR = 0.00 ± 0.00 vs male WT TRP+ = 58.34 ± 8.33, p=0.0498) and there was an effect of strategy x diet ($F(2, 9) = 19.70$, p=0.0005). Meanwhile on the other days of training and the test (Figure 68 B, C, D), no significant differences were observed between any other groups. It should be noted that on the day of the test, none of the mice explored the platform in a mixed strategy manner.


A
Day 1
&
% animals
100
80
60
40
20
0
Direct
Serial
Mixed
Strategies
B
Day 2
% animals
Direct
Serial
Mixed
Strategies
C
Day 3
% animals
Direct
Serial
Mixed
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Direct
Serial
Mixed
Strategies
Female WT CTR
Male WT CTR
Female Nf1+/- CTR
Male Nf1+/- CTR
Female WT TRP+
Male WT TRP+
Female Nf1+/- TRP+
Male Nf1+/- TRP+

**Figure 68 | Males WT TRP+ showed an improve in learning regarding search strategy of offspring in ASD condition**

Males WT TRP+ searched more directly than males WT CTR on (A) day 1, thus showing that the diet improved the search strategy in WT males. However, on (B) day 2, (C) day 3 and (D) probe day the diet did not influence the search strategy. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; &: significant difference between male WT TRP+ and male WT TRP+. Data represented as mean ± SEM; n (female WT CTR) = 7; n (female *Nf1*$^{+/-}$ CTR) = 3; n (female WT TRP+) = 5; n (female *Nf1*$^{+/-}$ TRP+) = 7; n (male WT CTR) = 1; n (male *Nf1*$^{+/-}$ CTR) = 3; n (male WT TRP+) = 6; n (male *Nf1*$^{+/-}$ TRP+) = 3.

Taking into account these results, in which the TRP+ diet was shown to influence and improve performance in the experimental groups in terms of time in the target zone, exploring errors and the type of search strategy in the training sessions, especially in the WT TRP+ males, we can conclude that the TRP+ diet can improve learning abilities. However, this diet has not been shown to influence memory capacity.

### 4.2.7. Prenatal tryptophan suplemented diet inreased levels of tryptophan and kynurenine and decreased serotonin levels in hypothalamus in females *Nf1*$^{+/-}$ TRP+ in ASD condition

We analyzed levels of Trp, kynurenine and serotonin in hypothalamus at P42 through ELISA to understand how their molecular systems may be related to the observed behavioral outcomes.

Concerning Trp levels (Figure 69A), female *Nf1*$^{+/-}$ TRP+ had a higher concentration of this amino acid than females WT TRP+ and then males *Nf1*$^{+/-}$ TRP+ (female WT TRP+ = 0.2873 ± 0.01421 vs female *Nf1*$^{+/-}$ TRP+ = 0.6503 ± 0.1543, p=0.0211; female *Nf1*$^{+/-}$ TRP+ = 0.6503 ± 0.1543 vs male *Nf1*$^{+/-}$ TRP+ = 0.266 ± 0.05433, p= 0.0211). Overall, there was a sex-dependent effect (F (1, 28) = 10.47, p=0.0031).

Same as Trp, females *Nf1*$^{+/-}$ TRP+ had a higher concentration of kynurenine (Figure 69B) than females WT TRP+ and then males *Nf1*$^{+/-}$ TRP+ (female WT TRP+ = 1.478 ± 0.1117 vs female *Nf1*$^{+/-}$ TRP+ = 3.233 ± 0.7209, p=0.0301; female *Nf1*$^{+/-}$ TRP+ = 3.233 ± 0.7209 vs male *Nf1*$^{+/-}$ TRP+ = 1.249 ± 00.306, p= 0.0188). There was also a sex-dependent effect (F (1, 28) = 5.167, p=0.0309) and, in addition, a sex x genotype effect (F (1, 28) = 6.437, p=0.0170).

On the other hand, females *Nf1*$^{+/-}$ CTR had higher serotonin levels (Figure 69C) than females *Nf1*$^{+/-}$ TRP+ and also higher than males *Nf1*$^{+/-}$ CTR (female *Nf1*$^{+/-}$ CTR = 3.346 ± 0.6988 vs female *Nf1*$^{+/-}$ TRP+ = 1.392 ± 0.7417 ,p=0.0362; female *Nf1*$^{+/-}$ CTR = 3.346 ± 0.6988 vs male *Nf1*$^{+/-}$ CTR = 0.67 ± 0.608, p=0.0154). It's important to noting that there was sex- (F (1, 25) = 10.46, p=0.0034) and diet-dependent effect (F (1, 25) = 4.448, p=0.0451), and also, a sex x diet effect (F (1, 25) = 5.596, p=0.0261).

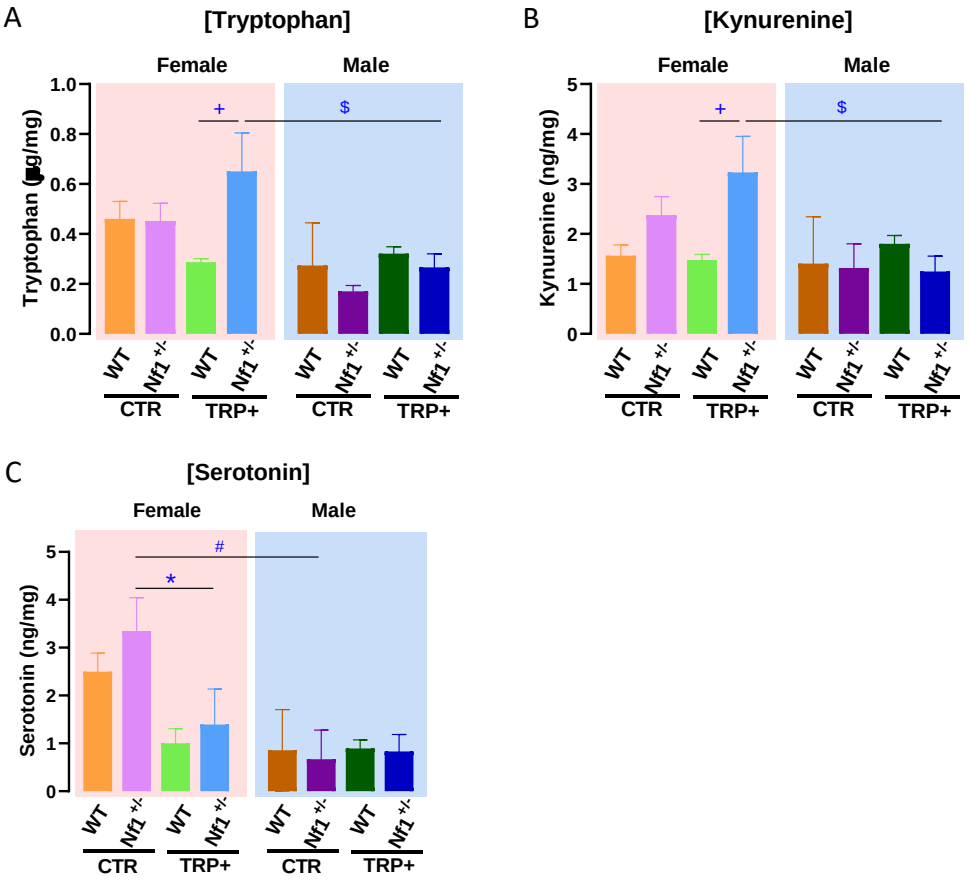



**Figure 69 | Females *Nf1*$^{+/-}$ TRP+ showed high levels of tryptophan and kynurenine and low serotonin levels in hypothalamus of offspring in ASD condition**

In both (A) Trp and (B) kynurenine concentrations in hypothalamus, female *Nf1*$^{+/-}$ TRP+ showed higher levels than female WT TRP+ and than male *Nf1*$^{+/-}$ TRP+. In the case of (C) concentrations of serotonin, female *Nf1*$^{+/-}$ TRP+ showed lower levels than female WT TRP+ and the dimorphism observed in Trp and kynurenine no longer exists. On the other hand, there was differences between sex in mice *Nf1*$^{+/-}$ CTR. Three-way ANOVA followed by Sidak's multiple comparisons test; $p<0.05$; *: significant difference between female *Nf1*$^{+/-}$ CTR and female *Nf1*$^{+/-}$ TRP+; #: significant difference between female *Nf1*$^{+/-}$ CTR and male *Nf1*$^{+/-}$ CTR; $: significant difference between female *Nf1*$^{+/-}$ TRP+ and male *Nf1*$^{+/-}$ TRP+; +: significant difference between female WT TRP+ and female *Nf1*$^{+/-}$ TRP+. Data represented as mean ± SEM; n (female WT CTR) = 5; n (female *Nf1*$^{+/-}$ CTR) = 6; n (female WT TRP+) = 4; n (female *Nf1*$^{+/-}$ TRP+) = 5; n (male WT CTR) = 2; n (male *Nf1*$^{+/-}$ CTR) = 4; n (male WT TRP+) = 7; n (male *Nf1*$^{+/-}$ TRP+) = 3.

Thus, the group most affected by the diet was female *Nf1*$^{+/-}$ TRP+ which caused a decrease in serotonin levels.

## 4.3. EFFECT OF TRYPTOPHAN DIET IN MATERNAL CARE AND BEHAVIOR

In this work, we also investigated maternal behavior, and whether it was influenced by Trp diet intake. For this purpose, we analyzed the nesting behavior (3 days before and 3 days after litter birth) and pup retrieval behavior (after separation for milestones until P10) of each mother used in this work, and we performed a reunion test on P9. Due to the small number of litters and mothers, there were not many results with statistical significance, although some tendencies can be observed.

In this part of the study, the results are only regarding dams' behavior and so, we will have 4 experimental groups:

- WT dams with CTR diet (WT dams CTR);
- *Nf1*$^{+/-}$ dams with CTR diet (*Nf1*$^{+/-}$ dams CTR);
- WT dams with TRP+ diet (WT dams TRP+);
- *Nf1*$^{+/-}$ dams with TRP+ diet (*Nf1*$^{+/-}$ dams TRP+);

### 4.3.1. Tryptophan supplemented diet appeared to improve maternal instinct in *Nf1*$^{+/-}$ dams in nest behavior

The nesting skills was scored through the state of the nests from G19 to P3 to measure the maternal instinct (Figure 70). As mentioned thus, due to the small number of litters it was not possible to obtain significant differences however there were some interesting observations.

There seemed to be a tendency for *Nf1*$^{+/-}$ dams CTR to have lower scores from the day of birth of the litter, both compared to WT dams CTR and also compared to *Nf1*$^{+/-}$ dams TRP+. In general, there appeared to be an interaction between the genotype of the mother and the diet consumed on nest scores and, consequently, on the maternal instinct.

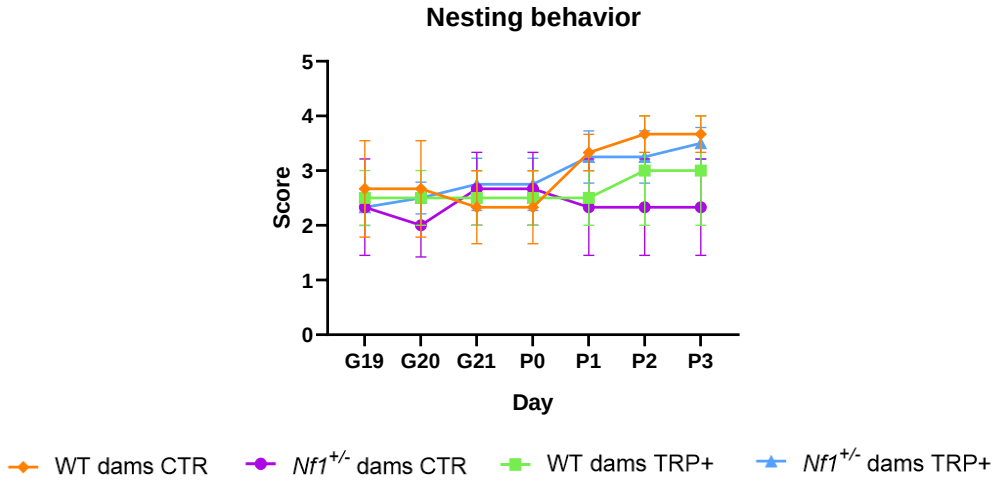


**Figure 70 | TRP+ diet seemed to increase nesting scores in *Nf1*$^{+/-}$ dams**
*Nf1*$^{+/-}$ dams TRP+ appears to have scored better regarding nesting skills than *Nf1*$^{+/-}$ dams CTR. Moreover, these last dams also seem to had lower score than WT dams CTR. Three-way ANOVA followed by Turkey's multiple comparisons test No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT dam CTR) = 3; n (*Nf1*$^{+/-}$ dam CTR) = 2; n (WT dam TRP+) = 3; n (*Nf1*$^{+/-}$ dam TRP+) = 3.

### 4.3.2. Tryptophan supplemented diet improved maternal behavior according to pup retrieval

We also observed the time of retrieval of each pup by the dam to the nest after the milestones, from P4 to P10 (Figure 71).

We observed that WT dams TRP+ recovered their pups faster after separation than WT dams CTR at P4 (WT dams CTR = 68.29 ± 7.953 vs WT dams TRP+ = 30.32 ± 4.990, p<0.0001). In addition, at P4 WT dams CTR performed worse than *Nf1*$^{+/-}$ dams with same diet (WT dams CTR = 68.29 ± 7.953 vs *Nf1*$^{+/-}$ dams CTR = 25.84 ± 3.912, <0.0001). However, this difference was reversed at P6, *Nf1*$^{+/-}$ dams CTR took longer to recover their pups than WT dams CTR (WT dams CTR = 16.45 ± 2.368 vs *Nf1*$^{+/-}$ dams CTR = 31.33 ± 5.012, <0.0001). Also, at P6, we found that *Nf1*$^{+/-}$ dams TRP+ diet performed better compared to *Nf1*$^{+/-}$ dams CTR (*Nf1*$^{+/-}$ dams CTR = 31.33 ± 5.012 vs *Nf1*$^{+/-}$ dams TRP+ = 6.64 ± 0.899).

Overall, there were day- (F (3, 246) = 50.80, p<0.0001), genotype- (F (1, 246) = 18.94, p<0.0001) and diet-dependent effect (F (1, 246) = 32.94, p<0.0001). In addition, there was also an effect of day x genotype (F (3, 246) = 14.18, p<0.0001) and day x diet (F (3, 246) = 6.574, p=0.0003).

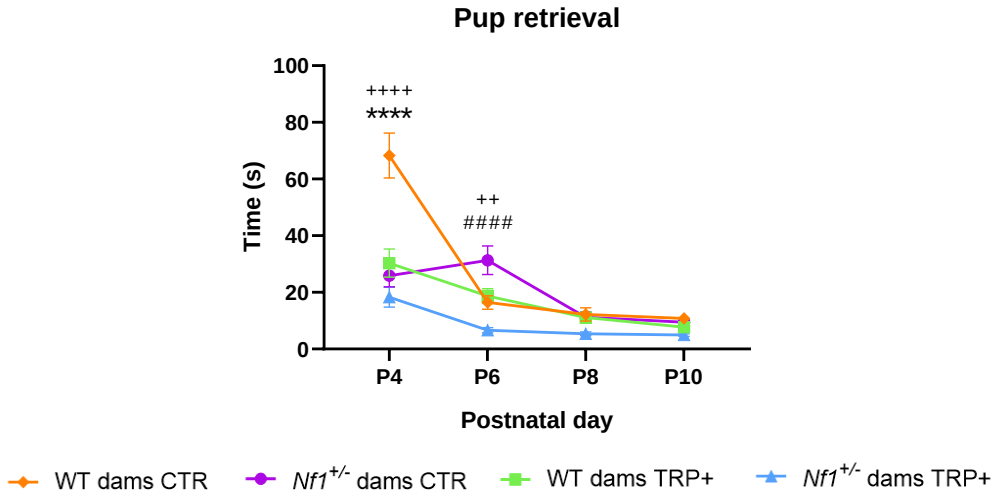



**Figure 71 | Dams TRP+ were faster to retrieve their pups after separation**
WT dams TRP+ and *Nf1*$^{+/-}$ dams TRP+ showed an improve in pup retrieval time compared to WT dams CTR and *Nf1*$^{+/-}$ dams CTR, respectively. In addition, there were also differences between genotypes in CTR diet. Three-way ANOVA followed by Turkey's multiple comparisons test p<0.05; *: significant difference between WT dams CTR and WT dams TRP+; #: significant difference between *Nf1*$^{+/-}$ dams CTR and *Nf1*$^{+/-}$ dams TRP+; +: significant difference between WT dams CTR and *Nf1*$^{+/-}$ dams CTR. Data represented as

mean ± SEM; n (dam WT CTR) = 19; n (dam *Nf1*$^{+/-}$ CTR) = 15; n (dam WT TRP+) = 19; n (dam *Nf1*$^{+/-}$ TRP+) = 21.

### 4.3.3. Tryptophan supplemented diet did not influenced maternal behavior in the reunion test

In order to analyze maternal instinct, we performed the reunion test on P9, to investigate whether a Trp diet affected the mothers' behavior. For the same reason as mentioned above, it was not possible to obtain significant results although it should be noted that there are some tendencies.

During the habituation phase (Figure 72A), there was an effect of behavior ($F(2, 24) = 135.8$, $p<0.0001$) and of behavior x diet ($F(2, 24) = 3.514$, $p=0.0459$).

In the next phase, separation phase (Figure 72B), there was also an effect of behavior ($F(2, 24) = 70.69$, $p<0.0001$) and of behavior x diet ($F(2, 24) = 4.205$, $p=0.0272$).

In the last phase, the reunion phase (Figure 72C), there was also the effect of behavior ($F(4, 40) = 86.19$, $p<0.0001$) and of behavior x diet ($F(4, 40) = 3.180$, $p=0.0233$). In addition, there was no significant differences regarding the time in the litter side (Figure 72D).

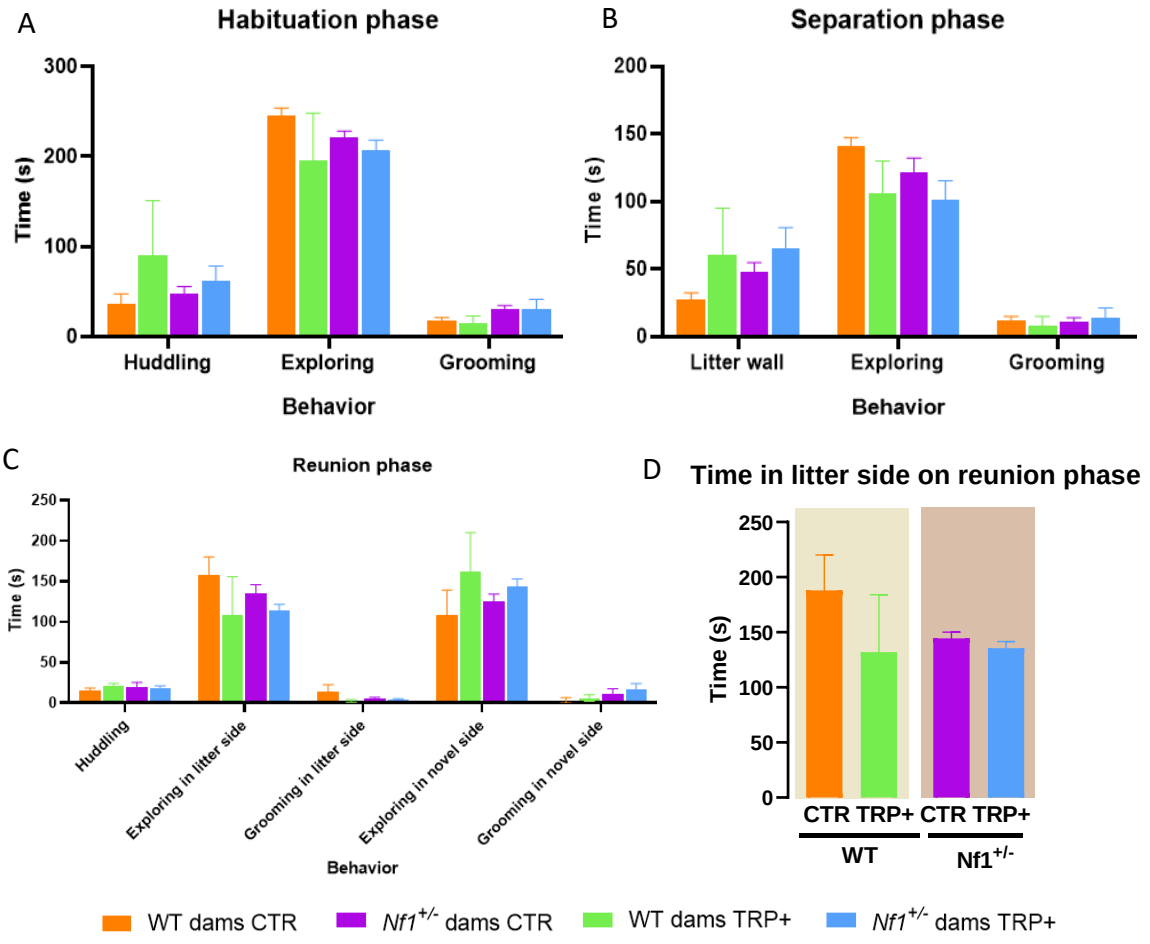


**Figure 72 | TRP+ diet did not influence maternal behavior in reunion test**
TRP+ diet did not influence dams' behavior in (A) habituation phase, (B) separation phase or (C) reunion phase. Also, did not influenced the (D) time that dams spent in litter side on reunion phase. Three-way ANOVA followed by Turkey's multiple comparisons test No significant differences found between any of the experimental groups. Data represented as mean ± SEM; n (WT dam CTR) = 3; n (*Nf1*$^{+/-}$ dam CTR) = 2; n (WT dam TRP+) = 3; n (*Nf1*$^{+/-}$ dam TRP+) = 3.

Even though there were only significant differences in the pup retrieval test, there seemed to be trends in the nest behavior test that collaborated with the pup retrieval results. Thus, considering the results of the maternal behavior tests, the TRP+ diet improved maternal behavior and care, especially in the *Nf1*$^{+/-}$ dams.

# CHAPTER 5 | **DISCUSSION**

Dietary Trp has been used as a therapeutic approach because changes in Trp levels in the brain are essential to serotonin synthesis. The central serotonergic system has an important role in regulating many physiological and behavioral processes, such as mood, cognition, activity, sleep, and appetite. A disruption of the central serotoninergic function due to inadequate Trp availability is recognized as a contributory factor to impaired affect, anxiety, stress, aggression, eating disorders and others (Le Floc'h et al., 2011).

## 5.1. EFFECT OF PRENATAL TRYPTOPHAN DIET IN HEALTH CONDITIONS

As mentioned above, Trp plays an important role in appetite and eating disorders, so it is to be expected that an increased availability of this amino acid will promote a greater appetite and, consequently, a higher body weight and weight gain (Le Floc'h et al., 2011). Thus, it is important to monitor the weight of the litter, where it was possible to observe that diet influence the body weight of the animals at juvenile age. Coupled with this, females TRP+ diet hah a higher weight, however, there was a sexual dimorphism where males TRP+ diet hah a lower weight compared to the respective prenatal CTR diet. Contradictory to what is reported in the literature (Spring et al., 2007), in both diets females brain index is higher. So, Trp metabolism may be affected by the sex chromosomes and, consequently, affect these measurements.

Since Trp is linked to serotonin and several studies showed the role of this neurotransmitter in the affective modulation of ultrasonic communication in rodents across the lifespan, including in isolation-induced USV in pups (Hård & Engel, 1988; Hodgson et al., 2008; Olivier et al., 1989; Simola, 2015), it is important to analyses the effects of diet in USVs. Mouse pups emit isolation-induced USVs when separated from their mother and littermates, so these USVs reflect a negative affective state due to isolation stress (Wöhr et al., 2015). At P4, males TRP+ diet had a much longer latency time compared to the other experimental groups, which mean, they took longer to call for their mother after separation. Also, at P4 females TRP+ had a longer call interval than the other groups. These differences suggest that an increase of Trp and consequently serotonin promote to a lower stress levels of pups when separate from their mother.

Later in life, a test was performed to analyze the effect of TRP+ prenatal diet in anxiety-like behavior (EPM). Our results are in agreement with the study of Browne et al. (2012), that a supplemented prenatal diet does not affect anxiety levels.

Meanwhile, several authors (aan het Rot et al., 2006; Moskowitz et al., 2001, 2003; Reuter et al., 2021) support the idea that Trp may have significant benefits for healthy individuals in improving social behavior. This hypothesis is supported due to the fact a supplementation with TRP will result in increased serotonin functions, leading to a potential promotion of positive social behaviors, i.e. agreeable and pro-social. In these

studies, above mentioned, they have demonstrated positive effects of a Trp-rich diet on social cognition, quarrelsome behaviors significantly decreased and increased the proper detection of emotions in our social interaction partners and by increasing the ability to properly judge social behavior agreeable behaviors. It is important to note that, however, in our study, no significant differences were observed (and this may be due to the low number of pairs to perform this test), there was some tendency for mice with supplemented prenatal diet to showed a higher number of interactions and vocalized more, both in social and non-social contexts. In addition, males on supplemented prenatal diet were the only experimental group with a positive score on the social index.

In the segment of this study males TRP+ diet buried more marbles compared to males CTR diet and so, a supplemented diet promotes an repetitive behavior in males. However, the same did not happen in females, there was no such difference between diets. Unlike the study of Browne et al. (2012), where no differences are observed between the CTR and TRP+ diets, it is possible to observe the beginning of a tendency. This can be explained due to the fact that in our study there is a sexual dimorphism of the diets and there are only differences in the diet in males, and in the referenced study they do not segregate the analysis of the results by sex.

Since Trp play a role in cognition, it is important to study the effects of Trp supplemented diet in learning and memory, especially when several studies in healthy individuals show an inconsistent pattern of no effects, impairments and improvements of memory functions (Harmer et al., 2002; J. A. Schmitt et al., 2001; Siepmann et al., 2003; Sobczak et al., 2003). Following to that, our study shows an impairment in learning and memory in males TRP+ diet.

Overall, our study indicated that prenatal Trp supplementation diet in health conditions leads to a learning and memory impaired and increases restricted and repetitive behavior in males. Therefore, this diet increased the amount of Trp to an excessive level and promote the dysregulation of this metabolism causing cognitive deficits.

## 5.2. EFFECT OF PRENATAL TRYPTOPHAN DIET IN ASD CONDITION

ASD is characterized by three main core traits - social interaction deficits, repetitive restricted behaviors and impairment in learning and memory (American Psychiatric Association, 2013). However, dysbiosis of the microbiota has been implicated in the pathogenesis of ASD (Beopoulos et al., 2022; Sorboni et al., 2022), therefore it is important to study the interaction between gut and brain. Since Trp is only obtained by diet and is the only precursor for serotonin, an important neurotransmitter in the brain, an dysregulation of Trp metabolism has been implicated in ASD (Ruddick et al., 2006).

Studies report that ASD males have more deficits in motor impairments (Fournier et al., 2010; van der Vaart et al., 2011). In our results there was a heterogeneity in motor coordination in developmental milestones tests, the locomotion test was in agreement with the literature however in the surface righting reflex and negative geotaxis tests *Nf1*$^{+/-}$ CTR diet had better scores than WT. Moreover, TRP+ diet only improved the development of females WT in the surface righting reflex. One explanation for this could be that the adequate intake of Trp from the diets to which our mice are submitted has positive effects.

Since USVs are associated with stress due to mother-pup separation, it is important to study the effect of Trp and their metabolism to serotonin. As other study (Maloney et al., 2018) has reported, *Nf1*$^{+/-}$ mice produced more vocalizations compared to their WT siblings (females *Nf1*$^{+/-}$ CTR vocalized more than females WT CTR). Differences were also observed in the interval time between calls, where females *Nf1*$^{+/-}$ CTR had a shorter interval compared to females WT CTR, i.e. they vocalized more repetitively. This behavior may indicate a sign of desperation and stress when separated from the mother. Furthermore, Maloney et al. (2018) report that USVS duration is not altered by genotype and our results are in agreement with pups CTR diet. However, in pups TRP+ diet, the diet caused a decrease in call length in females WT and males *Nf1*$^{+/-}$.

In our study, at juvenile age, it was possible to observe that TRP+ diet decreased number of open arms entries in *Nf1*$^{+/-}$ mice, however, since there was no difference in the other parameters analyzed, diet does not influence anxiety-like behavior. These results are not in line with other study (D. Wang et al., 2022) reporting that a rich-trp diet improve anxiety-like behavior.

Concerning social communication and interactions, Frazier et al. (2014) reported impairment in social communication in *Nf1*$^{+/-}$ mice. Meanwhile, as previously stated, to be able to observe more differences it would be necessary to increase the number of animals. However, it was possible to observe a tendency to TRP+ diet improves the number and time of social interactions in *Nf1*$^{+/-}$.

Regarding the symptoms of ASD, we found that *Nf1*$^{+/-}$ males showed a higher tendency for increased repetitive and hyperactivity behavior compared to *Nf1*$^{+/-}$ females. This was expected, as several studies (Hartley & Sikora, 2009; May et al., 2014, 2019; Santos et al., 2023; Van Wijngaarden-Cremers et al., 2014) report this sex-

difference. Besides this, we found that TRP+ diet reverses this repetitive behavior in males and so, could be a good approach to improve/decrease this type of behavior.

Finally, we found no effect of genotype in learning and memory in this work as expected regarding Silva et al. (1997), which demonstrated that *Nf1*$^{+/-}$ mice show impaired learning and memory. In our study, several parameters of the Barnes test were measured and TRP+ prenatal diet improved performance in *Nf1*$^{+/-}$ in the training sessions. However, this diet has not been shown to influence memory capacity.

Our study showed that in addition to the diet not influencing the brain index, there were no significant differences between genotype. Taking into account a study conducted in our group which reports that *Nf1*$^{+/-}$ mice have a higher brain index, we can assume that an adequate amount of trp in the diet (both control and supplemented) equalized the index between *Nf1*$^{+/-}$ and WT mice. Furthermore, females *Nf1*$^{+/-}$ TRP+ have a higher brain index than males *Nf1*$^{+/-}$TRP+ . This result correlates with the analysis of Trp concentration in the hypothalamus carried out by ELISA in this study. In other words, females *Nf1*$^{+/-}$ TRP+ had higher Trp levels than males *Nf1*$^{+/-}$ TRP+ and, therefore, high Trp concentrations in the brain promote brain growth in the offspring of ASD condition.

As already mentioned earlier, several studies reported deficiency of Trp, higher levels of kynurenine and hyperserotonemia in ASD individuals (Gabriele et al., 2014; Gevi et al., 2016; Murakami et al., 2019). Also, autistic males exhibit reduced levels and synthesis of serotonin in frontal cortex and thalamus (Chugani et al., 1997; Pinares-Garcia et al., 2018). We can observe that the females had an increase of levels of Trp and its metabolites, compared to males, as has been described in the literature (Brewerton et al., 2018; Carlsson & Carlsson, 1988; Guzmán et al., 2009).

In our study, no differences were observed in males, however, females *Nf1*$^{+/-}$ CTR exhibit levels of Trp and its two metabolites similar to WT with same diet. This indicates that the metabolic pathway of this amino acid, which is usually deregulated in *Nf1*$^{+/-}$ mice, can be normalized with this CTR diet, equivalent to neurotypical controls. On the other hand, the TRP+ diet in *Nf1*$^{+/-}$ females leads to an increase in Trp and kynurenine in the hypothalamus, while decreasing serotonin levels. This may be due to the fact that we are dealing with an excess of Trp, which will disturb the metabolic pathway of Trp by synthesizing low levels of serotonin.

Thus, these individuals who in their physiology have low concentrations of Trp, the TRP+ diet can equalize the normal concentrations of neurotypical individuals, providing a normal functioning of the metabolism.

Overall, our study indicated that prenatal Trp supplementation diet in ASD condition improve developmental in early life, tend to increase social interaction, decrease repetitive and restricted behaviors and improve learning abilities. Thus, this diet can be considered as a possible approach to reduce or improve of ASD symptoms severity.

## 5.3. EFFECT OF TRYPTOPHAN DIET IN MATERNAL CARE AND BEHAVIOR

As we know, maternal care is an indispensable component of offspring development (Angoa-Pérez et al., 2014) and therefore it is important to study if Trp administration influences maternal behavior.

As previously mentioned, it would be necessary to increase the number of litters to obtain statistical significance. Meanwhile, this study indicated that TRP+ diet improve maternal care in dams of both genotypes, showed a lower latency in pup retrieval test and a tendency to TRP+ diet increase nesting scores in *Nf1*$^{+/-}$ dams.

# CHAPTER 6 | **CONCLUDING REMARKS AND FUTURE DIRECTIONS**

The main focus of this study is to highlight the relationship between diet and behavior and cognition through the MGB axis, where modelling of Trp through diet influenced both offspring and maternal behavior (Figure 73).

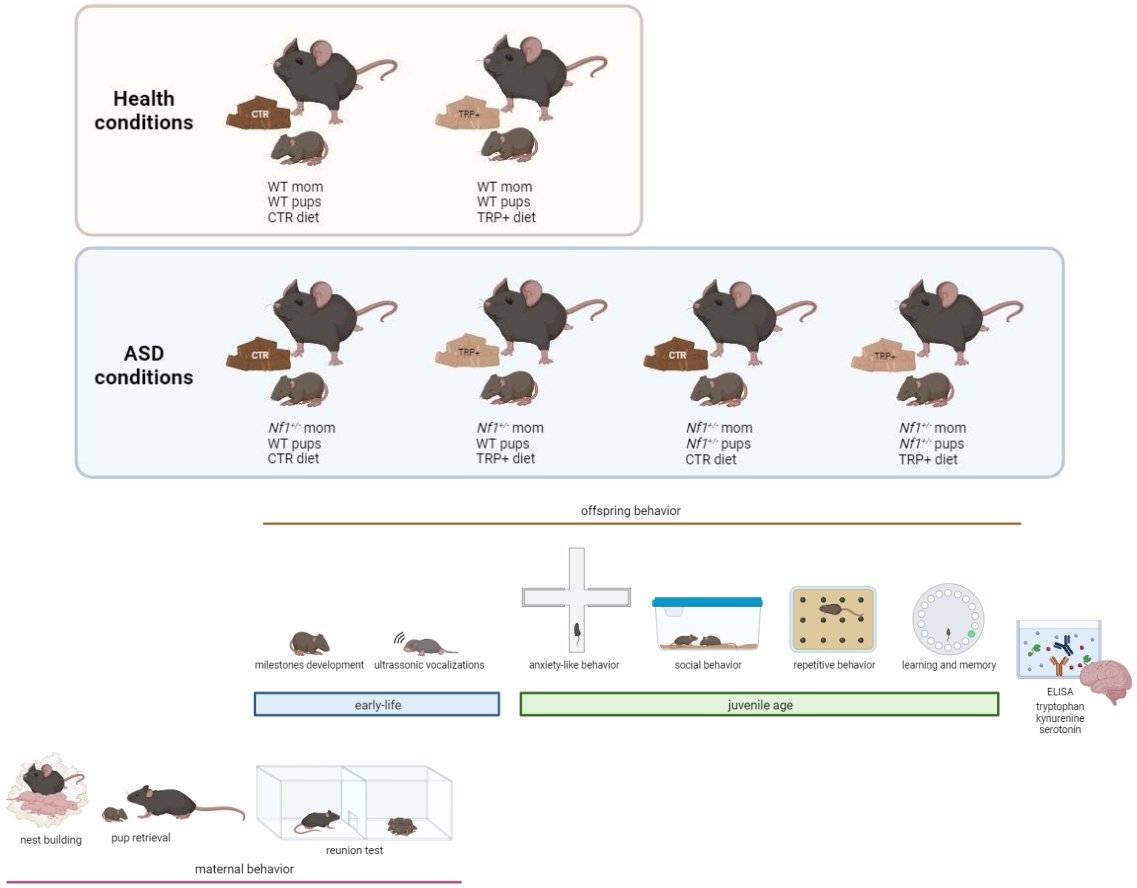
It has been tested the benefits of Trp supplementation in healthy individuals. Our results revealed that this excessive amount of Trp may lead to learning difficulties and an impaired memory (Figure 73). Furthermore, this diet increases repetitive behavior only in males, showing that attention needs to be given to sexual dimorphism in Trp metabolism.

On the other hand, we have also studied the effect of a Trp-enriched diet in individuals with ASD condition, who present an impairment in the serotoninergic system. Therefore, modulating the amount of Trp and consequently serotonin through diet could also have a positive effect on behavior and cognition. As such, in early-life, we detected improvements in development milestones tests in females WT, although they presented some influenced in USVs parameters, namely, shorter calls, higher power, lower tonality and higher principal and low frequencies. In juvenile age, TRP+ diet does not influence anxiety behavior; however, this diet seems to improved social interaction, decreased repetitive behavior and improved learning ability. Thus, this study demonstrated an early life approach to reduce/improve the risk of ASD symptoms later in life with Trp-supplemented diet (Figure 73).

In addition, the enriched diet has been shown to improve maternal care which may have benefits on offspring behavior.

Furthermore, it would be relevant to study the quantities of Trp and its metabolites in more brain regions, in the gut and in the blood to have a better understanding of this mechanism.

Moreover, since there are some discrepancies in the positive effects of Trp supplementation, it would also be important to study the effects of Trp depletion and try to find an adequate intake of Trp in both individuals with ASD and healthy individuals.




MAIN RESULTS

**HEALTH CONDITIONS:**

WT TRP+:

- learning and memory impaired;
- increases restricted and repetitive behavior in males.

**ASD CONDITION:**

*Nf1*$^{+/-}$ TRP+:

- improved developmental milestones tests;
- tendency to increased social interaction;
- decreased repetitive and restricted behaviors;
- improved learning ability;
- low levels of serotonin in brain.

CONCLUSION

**HEALTH CONDITIONS:**

TRP+: excessive amount of Trp leads to a dysregulation of this metabolism causing cognitive deficits

**ASD DISORDER:**

TRP+: possible approach in reducing and/or improving the severity of ASD symptoms.

**Figure 73 | Graphical abstract**

Graphical abstract representing the experimental groups, methods, main results and conclusions of this study.

# REFERENCES

aan het Rot, M., Moskowitz, D. S., Pinard, G., & Young, S. N. (2006). Social behaviour and mood in everyday life: The effects of tryptophan in quarrelsome individuals. *Journal of Psychiatry and Neuroscience*, *31*(4), 253–262.

Adams, J. B., Audhya, T., McDonough-Means, S., Rubin, R. A., Quig, D., Geis, E., Gehn, E., Loresto, M., Mitchell, J., Atwood, S., Barnhouse, S., & Lee, W. (2011). Nutritional and metabolic status of children with autism vs. Neurotypical children, and the association with autism severity. *Nutrition & Metabolism*, *8*(1), 34. https://doi.org/10.1186/1743-7075-8-34

Adams, J. B., & Holloway, C. (2004). Pilot study of a moderate dose multivitamin/mineral supplement for children with autistic spectrum disorder. *Journal of Alternative and Complementary Medicine (New York, N.Y.)*, *10*(6), 1033–1039. https://doi.org/10.1089/acm.2004.10.1033

Alharthi, A., Alhazmi, S., Alburae, N., & Bahieldin, A. (2022). The Human Gut Microbiome as a Potential Factor in Autism Spectrum Disorder. *International Journal of Molecular Sciences*, *23*(3), 1363. https://doi.org/10.3390/ijms23031363

Aman, M. G., Farmer, C. A., Hollway, J., & Arnold, L. E. (2008). Treatment of inattention, overactivity, and impulsiveness in autism spectrum disorders. *Child and Adolescent Psychiatric Clinics of North America*, *17*(4), 713–738, vii. https://doi.org/10.1016/j.chc.2008.06.009

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders: DSM-5™, 5th ed* (pp. xliv, 947). American Psychiatric Publishing, Inc. https://doi.org/10.1176/appi.books.9780890425596

Angoa-Pérez, M., Kane, M. J., Briggs, D. I., Francescutti, D. M., & Kuhn, D. M. (2013). Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *Journal of Visualized Experiments: JoVE*, *82*, 50978. https://doi.org/10.3791/50978

Angoa-Pérez, M., Kane, M. J., Sykes, C. E., Perrine, S. A., Church, M. W., & Kuhn, D. M. (2014). Brain serotonin determines maternal behavior and offspring survival. *Genes, Brain and Behavior*, *13*(7), 579–591. https://doi.org/10.1111/gbb.12159

Attar, A., Liu, T., Chan, W.-T. C., Hayes, J., Nejad, M., Lei, K., & Bitan, G. (2013). A Shortened Barnes Maze Protocol Reveals Memory Deficits at 4-Months of Age in the Triple-Transgenic Mouse Model of Alzheimer's Disease. *PLoS ONE*, *8*(11), e80355. https://doi.org/10.1371/journal.pone.0080355

Ayeni, F. A., Biagi, E., Rampelli, S., Fiori, J., Soverini, M., Audu, H. J., Cristino, S., Caporali, L., Schnorr, S. L., Carelli, V., Brigidi, P., Candela, M., & Turroni, S. (2018). Infant and Adult Gut Microbiome and Metabolome in Rural Bassa and Urban Settlers from Nigeria. *Cell Reports*, *23*(10), 3056–3067. https://doi.org/10.1016/j.celrep.2018.05.018

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., & Collins, F. (1990). The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell*, *63*(4), 851–859. https://doi.org/10.1016/0092-8674(90)90151-4

Baron-Cohen, S. (2002). The extreme male brain theory of autism. *Trends in Cognitive Sciences*, *6*(6), 248–254. https://doi.org/10.1016/s1364-6613(02)01904-6

Baron-Cohen, S., Knickmeyer, R. C., & Belmonte, M. K. (2005). Sex differences in the brain: Implications for explaining autism. *Science (New York, N.Y.)*, *310*(5749), 819–823. https://doi.org/10.1126/science.1115455

Barrett, H. L., Gomez-Arango, L. F., Wilkinson, S. A., McIntyre, H. D., Callaway, L. K., Morrison, M., & Dekker Nitert, M. (2018). A Vegetarian Diet Is a Major Determinant of Gut Microbiota Composition in Early Pregnancy. *Nutrients*, *10*(7), 890. https://doi.org/10.3390/nu10070890

Baumgarten, H. G., & Göthert, M. (Eds.). (2000). Serotoninergic Neurons and 5-HT Receptors in the CNS (Vol. 129). *Springer Berlin Heidelberg*. https://doi.org/10.1007/978-3-642-60921-3

Bell, C., Abrams, J., & Nutt, D. (2001). Tryptophan depletion and its implications for psychiatry. *The British Journal of Psychiatry: The Journal of Mental Science*, *178*, 399–405. https://doi.org/10.1192/bjp.178.5.399

Beopoulos, A., Gea, M., Fasano, A., & Iris, F. (2022). Autonomic Nervous System Neuroanatomical Alterations Could Provoke and Maintain Gastrointestinal Dysbiosis in Autism Spectrum Disorder (ASD): A Novel Microbiome–Host Interaction Mechanistic Hypothesis. *Nutrients*, *14*(1), Article 1. https://doi.org/10.3390/nu14010065

Beretich, G. R. (2009). Reversal of autistic symptoms by removal of low-relative tryptophan foods: Case report. *Medical Hypotheses*, *73*(5), 856–857. https://doi.org/10.1016/j.mehy.2009.05.001

Bernardino, I., Dionísio, A., & Castelo-Branco, M. (2022). Cortical inhibition in neurofibromatosis type 1 is modulated by lovastatin, as demonstrated by a randomized, triple-blind, placebo-controlled clinical trial. *Scientific Reports*, *12*(1), 13814. https://doi.org/10.1038/s41598-022-17873-x

Boadle-Biber, M. C. (1993). Regulation of serotonin synthesis. *Progress in Biophysics and Molecular Biology*, *60*(1), 1–15. https://doi.org/10.1016/0079-6107(93)90009-9

Bodnar, T. S., Lee, C., Wong, A., Rubin, I., Wegener Parfrey, L., & Weinberg, J. (2022). Evidence for long-lasting alterations in the fecal microbiota following prenatal alcohol exposure.

*Alcoholism, Clinical and Experimental Research*, *46*(4), 542–555. https://doi.org/10.1111/acer.14784

Borre, Y. E., O'Keeffe, G. W., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2014). Microbiota and neurodevelopmental windows: Implications for brain disorders. *Trends in Molecular Medicine*, *20*(9), 509–518. https://doi.org/10.1016/j.molmed.2014.05.002

Bosi, A., Banfi, D., Bistoletti, M., Giaroni, C., & Baj, A. (2020). Tryptophan Metabolites Along the Microbiota-Gut-Brain Axis: An Interkingdom Communication System Influencing the Gut in Health and Disease. *International Journal of Tryptophan Research : IJTR*, *13*, 1178646920928984. https://doi.org/10.1177/1178646920928984

Brannan, C. I., Perkins, A. S., Vogel, K. S., Ratner, N., Nordlund, M. L., Reid, S. W., Buchberg, A. M., Jenkins, N. A., Parada, L. F., & Copeland, N. G. (1994). Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes & Development*, *8*(9), 1019–1029. https://doi.org/10.1101/gad.8.9.1019

Brewerton, T. D., Putnam, K. T., Lewine, R. R. J., & Risch, S. C. (2018). Seasonality of cerebrospinal fluid monoamine metabolite concentrations and their associations with meteorological variables in humans. *Journal of Psychiatric Research*, *99*, 76–82. https://doi.org/10.1016/j.jpsychires.2018.01.004

Browne, C. A., Clarke, G., Dinan, T. G., & Cryan, J. F. (2012). An effective dietary method for chronic tryptophan depletion in two mouse strains illuminates a role for 5-HT in nesting behaviour. *Neuropharmacology*, *62*(5–6), 1903–1915. https://doi.org/10.1016/j.neuropharm.2011.12.009

Buchberg, A. M., Cleveland, L. S., Jenkins, N. A., & Copeland, N. G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. *Nature*, *347*(6290), 291–294. https://doi.org/10.1038/347291a0

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell*, *165*(7), 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001

Carlson, A. L., Xia, K., Azcarate-Peril, M. A., Goldman, B. D., Ahn, M., Styner, M. A., Thompson, A. L., Geng, X., Gilmore, J. H., & Knickmeyer, R. C. (2018). Infant Gut Microbiome Associated With Cognitive Development. *Biological Psychiatry*, *83*(2), 148–159. https://doi.org/10.1016/j.biopsych.2017.06.021

Carlsson, M., & Carlsson, A. (1988). A regional study of sex differences in rat brain serotonin. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *12*(1), 53–61. https://doi.org/10.1016/0278-5846(88)90061-9

Carter, A. S., Black, D. O., Tewani, S., Connolly, C. E., Kadlec, M. B., & Tager-Flusberg, H. (2007). Sex Differences in Toddlers with Autism Spectrum Disorders. *Journal of Autism and Developmental Disorders*, *37*(1), 86–97. https://doi.org/10.1007/s10803-006-0331-7

Champion, J. A., Rose, K. J., Payne, J. M., Burns, J., & North, K. N. (2014). Relationship between cognitive dysfunction, gait, and motor impairment in children and adolescents with neurofibromatosis type 1. *Developmental Medicine & Child Neurology*, *56*(5), 468–474. https://doi.org/10.1111/dmcn.12361

Charbonneau, M. R., O'Donnell, D., Blanton, L. V., Totten, S. M., Davis, J. C. C., Barratt, M. J., Cheng, J., Guruge, J., Talcott, M., Bain, J. R., Muehlbauer, M. J., Ilkayeva, O., Wu, C., Struckmeyer, T., Barile, D., Mangani, C., Jorgensen, J., Fan, Y., Maleta, K., … Gordon, J. I. (2016). Sialylated Milk Oligosaccharides Promote Microbiota-Dependent Growth in Models of Infant Undernutrition. *Cell*, *164*(5), 859–871. https://doi.org/10.1016/j.cell.2016.01.024

Chen, Y., Xu, J., & Chen, Y. (2021). Regulation of Neurotransmitters by the Gut Microbiota and Effects on Cognition in Neurological Disorders. *Nutrients*, *13*(6), Article 6. https://doi.org/10.3390/nu13062099

Chugani, D. C. (2004). Serotonin in autism and pediatric epilepsies. *Mental Retardation and Developmental Disabilities Research Reviews*, *10*(2), 112–116. https://doi.org/10.1002/mrdd.20021

Chugani, D. C., Muzik, O., Rothermel, R., Behen, M., Chakraborty, P., Mangner, T., da Silva, E. A., & Chugani, H. T. (1997). Altered serotonin synthesis in the dentatothalamocortical pathway in autistic boys. *Annals of Neurology*, *42*(4), 666–669. https://doi.org/10.1002/ana.410420420

Codagnone, M. G., Spichak, S., O'Mahony, S. M., O'Leary, O. F., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2019). Programming Bugs: Microbiota and the Developmental Origins of Brain Health and Disease. *Biological Psychiatry*, *85*(2), 150–163. https://doi.org/10.1016/j.biopsych.2018.06.014

Codagnone, M. G., Stanton, C., O'Mahony, S. M., Dinan, T. G., & Cryan, J. F. (2019). Microbiota and Neurodevelopmental Trajectories: Role of Maternal and Early-Life Nutrition. *Annals of Nutrition & Metabolism*, *74 Suppl 2*, 16–27. https://doi.org/10.1159/000499144

Cohen, H., Matar, M. A., & Joseph, Z. (2013). Animal Models of Post-Traumatic Stress Disorder. *Current Protocols in Neuroscience*, *64*(1). https://doi.org/10.1002/0471142301.ns0945s64

Comai, S., Bertazzo, A., Brughera, M., & Crotti, S. (2020). Tryptophan in health and disease. *Advances in Clinical Chemistry*, *95*, 165–218. https://doi.org/10.1016/bs.acc.2019.08.005

Costa, R. M., Federov, N. B., Kogan, J. H., Murphy, G. G., Stern, J., Ohno, M., Kucherlapati, R., Jacks, T., & Silva, A. J. (2002). Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. *Nature*, *415*(6871), 526–530. https://doi.org/10.1038/nature711

Costa, R. M., & Silva, A. J. (2003). Mouse models of neurofibromatosis type I: Bridging the GAP. *Trends in Molecular Medicine*, *9*(1), 19–23. https://doi.org/10.1016/s1471-4914(02)00008-4

Cowan, C. S. M., Dinan, T. G., & Cryan, J. F. (2020). Annual Research Review: Critical windows – the microbiota–gut–brain axis in neurocognitive development. *Journal of Child Psychology and Psychiatry*, *61*(3), 353–371. https://doi.org/10.1111/jcpp.13156

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews. Neuroscience*, *13*(10), 701–712. https://doi.org/10.1038/nrn3346

Cryan, J. F., & O'Mahony, S. M. (2011). The microbiome-gut-brain axis: From bowel to behavior. *Neurogastroenterology and Motility*, *23*(3), 187–192. https://doi.org/10.1111/j.1365-2982.2010.01664.x

Damiani, F., Cornuti, S., & Tognini, P. (2023). The gut-brain connection: Exploring the influence of the gut microbiota on neuroplasticity and neurodevelopmental disorders. *Neuropharmacology*, *231*, 109491. https://doi.org/10.1016/j.neuropharm.2023.109491

De Filippo, C., Di Paola, M., Ramazzotti, M., Albanese, D., Pieraccini, G., Banci, E., Miglietta, F., Cavalieri, D., & Lionetti, P. (2017). Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. *Frontiers in Microbiology*, *8*, 1979. https://doi.org/10.3389/fmicb.2017.01979

Dean, M., Harwood, R., & Kasari, C. (2017). The art of camouflage: Gender differences in the social behaviors of girls and boys with autism spectrum disorder. *Autism: The International Journal of Research and Practice*, *21*(6), 678–689. https://doi.org/10.1177/1362361316671845

Delgado, P. L., Price, L. H., Miller, H. L., Salomon, R. M., Aghajanian, G. K., Heninger, G. R., & Charney, D. S. (1994). Serotonin and the neurobiology of depression. Effects of tryptophan depletion in drug-free depressed patients. *Archives of General Psychiatry*, *51*(11), 865–874. https://doi.org/10.1001/archpsyc.1994.03950110025005

Delgado, P. L., Price, L. H., Miller, H. L., Salomon, R. M., Licinio, J., Krystal, J. H., Heninger, G. R., & Charney, D. S. (1991). Rapid serotonin depletion as a provocative challenge test for patients with major depression: Relevance to antidepressant action and the neurobiology of depression. *Psychopharmacology Bulletin*, *27*(3), 321–330.

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., Schultz, R. T., Crawley, J., & Young, L. J. (2006). The developmental neurobiology of autism spectrum disorder. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(26), 6897–6906. https://doi.org/10.1523/JNEUROSCI.1712-06.2006

Diggs-Andrews, K. A., & Gutmann, D. H. (2013). Modeling Cognitive Dysfunction in Neurofibromatosis-1. *Trends in Neurosciences*, *36*(4), 237–247. https://doi.org/10.1016/j.tins.2012.12.002

Dinan, T. G., & Cryan, J. F. (2017). The Microbiome-Gut-Brain Axis in Health and Disease. *Gastroenterology Clinics of North America*, *46*(1), 77–89. https://doi.org/10.1016/j.gtc.2016.09.007

Erdoğan-Bakar, E., Cinbiş, M., Ozyürek, H., Kiriş, N., Altunbaşak, S., & Anlar, B. (2009). Cognitive functions in neurofibromatosis type 1 patients and unaffected siblings. *The Turkish Journal of Pediatrics*, *51*(6), 565–571.

Ey, E., Leblond, C. S., & Bourgeron, T. (2011). Behavioral profiles of mouse models for autism spectrum disorders. *Autism Research: Official Journal of the International Society for Autism Research*, *4*(1), 5–16. https://doi.org/10.1002/aur.175

Feather-Schussler, D. N., & Ferguson, T. S. (2016). A Battery of Motor Tests in a Neonatal Mouse Model of Cerebral Palsy. *Journal of Visualized Experiments : JoVE*, *117*, 53569. https://doi.org/10.3791/53569

Fernstrom, J. D. (2012). Effects and side effects associated with the non-nutritional use of tryptophan by humans. *The Journal of Nutrition*, *142*(12), 2236S-2244S. https://doi.org/10.3945/jn.111.157065

Fernstrom, J. D. (2013). Large neutral amino acids: Dietary effects on brain neurochemistry and function. *Amino Acids*, *45*(3), 419–430. https://doi.org/10.1007/s00726-012-1330-y

Fernstrom, M. H., & Fernstrom, J. D. (1995). Brain tryptophan concentrations and serotonin synthesis remain responsive to food consumption after the ingestion of sequential meals. *The American Journal of Clinical Nutrition*, *61*(2), 312–319. https://doi.org/10.1093/ajcn/61.2.312

Ferreira, H., Sousa, A. C., Sereno, J., Martins, J., Castelo-Branco, M., & Gonçalves, J. (2022). Sex-Dependent Social and Repetitive Behavior and Neurochemical Profile in Mouse Model of Autism Spectrum Disorder. *Metabolites*, *12*(1), Article 1. https://doi.org/10.3390/metabo12010071

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., Armanini, F., Truong, D. T., Manara, S., Zolfo, M., Beghini, F., Bertorelli, R., De Sanctis, V., Bariletti, I., Canto, R., Clementi, R., Cologna, M., Crifò, T., Cusumano, G., … Segata, N. (2018). Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host & Microbe*, *24*(1), 133-145.e5. https://doi.org/10.1016/j.chom.2018.06.005

Fett-Conte, A. C., Bossolani-Martins, A. L., Rosan, D. B. A., Fett-Conte, A. C., Bossolani-Martins, A. L., & Rosan, D. B. A. (2015). Etiology of Autism the Complexity of Risk Factors in Autism Spectrum Disorder. In *Autism Spectrum Disorder—Recent Advances*. IntechOpen. https://doi.org/10.5772/59109

Fournier, K. A., Hass, C. J., Naik, S. K., Lodha, N., & Cauraugh, J. H. (2010). Motor coordination in autism spectrum disorders: A synthesis and meta-analysis. *Journal of Autism and Developmental Disorders*, *40*(10), 1227–1240. https://doi.org/10.1007/s10803-010-0981-3

Frazier, T. W., Georgiades, S., Bishop, S. L., & Hardan, A. Y. (2014). Behavioral and cognitive characteristics of females and males with autism in the Simons Simplex Collection. *Journal of the American Academy of Child and Adolescent Psychiatry*, *53*(3), 329-340.e1-3. https://doi.org/10.1016/j.jaac.2013.12.004

Freeman, H. E., Klein, R. E., Townsend, J. W., & Lechtig, A. (1980). Nutrition and cognitive development among rural Guatemalan children. *American Journal of Public Health*, *70*(12), 1277–1285.

Fundora, J., Guha, P., Shores, D., Pammi, M., & Maheshwari, A. (2019). Intestinal dysbiosis and necrotizing enterocolitis: Assessment for causality using Bradford Hill criteria. *Pediatric Research*, *87*. https://doi.org/10.1038/s41390-019-0482-9

Gabriele, S., Sacco, R., & Persico, A. M. (2014). Blood serotonin levels in autism spectrum disorder: A systematic review and meta-analysis. *European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology*, *24*(6), 919–929. https://doi.org/10.1016/j.euroneuro.2014.02.004

Galley, J. D., Chen, H. J., Antonson, A. M., & Gur, T. L. (2021). Prenatal stress-induced disruptions in microbial and host tryptophan metabolism and transport. *Behavioural Brain Research*, *414*, 113471. https://doi.org/10.1016/j.bbr.2021.113471

Garg, S., Green, J., Leadbitter, K., Emsley, R., Lehtonen, A., Evans, D. G., & Huson, S. M. (2013a). Neurofibromatosis Type 1 and Autism Spectrum Disorder. *Pediatrics*, *132*(6), e1642–e1648. https://doi.org/10.1542/peds.2013-1868

Garg, S., Green, J., Leadbitter, K., Emsley, R., Lehtonen, A., Evans, D. G., & Huson, S. M. (2013b). Neurofibromatosis type 1 and autism spectrum disorder. *Pediatrics*, *132*(6), e1642-1648. https://doi.org/10.1542/peds.2013-1868

Garg, S., Lehtonen, A., Huson, S. M., Emsley, R., Trump, D., Evans, D. G., & Green, J. (2013). Autism and other psychiatric comorbidity in neurofibromatosis type 1: Evidence from a population-based study. *Developmental Medicine and Child Neurology*, *55*(2), 139–145. https://doi.org/10.1111/dmcn.12043

Gawel, K., Gibula, E., Marszalek-Grabska, M., Filarowska, J., & Kotlinska, J. H. (2019). Assessment of spatial learning and memory in the Barnes maze task in rodents-methodological consideration. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *392*(1), 1–18. https://doi.org/10.1007/s00210-018-1589-y

Gevi, F., Zolla, L., Gabriele, S., & Persico, A. M. (2016). Urinary metabolomics of young Italian autistic children supports abnormal tryptophan and purine metabolism. *Molecular Autism*, *7*, 47. https://doi.org/10.1186/s13229-016-0109-5

Gheorghe, C. E., Martin, J. A., Manriquez, F. V., Dinan, T. G., Cryan, J. F., & Clarke, G. (2019). Focus on the essentials: Tryptophan metabolism and the microbiome-gut-brain axis. *Current Opinion in Pharmacology*, *48*, 137–145. https://doi.org/10.1016/j.coph.2019.08.004

Ghozy, S., Tran, L., Naveed, S., Quynh, T. T. H., Helmy Zayan, A., Waqas, A., Sayed, A. K. H., Karimzadeh, S., Hirayama, K., & Huy, N. T. (2020). Association of breastfeeding status with risk of autism spectrum disorder: A systematic review, dose-response analysis and meta-analysis. *Asian Journal of Psychiatry*, *48*, 101916. https://doi.org/10.1016/j.ajp.2019.101916

Gibson, E. L. (2018). Tryptophan supplementation and serotonin function: Genetic variations in behavioural effects. *Proceedings of the Nutrition Society*, *77*(2), 174–188. https://doi.org/10.1017/S0029665117004451

Gohir, W., Whelan, F. J., Surette, M. G., Moore, C., Schertzer, J. D., & Sloboda, D. M. (2015). Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes*, *6*(5), 310–320. https://doi.org/10.1080/19490976.2015.1086056

Gonçalves, J., Violante, I. R., Sereno, J., Leitão, R. A., Cai, Y., Abrunhosa, A., Silva, A. P., Silva, A. J., & Castelo-Branco, M. (2017). Testing the excitation/inhibition imbalance hypothesis in a mouse model of the autism spectrum disorder: In vivo neurospectroscopy and molecular evidence for regional phenotypes. *Molecular Autism*, *8*(1), 47. https://doi.org/10.1186/s13229-017-0166-4

Gostner, J. M., Geisler, S., Stonig, M., Mair, L., Sperner-Unterweger, B., & Fuchs, D. (2020). Tryptophan Metabolism and Related Pathways in Psychoneuroimmunology: The Impact of Nutrition and Lifestyle. *Neuropsychobiology*, *79*(1), 89–99. https://doi.org/10.1159/000496293

Guoynes, C. D., & Marler, C. A. (2021). An acute dose of intranasal oxytocin rapidly increases maternal communication and maintains maternal care in primiparous postpartum California mice. *PLOS ONE*, *16*(4), e0244033. https://doi.org/10.1371/journal.pone.0244033

Gutmann, D. H., Ferner, R. E., Listernick, R. H., Korf, B. R., Wolters, P. L., & Johnson, K. J. (2017). Neurofibromatosis type 1. *Nature Reviews Disease Primers*, *3*(1), 17004. https://doi.org/10.1038/nrdp.2017.4

Guzmán, D. C., Ruíz, N. L., García, E. H., Mejía, G. B., Téllez, P. P., Jimenez, G. E., De la Rosa Apreza, M., & Olguín, H. J. (2009). Effect of sibutramine on Na+, K+ ATPase activity and tryptophan levels on male and female rat brain. *Hormone and Metabolic Research, 41*(5), 363–367. https://doi.org/10.1055/s-0028-1128145

Gyurak, A., Haase, C. M., Sze, J., Goodkind, M. S., Coppola, G., Lane, J., Miller, B. L., & Levenson, R. W. (2013). The effect of the serotonin transporter (5-HTTLPR) polymorphism on empathic and self-conscious emotional reactivity. *Emotion (Washington, D.C.)*, *13*(1), 25–35. https://doi.org/10.1037/a0029616

Hallam, M. C., Barile, D., Meyrand, M., German, J. B., & Reimer, R. A. (2014). Maternal high-protein or high-prebiotic-fiber diets affect maternal milk composition and gut microbiota in rat dams and their offspring. *Obesity (Silver Spring, Md.)*, *22*(11), 2344–2351. https://doi.org/10.1002/oby.20849

Hård, E., & Engel, J. (1988). Effects of 8-OH-DPAT on ultrasonic vocalization and audiogenic immobility reaction in pre-weanling rats. *Neuropharmacology*, *27*(10), 981–986. https://doi.org/10.1016/0028-3908(88)90056-1

Harmer, C. J., Bhagwagar, Z., Cowen, P. J., & Goodwin, G. M. (2002). Acute administration of citalopram facilitates memory consolidation in healthy volunteers. *Psychopharmacology*, *163*(1), 106–110. https://doi.org/10.1007/s00213-002-1151-x

Harrop, C., Jones, D., Zheng, S., Nowell, S., Schultz, R., & Parish-Morris, J. (2019). Visual attention to faces in children with autism spectrum disorder: Are there sex differences? *Molecular Autism*, *10*(1), 28. https://doi.org/10.1186/s13229-019-0276-2

Hartley, S. L., & Sikora, D. M. (2009). Sex Differences in Autism Spectrum Disorder: An Examination of Developmental Functioning, Autistic Symptoms, and Coexisting Behavior Problems in Toddlers. *Journal of Autism and Developmental Disorders*, *39*(12), 1715–1722. https://doi.org/10.1007/s10803-009-0810-8

Hiller, R. M., Young, R. L., & Weber, N. (2014). Sex Differences in Autism Spectrum Disorder based on DSM-5 Criteria: Evidence from Clinician and Teacher Reporting. *Journal of Abnormal Child Psychology*, *42*(8), 1381–1393. https://doi.org/10.1007/s10802-014-9881-x

Hodges, H., Fealko, C., & Soares, N. (2020). Autism spectrum disorder: Definition, epidemiology, causes, and clinical evaluation. *Translational Pediatrics*, *9*(Suppl 1), S55–S65. https://doi.org/10.21037/tp.2019.09.09

Hodgson, R. A., Guthrie, D. H., & Varty, G. B. (2008). Duration of ultrasonic vocalizations in the isolated rat pup as a behavioral measure: Sensitivity to anxiolytic and antidepressant drugs. *Pharmacology, Biochemistry, and Behavior*, *88*(3), 341–348. https://doi.org/10.1016/j.pbb.2007.09.005

Horn, J., Mayer, D. E., Chen, S., & Mayer, E. A. (2022). Role of diet and its effects on the gut microbiome in the pathophysiology of mental disorders. *Translational Psychiatry*, *12*, 164. https://doi.org/10.1038/s41398-022-01922-0

Hornef, M., & Penders, J. (2017). Does a prenatal bacterial microbiota exist? *Mucosal Immunology*, *10*(3), 598–601. https://doi.org/10.1038/mi.2016.141

Hoshino, Y., Yamamoto, T., Kaneko, M., & Kumashiro, H. (1986). Plasma free tryptophan concentration in autistic children. *Brain & Development*, *8*(4), 424–427. https://doi.org/10.1016/s0387-7604(86)80064-x

Howlin, P., & Magiati, I. (2017). Autism spectrum disorder: Outcomes in adulthood. *Current Opinion in Psychiatry*, *30*(2), 69–76. https://doi.org/10.1097/YCO.0000000000000308

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., Codelli, J. A., Chow, J., Reisman, S. E., Petrosino, J. F., Patterson, P. H., & Mazmanian, S. K. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*, *155*(7), 1451–1463. https://doi.org/10.1016/j.cell.2013.11.024

Huang, X., Feng, Z., & Cheng, H. (2022). Perspective: Gestational Tryptophan Fluctuation Altering Neuroembryogenesis and Psychosocial Development. *Cells*, *11*(8), 1270. https://doi.org/10.3390/cells11081270

Huerta, M., & Lord, C. (2012). Diagnostic evaluation of autism spectrum disorders. *Pediatric Clinics of North America*, *59*(1), 103–111, xi. https://doi.org/10.1016/j.pcl.2011.10.018

Hyman, S. L., Arthur Shores, E., & North, K. N. (2006). Learning disabilities in children with neurofibromatosis type 1: Subtypes, cognitive profile, and attention-deficit-hyperactivity disorder. *Developmental Medicine and Child Neurology*, *48*(12), 973–977. https://doi.org/10.1017/S0012162206002131

Hyman, S. L., Levy, S. E., Myers, S. M., & COUNCIL ON CHILDREN WITH DISABILITIES, SECTION ON DEVELOPMENTAL AND BEHAVIORAL PEDIATRICS. (2020). Identification, Evaluation, and Management of Children With Autism Spectrum Disorder. *Pediatrics*, *145*(1), e20193447. https://doi.org/10.1542/peds.2019-3447

Hyman, S. L., Shores, A., & North, K. N. (2005). The nature and frequency of cognitive deficits in children with neurofibromatosis type 1. *Neurology*, *65*(7), 1037–1044. https://doi.org/10.1212/01.wnl.0000179303.72345.ce

Isaacs, E. B., Morley, R., & Lucas, A. (2009). Early diet and general cognitive outcome at adolescence in children born at or below 30 weeks gestation. *The Journal of Pediatrics*, *155*(2), 229–234. https://doi.org/10.1016/j.jpeds.2009.02.030

Ishimwe, J. A., Akinleye, A., Johnson, A. C., Garrett, M. R., & Sasser, J. M. (2021). Gestational gut microbial remodeling is impaired in a rat model of preeclampsia superimposed on chronic hypertension. *Physiological Genomics*, *53*(3), 125–136. https://doi.org/10.1152/physiolgenomics.00121.2020

Jacquemont, S., Coe, B. P., Hersch, M., Duyzend, M. H., Krumm, N., Bergmann, S., Beckmann, J. S., Rosenfeld, J. A., & Eichler, E. E. (2014). A Higher Mutational Burden in Females Supports a "Female Protective Model" in Neurodevelopmental Disorders. *The American Journal of Human Genetics*, *94*(3), 415–425. https://doi.org/10.1016/j.ajhg.2014.02.001

Jenkins, T. A., Nguyen, J. C. D., Polglaze, K. E., & Bertrand, P. P. (2016). Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis. *Nutrients*, *8*(1), 56. https://doi.org/10.3390/nu8010056

Jett, K., & Friedman, J. M. (2010). Clinical and genetic aspects of neurofibromatosis 1. *Genetics in Medicine*, *12*(1), 1–11. https://doi.org/10.1097/GIM.0b013e3181bf15e3

Johnson, C. P., Myers, S. M., & American Academy of Pediatrics Council on Children With Disabilities. (2007). Identification and evaluation of children with autism spectrum disorders. *Pediatrics*, *120*(5), 1183–1215. https://doi.org/10.1542/peds.2007-2361

Kałużna-Czaplińska, J., Gątarek, P., Chirumbolo, S., Chartrand, M. S., & Bjørklund, G. (2019). How important is tryptophan in human health? *Critical Reviews in Food Science and Nutrition*, *59*(1), 72–88. https://doi.org/10.1080/10408398.2017.1357534

Kałuzna-Czaplinska, J., Michalska, M., & Rynkowski, J. (2010). Determination of tryptophan in urine of autistic and healthy children by gas chromatography/mass spectrometry. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *16*(10), CR488-492.

Kałużna-Czaplińska, J., Żurawicz, E., Struck, W., & Markuszewski, M. (2014). Identification of organic acids as potential biomarkers in the urine of autistic children using gas chromatography/mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *966*, 70–76. https://doi.org/10.1016/j.jchromb.2014.01.041

Kang, D.-W., Adams, J. B., Gregory, A. C., Borody, T., Chittick, L., Fasano, A., Khoruts, A., Geis, E., Maldonado, J., McDonough-Means, S., Pollard, E. L., Roux, S., Sadowsky, M. J., Lipson, K. S., Sullivan, M. B., Caporaso, J. G., & Krajmalnik-Brown, R. (2017). Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: An open-label study. *Microbiome*, *5*(1), 10. https://doi.org/10.1186/s40168-016-0225-7

Keane, J. M., Khashan, A. S., McCarthy, F. P., Kenny, L. C., Collins, J. M., O'Donovan, S., Brown, J., Cryan, J. F., Dinan, T. G., Clarke, G., & O'Mahony, S. M. (2021). Identifying a biological signature of prenatal maternal stress. *JCI Insight*, *6*(2), e143007, 143007. https://doi.org/10.1172/jci.insight.143007

Kodak, T., & Bergmann, S. (2020). Autism Spectrum Disorder. *Pediatric Clinics of North America*, *67*(3), 525–535. https://doi.org/10.1016/j.pcl.2020.02.007

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O., & Vaiserman, A. (2017). Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiology*, *17*(1), 120. https://doi.org/10.1186/s12866-017-1027-1

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., Gonzalez, A., Werner, J. J., Angenent, L. T., Knight, R., Bäckhed, F., Isolauri, E., Salminen, S., & Ley, R. E. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*, *150*(3), 470–480. https://doi.org/10.1016/j.cell.2012.07.008

Lai, M.-C., Lombardo, M. V., Auyeung, B., Chakrabarti, B., & Baron-Cohen, S. (2015). Sex/Gender Differences and Autism: Setting the Scene for Future Research. *Journal of the American Academy of Child and Adolescent Psychiatry*, *54*(1), 11–24. https://doi.org/10.1016/j.jaac.2014.10.003

Landrigan, P. J. (2010). What causes autism? Exploring the environmental contribution. *Current Opinion in Pediatrics*, *22*(2), 219–225. https://doi.org/10.1097/MOP.0b013e328336eb9a

Le Floc'h, N., Otten, W., & Merlot, E. (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids*, *41*(5), 1195–1205. https://doi.org/10.1007/s00726-010-0752-7

Lieberman, H. R., Agarwal, S., & Fulgoni, V. L. (2016). Tryptophan Intake in the US Adult Population Is Not Related to Liver or Kidney Function but Is Associated with Depression and Sleep Outcomes. *The Journal of Nutrition*, *146*(12), 2609S-2615S. https://doi.org/10.3945/jn.115.226969

Linehan, K., Dempsey, E. M., Ryan, C. A., Ross, R. P., & Stanton, C. (2022). First encounters of the microbial kind: Perinatal factors direct infant gut microbiome establishment. *Microbiome Research Reports*. https://doi.org/10.20517/mrr.2021.09

Loomes, R., Hull, L., & Mandy, W. P. L. (2017). What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *Journal of the American Academy of Child and Adolescent Psychiatry*, *56*(6), 466–474. https://doi.org/10.1016/j.jaac.2017.03.013

Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E. E., Brochado, A. R., Fernandez, K. C., Dose, H., Mori, H., Patil, K. R., Bork, P., & Typas, A. (2018). Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*, *555*(7698), Article 7698. https://doi.org/10.1038/nature25979

Maloney, S. E., Chandler, K. C., Anastasaki, C., Rieger, M. A., Gutmann, D. H., & Dougherty, J. D. (2018). Characterization of early communicative behavior in mouse models of Neurofibromatosis type 1. *Autism Research : Official Journal of the International Society for Autism Research*, *11*(1), 44–58. https://doi.org/10.1002/aur.1853

Mangiola, F., Ianiro, G., Franceschi, F., Fagiuoli, S., Gasbarrini, G., & Gasbarrini, A. (2016). Gut microbiota in autism and mood disorders. *World Journal of Gastroenterology*, *22*(1), 361–368. https://doi.org/10.3748/wjg.v22.i1.361

Masi, A., DeMayo, M. M., Glozier, N., & Guastella, A. J. (2017). An Overview of Autism Spectrum Disorder, Heterogeneity and Treatment Options. *Neuroscience Bulletin*, *33*(2), 183–193. https://doi.org/10.1007/s12264-017-0100-y

May, T., Adesina, I., McGillivray, J., & Rinehart, N. J. (2019). Sex differences in neurodevelopmental disorders. *Current Opinion in Neurology*, *32*(4), 622–626. https://doi.org/10.1097/WCO.0000000000000714

May, T., Cornish, K., & Rinehart, N. (2014). Does Gender Matter? A One Year Follow-up of Autistic, Attention and Anxiety Symptoms in High-Functioning Children with Autism Spectrum Disorder. *Journal of Autism and Developmental Disorders*, *44*(5), 1077–1086. https://doi.org/10.1007/s10803-013-1964-y

May, T., Cornish, K., & Rinehart, N. J. (2016). Gender Profiles of Behavioral Attention in Children With Autism Spectrum Disorder. *Journal of Attention Disorders*, *20*(7), 627–635. https://doi.org/10.1177/1087054712455502

McDougle, C. J., Naylor, S. T., Cohen, D. J., Aghajanian, G. K., Heninger, G. R., & Price, L. H. (1996). Effects of tryptophan depletion in drug-free adults with autistic disorder. *Archives of General Psychiatry*, *53*(11), 993–1000. https://doi.org/10.1001/archpsyc.1996.01830110029004

McElhanon, B. O., McCracken, C., Karpen, S., & Sharp, W. G. (2014). Gastrointestinal symptoms in autism spectrum disorder: A meta-analysis. *Pediatrics*, *133*(5), 872–883. https://doi.org/10.1542/peds.2013-3995

McKeever, K., Shepherd, C. W., Crawford, H., & Morrison, P. J. (2008). An epidemiological, clinical and genetic survey of Neurofibromatosis type 1 in children under sixteen years of age. *The Ulster Medical Journal*, *77*(3), 160–163.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiology and Molecular Biology Reviews : MMBR*, *81*(4), e00036-17. https://doi.org/10.1128/MMBR.00036-17

Molosh, A. I., & Shekhar, A. (2018). Neurofibromatosis type 1 as a model system to study molecular mechanisms of autism spectrum disorder symptoms. *Progress in Brain Research*, *241*, 37–62. https://doi.org/10.1016/bs.pbr.2018.09.014

Mortensen, M. S., Hebbelstrup Jensen, B., Williams, J., Brejnrod, A. D., O'Brien Andersen, L., Röser, D., Andreassen, B. U., Petersen, A. M., Stensvold, C. R., Sørensen, S. J., & Krogfelt, K. A. (2018). Stability and resilience of the intestinal microbiota in children in daycare—A 12 month cohort study. *BMC Microbiology*, *18*(1), 223. https://doi.org/10.1186/s12866-018-1367-5

Moskowitz, D. S., Pinard, G., Zuroff, D. C., Annable, L., & Young, S. N. (2001). The effect of tryptophan on social interaction in everyday life: A placebo-controlled study. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *25*(2), 277–289. https://doi.org/10.1016/S0893-133X(01)00219-6

Moskowitz, D. S., Pinard, G., Zuroff, D. C., Annable, L., & Young, S. N. (2003). Tryptophan, Serotonin and Human Social Behavior. In G. Allegri, C. V. L. Costa, E. Ragazzi, H. Steinhart, & L. Varesio (Eds.), *Developments in Tryptophan and Serotonin Metabolism* (Vol. 527, pp. 215–224). Springer US. https://doi.org/10.1007/978-1-4615-0135-0_25

Moussa, H. N., Srikrishnan, A., Blackwell, S. C., Dash, P., & Sibai, B. M. (2016). Fetal origins of autism spectrum disorders: The non-associated maternal factors. *Future Science OA*, *2*(2), FSO114. https://doi.org/10.4155/fsoa-2015-0001

Moy, S. S., Nadler, J. J., Magnuson, T. R., & Crawley, J. N. (2006). Mouse models of autism spectrum disorders: The challenge for behavioral genetics. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *142C*(1), 40–51. https://doi.org/10.1002/ajmg.c.30081

Mukherjee, S. B. (2017). Autism Spectrum Disorders—Diagnosis and Management. *Indian Journal of Pediatrics*, *84*(4), 307–314. https://doi.org/10.1007/s12098-016-2272-2

Murakami, Y., Imamura, Y., Saito, K., Sakai, D., & Motoyama, J. (2019). Altered kynurenine pathway metabolites in a mouse model of human attention-deficit hyperactivity/autism spectrum disorders: A potential new biological diagnostic marker. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-49781-y

Nagpal, R., Mainali, R., Ahmadi, S., Wang, S., Singh, R., Kavanagh, K., Kitzman, D. W., Kushugulova, A., Marotta, F., & Yadav, H. (2018). Gut microbiome and aging: Physiological and mechanistic insights. *Nutrition and Healthy Aging*, *4*(4), 267–285. https://doi.org/10.3233/NHA-170030

Napolitano, A., Schiavi, S., La Rosa, P., Rossi-Espagnet, M. C., Petrillo, S., Bottino, F., Tagliente, E., Longo, D., Lupi, E., Casula, L., Valeri, G., Piemonte, F., Trezza, V., & Vicari, S. (2022). Sex Differences in Autism Spectrum Disorder: Diagnostic, Neurobiological, and Behavioral Features. *Frontiers in Psychiatry*, *13*, 889636. https://doi.org/10.3389/fpsyt.2022.889636

Naushad, S. M., Jain, J. M. N., Prasad, C. K., Naik, U., & Akella, R. R. D. (2013). Autistic children exhibit distinct plasma amino acid profile. *Indian Journal of Biochemistry & Biophysics*, *50*(5), 474–478.

Nayak, B., Singh, R., Buttar, H. (2019). Role of Tryptophan in Health and Disease: Systematic Review of the Anti-Oxidant, Anti-Inflammation, and Nutritional Aspects of Tryptophan and Its Metabolites. *World Heart Journal*, *Vol. 11*, 161.

Nix, J. S., Blakeley, J., & Rodriguez, F. J. (2020). An update on the central nervous system manifestations of neurofibromatosis type 1. *Acta Neuropathologica*, *139*(4), 625–641. https://doi.org/10.1007/s00401-019-02002-2

Olivier, B., Mos, J., van der Heyden, J., & Hartog, J. (1989). Serotonergic modulation of social interactions in isolated male mice. *Psychopharmacology*, *97*(2), 154–156. https://doi.org/10.1007/BF00442239

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., & Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural Brain Research*, *277*, 32–48. https://doi.org/10.1016/j.bbr.2014.07.027

Pais, M. L., Martins, J., Castelo-Branco, M., & Gonçalves, J. (2023). Sex Differences in Tryptophan Metabolism: A Systematic Review Focused on Neuropsychiatric Disorders. *International Journal of Molecular Sciences*, *24*(6), 6010. https://doi.org/10.3390/ijms24066010

Palladino, E., Van Mieghem, T., & Connor, K. L. (2021). Diet Alters Micronutrient Pathways in the Gut and Placenta that Regulate Fetal Growth and Development in Pregnant Mice. *Reproductive Sciences (Thousand Oaks, Calif.)*, *28*(2), 447–461. https://doi.org/10.1007/s43032-020-00297-1

Pinares-Garcia, P., Stratikopoulos, M., Zagato, A., Loke, H., & Lee, J. (2018). Sex: A Significant Risk Factor for Neurodevelopmental and Neurodegenerative Disorders. *Brain Sciences*, *8*(8), 154. https://doi.org/10.3390/brainsci8080154

Plasschaert, E., Descheemaeker, M.-J., Van Eylen, L., Noens, I., Steyaert, J., & Legius, E. (2015). Prevalence of Autism Spectrum Disorder symptoms in children with neurofibromatosis type 1. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, *168*(1), 72–80. https://doi.org/10.1002/ajmg.b.32280

Pusceddu, M. M., El Aidy, S., Crispie, F., O'Sullivan, O., Cotter, P., Stanton, C., Kelly, P., Cryan, J. F., & Dinan, T. G. (2015). N-3 Polyunsaturated Fatty Acids (PUFAs) Reverse the Impact of Early-Life Stress on the Gut Microbiota. *PloS One*, *10*(10), e0139721. https://doi.org/10.1371/journal.pone.0139721

Ratto, A. B., Kenworthy, L., Yerys, B. E., Bascom, J., Wieckowski, A. T., White, S. W., Wallace, G. L., Pugliese, C., Schultz, R. T., Ollendick, T. H., Scarpa, A., Seese, S., Register-Brown, K., Martin, A., & Anthony, L. G. (2018). What About the Girls? Sex-Based Differences in Autistic Traits and Adaptive Skills. *Journal of Autism and Developmental Disorders*, *48*(5), 1698–1711. https://doi.org/10.1007/s10803-017-3413-9

Reichow, B., Hume, K., Barton, E. E., & Boyd, B. A. (2018). Early intensive behavioral intervention (EIBI) for young children with autism spectrum disorders (ASD). *The Cochrane Database of Systematic Reviews*, *2018*(5), CD009260. https://doi.org/10.1002/14651858.CD009260.pub3

Reuter, M., Zamoscik, V., Plieger, T., Bravo, R., Ugartemendia, L., Rodriguez, A. B., & Kirsch, P. (2021). Tryptophan-rich diet is negatively associated with depression and positively linked to social cognition. *Nutrition Research (New York, N.Y.)*, *85*, 14–20. https://doi.org/10.1016/j.nutres.2020.10.005

Ribeiro, M. J., Violante, I. R., Bernardino, I., Edden, R. A. E., & Castelo-Branco, M. (2015). Abnormal relationship between GABA, neurophysiology and impulsive behavior in neurofibromatosis type 1. *Cortex*, *64*, 194–208. https://doi.org/10.1016/j.cortex.2014.10.019

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *International Journal of Tryptophan Research: IJTR*, *2*, 45–60. https://doi.org/10.4137/ijtr.s2129

Robertson, R. C., Seira Oriach, C., Murphy, K., Moloney, G. M., Cryan, J. F., Dinan, T. G., Paul Ross, R., & Stanton, C. (2017). Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood. *Brain, Behavior, and Immunity*, *59*, 21–37. https://doi.org/10.1016/j.bbi.2016.07.145

Robertson, R. C., Seira Oriach, C., Murphy, K., Moloney, G. M., Cryan, J. F., Dinan, T. G., Ross, R. P., & Stanton, C. (2017). Deficiency of essential dietary n-3 PUFA disrupts the caecal microbiome and metabolome in mice. *The British Journal of Nutrition*, *118*(11), 959–970. https://doi.org/10.1017/S0007114517002999

Romero, R. (2009). Prenatal medicine: The child is the father of the man. 1996. *The Journal of Maternal-Fetal & Neonatal Medicine: The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, *22*(8), 636–639. https://doi.org/10.1080/14767050902784171

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., Galuppi, M., Lamont, R. F., Chaemsaithong, P., Miranda, J., Chaiworapongsa, T., & Ravel, J. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome*, *2*(1), 4. https://doi.org/10.1186/2049-2618-2-4

Roth, W., Zadeh, K., Vekariya, R., Ge, Y., & Mohamadzadeh, M. (2021). Tryptophan Metabolism and Gut-Brain Homeostasis. *International Journal of Molecular Sciences*, *22*(6), Article 6. https://doi.org/10.3390/ijms22062973

Ruddick, J. P., Evans, A. K., Nutt, D. J., Lightman, S. L., Rook, G. A. W., & Lowry, C. A. (2006). Tryptophan metabolism in the central nervous system: Medical implications. *Expert Reviews in Molecular Medicine*, *8*(20), 1–27. https://doi.org/10.1017/S1462399406000068

Sakanaka, M., Gotoh, A., Yoshida, K., Odamaki, T., Koguchi, H., Xiao, J.-Z., Kitaoka, M., & Katayama, T. (2019). Varied Pathways of Infant Gut-Associated Bifidobacterium to Assimilate Human Milk Oligosaccharides: Prevalence of the Gene Set and Its Correlation with Bifidobacteria-Rich Microbiota Formation. *Nutrients*, *12*(1), 71. https://doi.org/10.3390/nu12010071

Santocchi, E., Guiducci, L., Fulceri, F., Billeci, L., Buzzigoli, E., Apicella, F., Calderoni, S., Grossi, E., Morales, M. A., & Muratori, F. (2016). Gut to brain interaction in Autism Spectrum Disorders: A randomized controlled trial on the role of probiotics on clinical, biochemical and neurophysiological parameters. *BMC Psychiatry*, *16*, 183. https://doi.org/10.1186/s12888-016-0887-5

Santos, S., Ferreira, H., Martins, J., Gonçalves, J., & Castelo-Branco, M. (2022). Male sex bias in early and late onset neurodevelopmental disorders: Shared aspects and differences in Autism Spectrum Disorder, Attention Deficit/hyperactivity Disorder, and Schizophrenia. *Neuroscience and Biobehavioral Reviews*, *135*, 104577. https://doi.org/10.1016/j.neubiorev.2022.104577

Santos, S., Martins, B., Sereno, J., Martins, J., Castelo-Branco, M., & Gonçalves, J. (2023). Neurobehavioral sex-related differences in Nf1+/− mice: Female show a “camouflaging”-type behavior. *Biology of Sex Differences*, *14*(1), 24. https://doi.org/10.1186/s13293-023-00509-8

Saurman, V., Margolis, K. G., & Luna, R. A. (2020). Autism Spectrum Disorder as a Brain-Gut-Microbiome Axis Disorder. *Digestive Diseases and Sciences*, *65*(3), 818–828. https://doi.org/10.1007/s10620-020-06133-5

Schmitt, J. a. J., Wingen, M., Ramaekers, J. G., Evers, E. a. T., & Riedel, W. J. (2006). Serotonin and human cognitive performance. *Current Pharmaceutical Design*, *12*(20), 2473–2486. https://doi.org/10.2174/138161206777698909

Schmitt, J. A., Kruizinga, M. J., & Riedel, W. J. (2001). Non-serotonergic pharmacological profiles and associated cognitive effects of serotonin reuptake inhibitors. *Journal of Psychopharmacology (Oxford, England)*, *15*(3), 173–179. https://doi.org/10.1177/026988110101500304

Seferovic, M. D., Mohammad, M., Pace, R. M., Engevik, M., Versalovic, J., Bode, L., Haymond, M., & Aagaard, K. M. (2020). Maternal diet alters human milk oligosaccharide composition with implications for the milk metagenome. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-79022-6

Serino, M., Luche, E., Gres, S., Baylac, A., Bergé, M., Cenac, C., Waget, A., Klopp, P., Iacovoni, J., Klopp, C., Mariette, J., Bouchez, O., Lluch, J., Ouarné, F., Monsan, P., Valet, P., Roques, C., Amar, J., Bouloumié, A., Burcelin, R. (2012). Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut*, *61*(4), 543–553. https://doi.org/10.1136/gutjnl-2011-301012

Sharma, S. R., Gonda, X., & Tarazi, F. I. (2018). Autism Spectrum Disorder: Classification, diagnosis and therapy. *Pharmacology & Therapeutics*, *190*, 91–104. https://doi.org/10.1016/j.pharmthera.2018.05.007

Sharon, G., Cruz, N. J., Kang, D.-W., Gandal, M. J., Wang, B., Kim, Y.-M., Zink, E. M., Casey, C. P., Taylor, B. C., Lane, C. J., Bramer, L. M., Isern, N. G., Hoyt, D. W., Noecker, C., Sweredoski, M. J., Moradian, A., Borenstein, E., Jansson, J. K., Knight, R., … Mazmanian, S. K. (2019). Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell*, *177*(6), 1600-1618.e17. https://doi.org/10.1016/j.cell.2019.05.004

Shin, N.-R., Whon, T. W., & Bae, J.-W. (2015). Proteobacteria: Microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology*, *33*(9), 496–503. https://doi.org/10.1016/j.tibtech.2015.06.011

Siepmann, M., Grossmann, J., Mück-Weymann, M., & Kirch, W. (2003). Effects of sertraline on autonomic and cognitive functions in healthy volunteers. *Psychopharmacology*, *168*(3), 293–298. https://doi.org/10.1007/s00213-003-1448-4

Silber, B. Y., & Schmitt, J. a. J. (2010). Effects of tryptophan loading on human cognition, mood, and sleep. *Neuroscience and Biobehavioral Reviews*, *34*(3), 387–407. https://doi.org/10.1016/j.neubiorev.2009.08.005

Silva, A. J., Frankland, P. W., Marowitz, Z., Friedman, E., Lazlo, G., Cioffi, D., Jacks, T., & Bourtchuladze, R. (1997). A mouse model for the learning and memory deficits associated with neurofibromatosis type I. *Nature Genetics*, *15*(3), Article 3. https://doi.org/10.1038/ng0397-281

Simola, N. (2015). Rat Ultrasonic Vocalizations and Behavioral Neuropharmacology: From the Screening of Drugs to the Study of Disease. *Current Neuropharmacology*, *13*(2), 164–179. https://doi.org/10.2174/1570159X13999150318113800

Sobczak, S., Honig, A., Schmitt, J. a. J., & Riedel, W. J. (2003). Pronounced Cognitive Deficits Following an Intravenous L-Tryptophan Challenge in First-Degree Relatives of Bipolar Patients Compared to Healthy Controls. *Neuropsychopharmacology*, *28*(4), Article 4. https://doi.org/10.1038/sj.npp.1300055

Sorboni, S. G., Moghaddam, H. S., Jafarzadeh-Esfehani, R., & Soleimanpour, S. (2022). A Comprehensive Review on the Role of the Gut Microbiome in Human Neurological Disorders. *Clinical Microbiology Reviews*, *35*(1), e0033820. https://doi.org/10.1128/CMR.00338-20

Spring, S., Lerch, J. P., & Henkelman, R. M. (2007). Sexual dimorphism revealed in the structure of the mouse brain using three-dimensional magnetic resonance imaging. *NeuroImage*, *35*(4), 1424–1433. https://doi.org/10.1016/j.neuroimage.2007.02.023

Stacy, M. E., Zablotsky, B., Yarger, H. A., Zimmerman, A., Makia, B., & Lee, L.-C. (2014). Sex differences in co-occurring conditions of children with autism spectrum disorders. *Autism*, *18*(8), 965–974. https://doi.org/10.1177/1362361313505719

Steegenga, W. T., Mischke, M., Lute, C., Boekschoten, M. V., Lendvai, A., Pruis, M. G. M., Verkade, H. J., van de Heijning, B. J. M., Boekhorst, J., Timmerman, H. M., Plösch, T., Müller, M., & Hooiveld, G. J. E. J. (2017). Maternal exposure to a Western-style diet causes differences in intestinal microbiota composition and gene expression of suckling mouse pups. *Molecular Nutrition & Food Research*, *61*(1), 1600141. https://doi.org/10.1002/mnfr.201600141

Steenbergen, L., Jongkees, B. J., Sellaro, R., & Colzato, L. S. (2016). Tryptophan supplementation modulates social behavior: A review. *Neuroscience and Biobehavioral Reviews*, *64*, 346–358. https://doi.org/10.1016/j.neubiorev.2016.02.022

Sunyer, B., Patil, S., Höger, H., & Luber, G. (2007). Barnes maze, a useful task to assess spatial reference memory in the mice. *Protocol Exchange*. https://doi.org/10.1038/nprot.2007.390

Supekar, K., & Menon, V. (2015). Sex differences in structural organization of motor systems and their dissociable links with repetitive/restricted behaviors in children with autism. *Molecular Autism*, *6*(1), 50. https://doi.org/10.1186/s13229-015-0042-z

Tabbai, S., Moreno-Fernández, R. D., Zambrana-Infantes, E., Nieto-Quero, A., Chun, J., García-Fernández, M., Estivill-Torrús, G., Rodríguez de Fonseca, F., Santín, L. J., Oliveira, T. G., Pérez-Martín, M., & Pedraza, C. (2019). Effects of the LPA1 Receptor Deficiency and Stress on the Hippocampal LPA Species in Mice. *Frontiers in Molecular Neuroscience*, *12*. https://doi.org/10.3389/fnmol.2019.00146.

Tamura, R. (2021). Current Understanding of Neurofibromatosis Type 1, 2, and Schwannomatosis. *International Journal of Molecular Sciences*, *22*(11), 5850. https://doi.org/10.3390/ijms22115850

Tandon, P. S., Tovar, A., Jayasuriya, A. T., Welker, E., Schober, D. J., Copeland, K., Dev, D. A., Murriel, A. L., Amso, D., & Ward, D. S. (2016). The relationship between physical activity and diet and young children's cognitive development: A systematic review. *Preventive Medicine Reports*, *3*, 379–390. https://doi.org/10.1016/j.pmedr.2016.04.003

Taniya, M. A., Chung, H.-J., Al Mamun, A., Alam, S., Aziz, M. A., Emon, N. U., Islam, M. M., Hong, S.-T. S., Podder, B. R., Ara Mimi, A., Aktar Suchi, S., & Xiao, J. (2022). Role of Gut Microbiome in Autism Spectrum Disorder and Its Therapeutic Regulation. *Frontiers in Cellular and Infection Microbiology*, *12*, 915701. https://doi.org/10.3389/fcimb.2022.915701

Trovó-Marqui, A., & Tajara, E. (2006). Neurofibromin: A general outlook. *Clinical Genetics*, *70*(1), 1–13. https://doi.org/10.1111/j.1399-0004.2006.00639.x

Tsopmo, A., Diehl-Jones, B. W., Aluko, R. E., Kitts, D. D., Elisia, I., & Friel, J. K. (2009). Tryptophan released from mother's milk has antioxidant properties. *Pediatric Research*, *66*(6), 614–618. https://doi.org/10.1203/PDR.0b013e3181be9e7e

Tun, H. M., Konya, T., Takaro, T. K., Brook, J. R., Chari, R., Field, C. J., Guttman, D. S., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Sears, M. R., Scott, J. A., Kozyrskyj, A. L., & CHILD Study Investigators. (2017). Exposure to household furry pets influences the gut microbiota of infant at 3-4 months following various birth scenarios. *Microbiome*, *5*(1), 40. https://doi.org/10.1186/s40168-017-0254-x

van der Vaart, T., van Woerden, G. M., Elgersma, Y., de Zeeuw, C. I., & Schonewille, M. (2011). Motor deficits in neurofibromatosis type 1 mice: The role of the cerebellum. *Genes, Brain, and Behavior*, *10*(4), 404–409. https://doi.org/10.1111/j.1601-183X.2011.00685.x

Van Wijngaarden-Cremers, P. J. M., van Eeten, E., Groen, W. B., Van Deurzen, P. A., Oosterling, I. J., & Van der Gaag, R. J. (2014). Gender and age differences in the core triad of impairments in autism spectrum disorders: A systematic review and meta-analysis. *Journal of Autism and Developmental Disorders*, *44*(3), 627–635. https://doi.org/10.1007/s10803-013-1913-9

VanRyzin, J. W., Yu, S. J., Perez-Pouchoulen, M., & McCarthy, M. M. (2016). Temporary Depletion of Microglia during the Early Postnatal Period Induces Lasting Sex-Dependent and Sex-Independent Effects on Behavior in Rats. *ENeuro*, *3*(6), ENEURO.0297-16.2016. https://doi.org/10.1523/ENEURO.0297-16.2016

Violante, I. R., Patricio, M., Bernardino, I., Rebola, J., Abrunhosa, A. J., Ferreira, N., & Castelo-Branco, M. (2016). GABA deficiency in NF1: A multimodal [11C]-flumazenil and spectroscopy study. *Neurology*, *87*(9), 897–904. https://doi.org/10.1212/WNL.0000000000003044

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*, *2*(2), Article 2. https://doi.org/10.1038/nprot.2007.44

Walsh, K. S., Vélez, J. I., Kardel, P. G., Imas, D. M., Muenke, M., Packer, R. J., Castellanos, F. X., & Acosta, M. T. (2013). Symptomatology of autism spectrum disorder in a population with neurofibromatosis type 1. *Developmental Medicine and Child Neurology*, *55*(2), 131–138. https://doi.org/10.1111/dmcn.12038

Wang, C., Geng, H., Liu, W., & Zhang, G. (2017). Prenatal, perinatal, and postnatal factors associated with autism. *Medicine*, *96*(18), e6696. https://doi.org/10.1097/MD.0000000000006696

Wang, D., Wu, J., Zhu, P., Xie, H., Lu, L., Bai, W., Pan, W., Shi, R., Ye, J., Xia, B., Zhao, Z., Wang, Y., Liu, X., & Zhao, B. (2022). Tryptophan-rich diet ameliorates chronic unpredictable mild stress induced depression- and anxiety-like behavior in mice: The potential involvement of gut-brain axis. *Food Research International*, *157*, 111289. https://doi.org/10.1016/j.foodres.2022.111289

Wang, H.-X., & Wang, Y.-P. (2016). Gut Microbiota-brain Axis. *Chinese Medical Journal*, *129*(19), 2373–2380. https://doi.org/10.4103/0366-6999.190667

Wang, J., Zheng, J., Shi, W., Du, N., Xu, X., Zhang, Y., Ji, P., Zhang, F., Jia, Z., Wang, Y., Zheng, Z., Zhang, H., & Zhao, F. (2018). Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. *Gut*, *67*(9), 1614–1625. https://doi.org/10.1136/gutjnl-2018-315988

Werling, D. M., & Geschwind, D. H. (2013). Sex differences in autism spectrum disorders. *Current Opinion in Neurology*, *26*(2), 146–153. https://doi.org/10.1097/WCO.0b013e32835ee548

Wöhr, M., Rippberger, H., Schwarting, R. K. W., & van Gaalen, M. M. (2015). Critical involvement of 5-HT2C receptor function in amphetamine-induced 50-kHz ultrasonic vocalizations in rats. *Psychopharmacology*, *232*(10), 1817–1829. https://doi.org/10.1007/s00213-014-3814-9

Wu, W.-L., Wang, C.-H., Huang, E. Y.-K., & Chen, C.-C. (2009). Asic3−/− Female Mice with Hearing Deficit Affects Social Development of Pups. *PLOS ONE*, *4*(8), e6508. https://doi.org/10.1371/journal.pone.0006508

Xiao, L., & Zhao, F. (2023). Microbial transmission, colonisation and succession: From pregnancy to infancy. *Gut*, *72*(4), 772–786. https://doi.org/10.1136/gutjnl-2022-328970

Xu, G. F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., & White, R. (1990). The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell*, *62*(3), 599–608. https://doi.org/10.1016/0092-8674(90)90024-9

Yao, Y., Cai, X., Ye, Y., Wang, F., Chen, F., & Zheng, C. (2021). The Role of Microbiota in Infant Health: From Early Life to Adulthood. *Frontiers in Immunology*, *12*, 708472. https://doi.org/10.3389/fimmu.2021.708472

Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A.-M., Härkönen, T., Ryhänen, S. J., Franzosa, E. A., Vlamakis, H., Huttenhower, C., Gevers, D., Lander, E. S., Knip, M., DIABIMMUNE Study Group, & Xavier, R. J. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Science Translational Medicine*, *8*(343), 343ra81. https://doi.org/10.1126/scitranslmed.aad0917

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), Article 7402. https://doi.org/10.1038/nature11053

Yoon, S. H., Choi, J., Lee, W. J., & Do, J. T. (2020). Genetic and Epigenetic Etiology Underlying Autism Spectrum Disorder. *Journal of Clinical Medicine*, *9*(4), 966. https://doi.org/10.3390/jcm9040966

Young, D. M., Schenk, A. K., Yang, S.-B., Jan, Y. N., & Jan, L. Y. (2010). Altered ultrasonic vocalizations in a tuberous sclerosis mouse model of autism. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(24), 11074–11079. https://doi.org/10.1073/pnas.1005620107

Yu, J., Liu, X., Li, Y., Meng, S., Wu, F., Yan, B., Xue, Y., Ma, T., Yang, J., & Liu, J. (2018). Maternal exposure to farming environment protects offspring against allergic diseases by modulating the neonatal TLR-Tregs-Th axis. *Clinical and Translational Allergy*, *8*, 34. https://doi.org/10.1186/s13601-018-0220-0

Yun, H., Park, E.-S., Choi, S., Shin, B., Yu, J., Yu, J., Amarasekara, D. S., Kim, S., Lee, N., Choi, J.-S., Choi, Y., & Rho, J. (2019). TDAG51 is a crucial regulator of maternal care and depressive-like behavior after parturition. *PLOS Genetics*, *15*(6), e1008214. https://doi.org/10.1371/journal.pgen.1008214

Zahar, S., Schneider, N., Makwana, A., Chapman, S., Corthesy, J., Amico, M., & Hudry, J. (2023). Dietary tryptophan-rich protein hydrolysate can acutely impact physiological and psychological measures of mood and stress in healthy adults. *Nutritional Neuroscience*, *26*(4), 303–312. https://doi.org/10.1080/1028415X.2022.2047435