



Dipartimento di Scienze e Innovazione Tecnologica - DISIT

Corso di Laurea Magistrale in Biologia

Curriculum Nutrizione e Ambiente

TESI DI LAUREA

**Effects of maternal low-protein and phytoestrogen-enriched diets
on the correlation between eating behaviors and anxiety in offspring**

Candidata: Sofia Lafaenza

Relatore: Prof.ssa Flavia Prodam

Correlatore: Prof.ssa Marilena Marraudino



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SUMMARY

Maternal nutrition during pregnancy and lactation represents a crucial determinant of offspring health, with long-term effects on metabolic and neurobehavioral development.

This study examined the effects of altered diets during pregnancy and lactation on adult rat pups that received a standard postnatal diet. We focus on the consequences of a maternal low-protein diet enriched with phytoestrogens, as well as the combination of two diets. Since in often altered low-protein diets, proteins are supplemented with foods rich in phytoestrogens, such as soy. Four groups were compared: low-protein maternal diet (LOW), genistein-enriched diet (GEN, phytoestrogen), a combination of both (LOWGEN), and a control group (CON). Maternal milk composition and various offspring parameters were analyzed, including metabolic measures (body weight and intake), behavioral assessments (EPM and OF tests), and neurochemical markers of the hypothalamic POMC system and serotonergic systems (immunohistochemistry for 5-HT in the Raphe, and POMC, 5-HT, and 5-HT_{2C} in the arcuate nucleus of the hypothalamus).

Maternal protein restriction significantly reduced milk protein and fat content, hindered neonatal growth, and decreased POMC expression, while also increasing 5-HT_{2C} receptors levels, making it more difficult for the neonate to adapt to changes and new stimuli. The phytoestrogen, Genistein, increased milk lipid content and neonatal weight, suggesting a potential obesogenic effect in female pups. The long-term effects of genistein were sex-specific: males exhibited enhanced serotonergic sensitivity, characterized by increased expression of the 5-HT system, and an anxiolytic behavioral profile. In contrast, females showed serotonergic hyperactivity. The combination of the two diets displayed milk composition and metabolic parameters similar to those of the LOW group, suggesting a predominant effect of protein restriction that overrides the potential benefits of genistein. Behaviorally, profiles were sexually dimorphic: males showed anxiety, whereas females displayed an anxiolytic profile similar to GEN females, highlighting greater female sensitivity to genistein's estrogenic effects.

Overall, the results demonstrate that early nutritional programming has a stable influence on the development of metabolic and emotional circuits in a sex-specific manner, underscoring the importance of adequate protein intake during pregnancy and lactation, as well as the cautious

evaluation of phytoestrogen consumption during critical periods, when maternal dietary choices can have lasting consequences for offspring health.

1. INTRODUCTION

1.1 Maternal Nutrition

Pregnancy and lactation are complex physiological stages marked by significant metabolic, hormonal, and immunological changes that support fetal and neonatal growth while maintaining maternal health. In this process, nutrition is crucial, as an inadequate diet can harm both pregnancy outcomes and the immediate and long-term health of the offspring (J. Stephenson et al., 2018).

A balanced diet, rich in both macro- and micronutrients, is essential for supporting the physiological changes in the mother's body, promoting proper fetal development, and ensuring optimal breast milk production during the postnatal period. However, in today's environment—shaped by extreme dietary trends and widespread misinformation on social media—many pregnant women follow self-imposed diets without medical guidance. This situation can lead to malnutrition with harmful effects on both the mother and the unborn child (S.V. Wrottesley et al., 2020; K. Fleischer Michaelsen et al., 2021), such as an increased risk of metabolic problems in childhood, including obesity, insulin resistance, and metabolic syndrome (D.M. Chu et al., 2016; O. Koren et al., 2012).

Protein deficiency, especially from high biological value sources, can happen in different situations. These include restrictive and unbalanced self-managed diets, food restrictions due to exaggerated fears of contamination (such as avoiding certain meats because of concerns about toxoplasmosis), or low-income situations where access to high-quality protein sources is limited (O. Koletzko et al., 2019).

A growing area of interest involves the increasing number of women adopting vegetarian or vegan diets during their reproductive years. While these diets can theoretically meet nutritional needs during pregnancy if well planned, they require careful attention to protein quality and supplementation of essential nutrients. Without proper guidance, these diets may lead to inadequate intake of essential amino acids, potentially causing serious effects on fetal development (L. Baroni et al., 2018).

Additionally, plant-based diets often rely heavily on soy-derived products, such as tofu, tempeh, and soy drinks, as primary protein sources (M. Messina, 2010). These foods, when consumed in excess, can lead to high levels of genistein (GEN), an isoflavone that mimics the effects of estrogen. Since GEN is a phytoestrogen, it can disrupt hormonal balance and affect fetal

development, as shown in several animal studies (M.Cimafranca et al., 2010; W.N. Jefferson et al., 2012)

1.1.1 Pregnancy nutrition

Pregnancy is a critical period nutritionally, as it involves a series of physiological changes essential for supporting embryonic and fetal development while protecting maternal health. During pregnancy, the mother's body experiences significant hormonal and metabolic shifts, including increased insulin sensitivity in the first trimester, followed by physiological insulin resistance in the second and third trimesters. There are also changes in lipid profile and protein metabolism (J.C. King, 2000; E. Herrera et al.; 2014) These adaptations aim to maximize nutrient transfer to the fetus; however, if the mother's diet is not adequate, they can increase the risk of complications for both mother and baby.

Energy needs during pregnancy vary based on the gestational stage and the woman's nutritional status. While the first trimester does not require a substantial increase in calorie intake, an extra 200–300 kcal/day is generally advised in the second and third trimesters, reaching up to 400 kcal/day for underweight women (Institute of Medicine, 2009).

The distribution of macronutrients in the maternal diet is crucial. Carbohydrates should make up about 45–60% of total energy intake, with simple sugars limited to less than 10%. It's better to choose low-glycemic index starchy foods and ensure a fiber intake of 25–30 grams per day to help reduce postprandial glucose spikes, lower the risk of gestational diabetes, and promote satiety (B. Koletzko, 2019).

Fats should make up 25–35% of daily energy intake, with special attention to fat quality: saturated fats should stay below 10%, monounsaturated fats (mainly oleic acid) around 10%, and polyunsaturated fatty acids—especially DHA—should be included at 100–200 mg/day, due to their crucial role in fetal brain and retinal development (J.A. Greenberg, 2008). The omega-6 to omega-3 ratio should ideally be kept near 4:1.

Proteins are especially important, with increasing daily requirements: +1 g/day in the first trimester, +7–9 g/day in the second, and +21–29 g/day in the third trimester (WHO/FAO/UNU Expert Consultation, 2007). High-quality protein sources are essential, considering socioeconomic conditions, dietary restrictions (e.g., vegetarian or vegan diets), and the potential need for supplementation.

Special attention must be paid to micronutrient intake, as several nutrient requirements increase significantly during pregnancy.

- **Folic acid** supplementation with 400 µg/day is recommended beginning in the preconception period to lower the risk of neural tube defects (L.M. De-Regil et al., 2015).
- **Iron.** Daily requirements increase from 18 to 27 mg to support expanded plasma volume and fetal needs. Despite increased intestinal absorption and the lack of menstrual losses, about 40% of pregnant women develop iron-deficiency anemia (J.P. Peña-Rosas et al., 2015).
- **Calcium.** Although the absolute need does not increase, intestinal absorption improves during pregnancy, making supplementation generally unnecessary for omnivorous individuals. However, restrictive diets or vitamin D deficiency can reduce calcium bioavailability.
- **Vitamin D.** Often deficient during pregnancy, particularly in winter or with limited sunlight. Supplementing is usually needed to maintain healthy levels for both mother and baby (B.W. Hollis et al., 2011).
- **Vitamin B12** is essential for women on vegan diets or with malabsorption disorders. A deficiency can impair fetal growth and neurological development.
- **Iodine.** Requirements increase by about 50%; supplementation with 100–200 µg/day is recommended to support fetal thyroid hormone production (M.B. Zimmermann, 2011).

1.1.2 Lactation nutrition

The postpartum period is equally crucial for both maternal and infant health. After childbirth, a mother's nutritional needs stay high to support breast milk production, which is the sole source of nutrition for the infant during the first months of life. Human milk is a dynamic biofluid, whose composition adjusts to the needs of the growing infant and is affected by the maternal diet—particularly concerning fatty acids, fat-soluble vitamins, and specific micronutrients such as iodine, vitamin B12, and selenium (F. Bravi et al., 2016; D.A. Gidrewicz et al., 2014).

Energy needs increase by about 500 kcal per day during the first six months of exclusive breastfeeding, and by roughly 400 kcal per day afterward if breastfeeding continues along with complementary feeding (P.R. Trumbo et al., 2020; Institute of Medicine, 2005). Protein requirements are also higher, needing an extra 15 g per day to support lean body mass and the production of milk proteins (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2017).

From a lipid perspective, docosahexaenoic acid (DHA) intake remains essential. The DHA level in breast milk heavily depends on maternal dietary intake and is vital for infant brain and visual development. A daily intake of at least 200–300 mg is recommended (B. Koletzko et al., 2008).

Several micronutrients require particular attention during lactation.

- **Iodine.** Excretion in breast milk significantly increases. WHO and national guidelines recommend an intake of 250 µg/day during lactation, typically achieved through supplementation (M.B. Zimmermann et al., 2020).
- **Vitamin D.** Levels in human milk are usually low; maternal or direct infant supplementation is often needed to maintain sufficient vitamin D levels (B.W. Hollis et al., 2019).
- **Vitamin B12.** Especially important in vegan or vegetarian mothers. Deficiency can lead to severe neurological impairment in the infant (L. Bärebring et al., 2022).

A balanced maternal diet during the postpartum period not only ensures proper nutrition for lactation but also aids maternal recovery, helps prevent postpartum depression, enhances sleep quality, and fosters positive mother–infant bonding (O. Orta et al., 2021).

1.1.3 Maternal low-protein diet

Proteins are vital macronutrients that play a crucial role in many physiological processes, including tissue growth, enzyme and hormone production, immune function, and nitrogen balance maintenance. During pregnancy, protein needs increase considerably to support maternal tissue growth, placental development, and fetal development (Institute of Medicine (US) Panel on Macronutrients, 2005).

Dietary proteins are made up of amino acids (AAs), nine of which are considered essential (EAAs) because they cannot be produced by the body and must be obtained through food. The quality of a protein source depends on its *biological value* (BV), which indicates its amino acid profile and how well it is digested. Proteins from animals—like eggs, dairy, meat, and fish—are classified as high biological value (HBV) because they contain all EAAs in proportions that closely match human needs. On the other hand, most plant-based proteins (such as legumes, grains, and nuts) are considered to have lower biological value, often lacking one or more EAAs—most commonly lysine or methionine (VR. Young, 1994).

People following vegetarian or vegan diets must carefully combine complementary plant proteins (like rice and beans) or depend on fortified foods and supplements to meet EAA needs. This is especially important during pregnancy, when not getting enough EAAs can hinder protein production and metabolic regulation in both mother and baby (GB. Piccoli et al., 2015).

Low-protein diets—whether caused by insufficient intake or dependence on low-quality protein sources—have been linked to negative pregnancy outcomes. Animal studies have shown that maternal protein restriction during pregnancy can lead to intrauterine growth restriction (IUGR), poor placental development, and long-term effects on metabolic and cardiovascular health in offspring, supporting the Developmental Origins of Health and Disease (DOHaD) hypothesis (SC. Langley-Evans et al., 2001; T. Van der Lende et al., 2006).

From a molecular perspective, inadequate protein intake during gestation has been linked to altered placental amino acid transport, reduced fetal cell proliferation, and epigenetic modifications that influence organ development and future disease risk (FJ. Rosario et al., 2011). Furthermore, insufficient intake of specific EAAs such as methionine and tryptophan may impair neurotransmitter synthesis and neurodevelopmental outcomes (G. Wu et al., 2004).

In humans, observational studies indicate that maternal diets low in protein are linked to lower birth weight, smaller head circumference, and higher risk of metabolic syndrome later in life (BS. Rubin et al., 2001; RR. Newbold et al., 2007). Vegan and vegetarian pregnant women, in particular, may face a risk of protein and EAA deficiencies if their diets are not well-planned and supported by nutritional counseling. According to the EFSA and the WHO, pregnant women should ensure an adequate protein intake of at least 1.1 g/kg/day, paying attention to both quantity and quality, especially during the second and third trimesters (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012).

Therefore, dietary protein adequacy during pregnancy is not solely a matter of caloric sufficiency, but of amino acid composition and bioavailability. Ensuring sufficient intake of HBV proteins—either through animal products or thoughtful plant-based combinations and supplementation—is essential to support fetal development and reduce the risk of long-term health consequences in the offspring (V. Melina et al., 2016).

In this context, the Academy of Nutrition and Dietetics and the United States Department of Agriculture recommend that vegetarian and vegan individuals include protein-rich legumes, especially soy-based foods and supplements, in their diets. Soy contains isoflavones, which are

phytoestrogens that not only enhance the amino acid quality of plant-based diets but also exhibit antioxidant and cardioprotective properties (M. Messina et al., 2010; G. Rizzo et al., 2018). Isoflavones such as genistein and daidzein, found abundantly in soy products like tofu, tempeh, and soy milk, are generally considered safe when consumed as part of a balanced diet. In supplement form, they are safe in non-pregnant adults up to 150 mg/day, though caution is advised in pregnancy due to their potential effects on thyroid function and hormone-sensitive pathways. Nevertheless, the intake of whole soy foods during pregnancy is widely regarded as safe and beneficial, particularly in vegan and vegetarian populations when integrated into a nutritionally adequate plan (EFSA Panel on Nutrition, 2015; I.M. Rietjens et al., 2013).

1.2 The phytoestrogens: natural endocrine-disrupting chemicals in the diet

1.2.1 Phytoestrogens

Over the past few decades, there has been growing scientific concern regarding Endocrine-Disrupting Chemicals (EDCs), substances capable of interfering with the endocrine system in humans and wildlife. EDCs can mimic, block, or alter the normal action of hormones, potentially leading to adverse effects on reproductive health, neurodevelopment, metabolism, and immune function (E. Diamanti-Kandarakis et al., 2009; E. Gore et al., 2015). While many EDCs are synthetic—such as pesticides, plasticizers, and flame retardants—there are also naturally occurring endocrine disruptors, with phytoestrogens being particularly notable. Phytoestrogens are plant-derived compounds with estrogen-like activity, commonly found in the human diet through foods like soy, legumes, whole grains, and seeds (H.B. Patisaul, W. Jefferson, 2010). Their ability to bind estrogen receptors has sparked both interest in their potential health benefits and concern about their disruptive effects, especially during critical developmental windows.

1.2.2 Genistein

Genistein is a naturally occurring isoflavone that belongs to the class of phytoestrogens—plant-derived compounds structurally similar to human estrogens. Its main dietary source is soy (*Glycine max*) and its most common derivatives, including tofu, tempeh, edamame, miso, soy-based beverages, and various processed plant-based products now widely used in vegetarian and vegan diets (KD. Setchell, A. Cassidy, 1999).

The rising popularity of plant-based diets—often unbalanced and influenced by unsupported nutritional trends on social media—has resulted in excessive and chronic genistein intake, especially when soy-based products serve as the primary protein source (AM Leung et al, 2020).

Another concern is that many soy-based foods, despite being marketed as "healthy" due to their plant origin, are highly processed. These “ultra-processed” foods are often enriched with additives, sugars, fats, and sodium, and can contain significant amounts of bioactive isoflavones, including genistein (CA. Monteiro et al., 2013). This raises questions about the true health value of these dietary choices when eaten regularly and without proper nutritional guidance.

On a molecular level, genistein closely resembles 17 β -estradiol, the most potent endogenous estrogen (Figure 1).

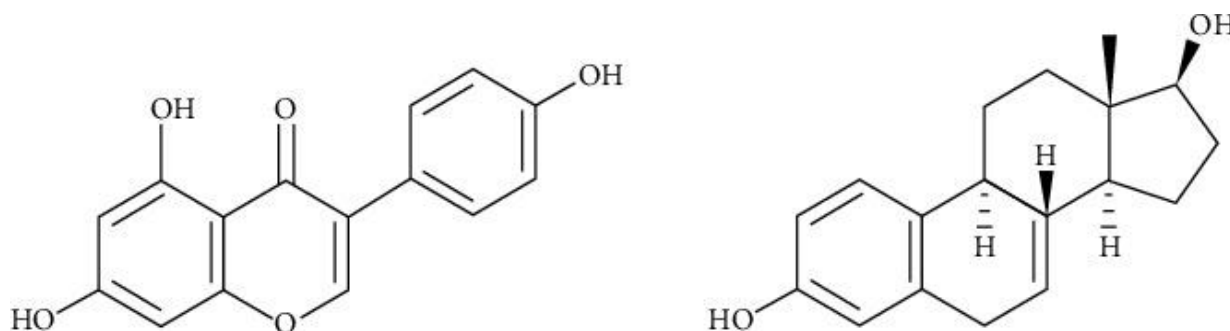


Figure 1: Genistein and 17 β -estradiol chemical structures.

(J. Sharifi-Rad J et al. 2021)

This structural similarity enables it to bind to estrogen receptors (ER α and, more prominently, ER β), thereby mimicking or modulating estrogenic activity (GGJM. Kuiper et al., 1998). Specifically, genistein functions as a Selective Estrogen Receptor Modulator (SERM), meaning it can act as either an agonist or antagonist depending on the target tissue and hormonal environment. This characteristic classifies genistein as a potential endocrine disruptor—capable of disturbing hormonal balance through both direct receptor interactions and indirect epigenetic or metabolic effects (E. Diamanti-Kandarakis et al., 2009; HB. Patisaul, W. Jefferson, 2010).

Although its estrogenic potency is much lower than that of estradiol, genistein's bioavailability and receptor affinity can still cause significant biological effects, especially with prolonged or high-dose exposure (WN. Jefferson et al., 2007). Several animal studies have shown the harmful effects of chronic or developmentally-timed exposure to genistein. In rodents, high genistein intake has been linked to disruptions in sexual maturation, changes in mammary and uterine tissues, and interference with the hypothalamic-pituitary-gonadal axis (RR. Newbold et al., 2009).

In recent years, there has been growing interest in soy-based proteins and their biologically active components, such as isoflavones. Among these, genistein (GEN) is a key phytoestrogen predominantly found in soy products in its glycosylated form, genistin (GIN). In the diet, genistein is mainly present as genistin, a precursor compound with minimal cytotoxic or cytostatic activity. However, GIN is enzymatically hydrolyzed by intestinal β -glycosidases and phenol sulfatases into its aglycone form, GEN, which has higher lipophilicity and biological activity (M.G. Busby et al., 2002; K.D.R. Setchell et al., 2002). GEN can be absorbed directly, affecting local tissues, or undergo further metabolism by gut microbiota (AA. Franke et al., 2014).

Experimental studies have shown that exposure to genistein mainly occurs through dietary intake of genistin (WN. Jefferson et al., 2006) and the extent of its bioactivity largely depends on individual gut flora and enzymatic conversion. Table 1 highlights genistin and genistein content in various soy-based foods (M. Fukutake et al., 1996):

FOOD SOURCE	GENISTIN (GIN) mg/g	GENISTEIN (GEN) mg/g
<i>Soybeans</i>	~0.602	~0.027
<i>Defatted soy flour</i>	~0.602	~0.027
<i>Soy milk, tofu</i>	0.095–0.138	0.002–0.019
<i>Fermented (miso, natto)</i>	0.071–0.493	0.039–0.229

Table 1. Genistin (GIN) and genistein (GEN) content in selected soy-based foods.

Values are expressed as mg/g of dry weight.

These values demonstrate that fermentation significantly enhances the conversion of GIN into GEN, thereby improving its bioavailability and potential physiological effects (Pingxu Qin et al., 2022).

1.2.3 Dietary Genistein: A Cross-Cultural Perspective

Genistein intake varies greatly across regions, reflecting different dietary customs and cultural influences. In Japan, for instance, soy consumption is deeply embedded in daily life, with traditional fermented soy products such as miso, natto, and soy sauce playing a central role in the diet. These foods not only provide significant amounts of genistin and genistein but also benefit from fermentation processes that enhance the bioavailability of isoflavones (M. Fukutake et al., 2016). The Japanese diet, characterized by frequent use of soy in soups, pickles, and side dishes, supports an average genistein intake ranging from 1.5 to 4.1 mg/day and genistin intake between 6.3 and 8.3 mg/day. This high level of consumption is linked to long-standing cultural

appreciation of soy as a healthful staple (Linus Pauling Institute, 2022), rooted in centuries of culinary tradition (Figure 2).

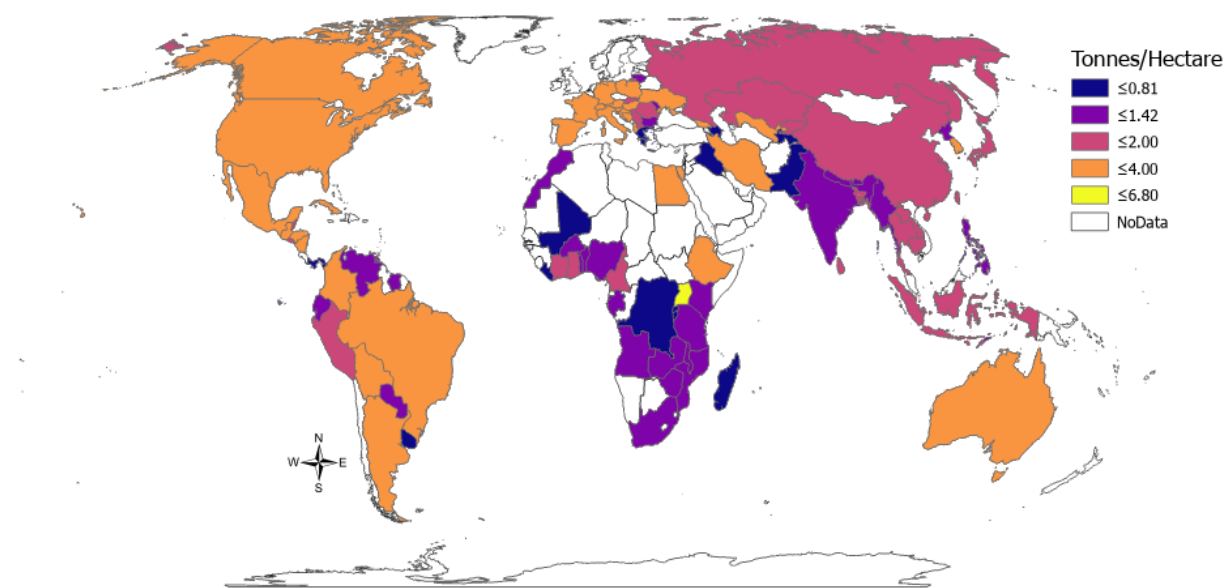


Figure 2: Global Soybean Yield Measured in Tonnes Per Hectare, 2022.

The map illustrates the global distribution of soy consumption intended for direct human intake (e.g., tofu, tempeh, soy milk), excluding use as animal feed.

(Food and Agriculture Organization of the United Nations, 2024)

Across a broader part of Asia, including China and Korea, soy remains a dietary staple, though consumption patterns vary slightly. In China, soy is eaten in both fermented forms like tofu and soy paste, and non-fermented forms like soy milk. This variety results in estimated daily isoflavone intakes ranging from 20 to 100 mg/day—much higher than averages in the West. These food traditions are closely linked to cultural values that emphasize plant-based diets and longevity (B.L Song et al., 2022).

In sharp contrast, Western countries such as the United States and Europe exhibit much lower soy and isoflavone intakes, reflecting different culinary histories and food preferences. In Europe, particularly in countries like Italy, Ireland, the Netherlands, and the UK, soy is typically consumed as a niche product, often associated with vegetarian or health-conscious groups. The VENUS study reports average daily isoflavone intakes below 1 mg/day in these countries, indicating limited integration of soy into traditional European diets (M.A.J. Van Erp-Baart et al. 2003).

Similarly, in the United States, soy consumption remains relatively modest at around 2.5 mg/day on average, with occasional higher intakes among consumers of soy-based health foods or supplements. This moderate intake aligns with a cultural dietary pattern heavily oriented toward animal protein and dairy, where soy often serves as a plant-based alternative rather than a staple (A. Kumar, B. Singh, 2019). The divergence in soy consumption is also visible in geographic consumption maps: East and Southeast Asian countries stand out with high per capita soy intake, whereas North America and Europe show lighter consumption zones (Sustainable Nutrition Initiative, 2023). These patterns reflect centuries of culinary evolution, agricultural production, and cultural attitudes toward soy foods, which in turn impact the nutritional exposure to genistein and its associated health effects.

1.2.4 Maternal diet rich in Genistein

During pregnancy, exposure to high levels of genistein may impact the fetal and placental microenvironment. Evidence shows that genistein can impair placental vascularization, alter gene expression in estrogen-sensitive tissues, and interfere with fetal metabolic programming—key concepts within the Developmental Origins of Health and Disease (DOHaD) framework (CR. Cederroth et al., 2012; J. Bouilly et al., 2018). At the cellular level, genistein can regulate important signaling pathways such as PI3K/Akt and MAPK, and affect epigenetic regulation through DNA methylation changes and microRNA expression (DC. Dolinoy et al., 2006; SL. Tilghman et al., 2010).

Experimental data suggest that prenatal exposure to genistein may negatively affect the development of the male reproductive system, thyroid function, neurogenesis, and neuronal differentiation (AB. Wisniewski et al., 2003). Additionally, estrogenic interference during critical developmental periods has been linked to increased risks of early puberty, ovarian dysfunction, and behavioral changes later in life (BS. Rubin et al., 2001; RR. Newbold et al., 2007).

Although human clinical studies are limited and sometimes inconclusive, regulatory agencies such as EFSA recommend caution with phytoestrogen intake during pregnancy, especially from concentrated sources or dietary supplements. It is important to recognize that genistein's effects may not follow a straightforward dose–response relationship: even low doses, if consumed during critical periods of fetal development, can lead to long-lasting physiological changes (HB. Patisaul, W. Jefferson, 2010; DC Dolinoy et al., 2006).

1.3 Function of the Serotonergic and POMC Systems in the Brain

1.3.1 Central Regulation of Feeding Behavior. Focus on the POMC System

The regulation of food intake is a complex and finely orchestrated process that operates across three functional levels: afferent, central, and efferent (Figure 3).

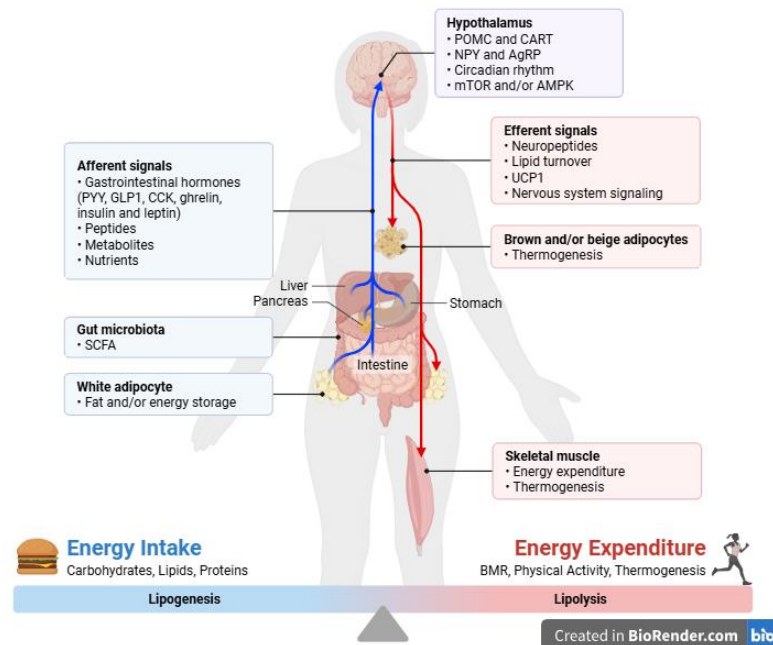


Figure 3: Regulation of energy balance.

Interaction between afferent signals (gastrointestinal hormones, metabolites, nutrients), hypothalamic activity, and efferent signals affecting peripheral tissues (adipocytes, skeletal muscle) to balance energy intake and expenditure. The system integrates inputs from gut microbiota, adipose tissue, and muscle, coordinating lipogenesis and lipolysis. (R.San-Cristobal et al., 2020)

At the **afferent level**, signals reflecting the body's nutritional and metabolic status are detected by peripheral receptors and transmitted to the central nervous system via both systemic circulation and vagal afferents. These signals include hormones such as leptin (secreted by white adipose tissue), insulin (from the pancreas), and ghrelin (from the stomach), as well as metabolites produced by the gut microbiota, particularly short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate.

At the **central level**, this information primarily converges in the hypothalamus, specifically in the arcuate nucleus (ARC), which contains two main populations of first-order neurons:

- POMC/CART neurons, which exert anorexigenic (appetite-suppressing) effects;
- NPY/AgRP neurons, which promote orexigenic (appetite-stimulating) signaling (D. Feifel, F.J. Vaccarino, 1989; M. Schwartz et al., 2000).

Both of these neuronal populations receive input from peripheral signals and project to second-order neurons in other hypothalamic nuclei such as the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), and the dorsomedial nucleus (DMH). Pro-opiomelanocortin (POMC) neurons, when activated, release alpha-melanocyte-stimulating hormone (α -MSH), a peptide that acts on melanocortin receptors, particularly MC4R, promoting satiety and increasing energy expenditure (R. Cone et al., 2005). In contrast, NPY/AgRP neurons release neuropeptide Y (NPY) and agouti-related peptide (AgRP), which antagonize melanocortin signaling and stimulate food intake (Y. Aponte et al., 2011), thereby creating a functional balance that finely regulates caloric intake (Figure 4).

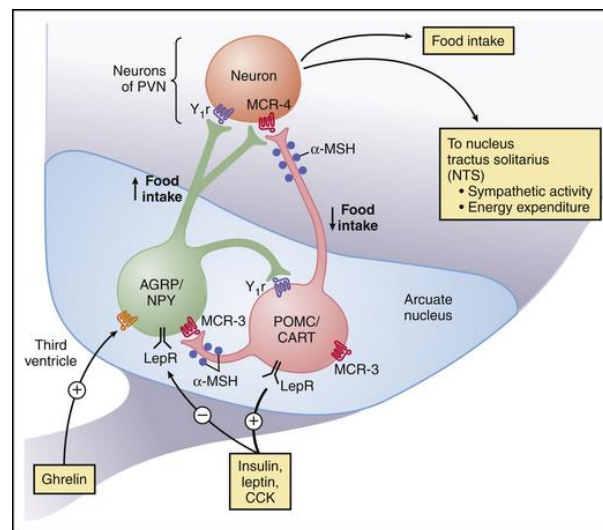


Figure 4: Schematic representation of hypothalamic pathways involved in the regulation of food intake and energy balance.

Peripheral signals such as insulin, leptin, CCK, and ghrelin influence neurons in the arcuate nucleus (ARC), particularly POMC/CART and AgRP/NPY neurons. POMC neurons release α -MSH, which activates melanocortin receptors (MC3R and MC4R) and promotes satiety. Conversely, AgRP/NPY neurons stimulate feeding behavior by antagonizing melanocortin signaling. These neurons project to key hypothalamic areas, including the paraventricular nucleus (PVN), contributing to the integration of metabolic signals and the regulation of energy homeostasis. (Redrawn from Barsh GS, Schwartz MW: *Nat Rev Genet* 3:589, 2002).

Insulin, beyond its well-known role in glucose metabolism, acts centrally as a satiety signal and can influence cognitive functions such as memory and learning (C. Benedict et al., 2004). Ghrelin stimulates appetite, promotes growth hormone secretion, influences motivational behavior, and can modulate mood (J. Van der Lely et al., 2004).

Moreover, short-chain fatty acids (SCFAs) produced by the gut microbiota—such as acetate and butyrate—exert systemic effects, regulating not only appetite but also inflammation, intestinal barrier integrity, and insulin sensitivity (E. Canfora et al., 2015).

In addition to their central role in the control of food intake, POMC neurons participate in several neuroendocrine and behavioral processes. POMC neurons, for instance, are not exclusively involved in anorexigenic signaling: they also contribute to the regulation of the hypothalamic–pituitary–adrenal (HPA) axis and the stress response, by modulating corticotropin (ACTH) release, another peptide derived from the POMC precursor protein (M.E. Keller-Wood, M. E. Dallman, 1984). Furthermore, recent evidence suggests a role for POMC neurons in endogenous analgesia, through the production of β -endorphin, another POMC-derived opioid peptide (H. Akil et al., 1984).

1.3.2 Serotonin system

Serotonin (5-HT) is one of the most versatile neurotransmitters in the central nervous system, involved in regulating mood, thermoregulation, sleep-wake cycles, pain perception, social behavior, and appetite modulation. Alterations in serotonergic transmission are strongly implicated in the pathophysiology of psychiatric disorders, including depression, anxiety, schizophrenia, and obsessive-compulsive disorder (M. Berger, J.A. Gray, B.L. Roth, 2009).

Serotonergic neurons originate mainly from the Raphe nuclei and project broadly to brain regions such as the hypothalamus, amygdala, prefrontal cortex, and limbic system (Figure 5). Serotonin regulates mood via its influence on limbic circuits, thereby modulating anxiety, depression, and social behaviors (M. Berger, J.A. Gray, B.L. Roth, 2009)

Finally, serotonin also modulates the nociceptive system, acting through specific receptors in the brain and spinal regions involved in pain transmission, thereby altering both pain perception and modulation (M.J.Millan, 2002).

Through its receptors, serotonin influences not only appetite but also stress regulation, mood, and pain perception, demonstrating the complex interconnectivity of these systems.

The 5-HT_{2C} receptor, expressed in several brain regions beyond the hypothalamus (including the amygdala and prefrontal cortex), modulates neuronal activity by influencing the release of other neurotransmitters such as dopamine and norepinephrine. This modulation impacts reward and motivation circuits, which are particularly important in the control of mood, emotional responses, impulsivity, and anxiety (L.K. Heisler, 2006).

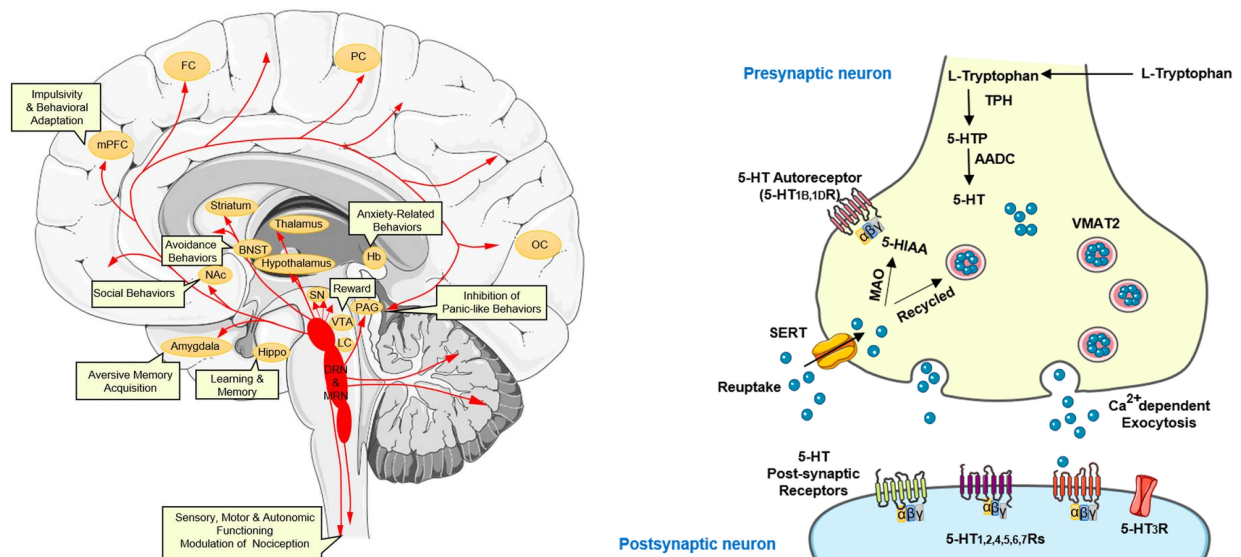


Figure 5: Interaction between the arcuate nucleus and serotonergic pathways in the regulation of energy balance. The arcuate nucleus (highlighted in red) integrates peripheral metabolic signals and communicates with other brain regions, including the raphe nuclei (in blue), through specific neural pathways (purple lines). These pathways are involved in the serotonergic modulation of appetite and energy expenditure. The inset diagram illustrates synaptic mechanisms, including serotonin (5-HT) release and receptor activity, that influence hypothalamic neurons such as POMC, affecting satiety and metabolic responses. (Pourhamzeh et al., 2021)

1.3.3 Serotonin System as a Modulator of Food Intake

Serotonin is a vital regulator of feeding-related hypothalamic circuits. Serotonergic neurons project widely to the hypothalamus, where they activate 5-HT_{2C} receptors located on POMC neurons (L.K. Heisler et al, 2002; Y. Xu et al., 2008). This activation stimulates the release of alpha-MSH, leading to the anorexigenic signaling cascade via MC4R receptors. Additionally, serotonin indirectly inhibits NPY/AgRP neurons, thereby enhancing the anorexigenic effect (AS. Garfield, LK. Heisler LK, 2009

In this context, the use of 5-HT, 5-HT receptors (especially 5-HT_{2C}), and POMC as molecular markers in the brain allows for monitoring the functional impact of different dietary interventions, providing an objective index of their influence on the central appetite regulation system. It is important to emphasize that many of the signals involved in appetite regulation exhibit pleiotropic effects, influencing numerous physiological processes beyond hunger and satiety control. For example, leptin not only inhibits food intake but also modulates immune function, fertility, and bone density (R.S. Ahima, J.S. Flier, 2000).

Recent studies have demonstrated that low-protein diets or diets enriched with phytoestrogens can profoundly alter the expression of genes involved in appetite regulation and central energy

metabolism (J. Moro et al., 2021). For instance, low-protein diets have been shown to reduce the activity of pro-opiomelanocortin (POMC) neurons and negatively modulate serotonergic receptors, resulting in increased caloric intake and decreased energy expenditure (A.K Gosby et al., 2011; CD. Morrison et al., 2015). Similarly, chronic exposure to phytoestrogens—bioactive plant-derived compounds with estrogenic activity, such as genistein and daidzein—has been associated with both epigenetic and transcriptional modifications in the hypothalamus. These changes can influence the expression of POMC and serotonergic receptors, potentially leading to anorexigenic or metabolic effects that are dose- and duration-dependent (HL. Bateman, HB Patisaul, 2008; E.D. Lephart, 2015).

2. AIM OF WORK

Adequate maternal nutrition during pregnancy and lactation is crucial for the proper development of the fetus and newborn, with long-lasting impacts on the child's health throughout their life. Specifically, the quality and composition of the maternal diet can significantly impact the development of neuroendocrine systems responsible for regulating appetite, maintaining energy balance, and influencing emotional behavior.

The aim of this study was to investigate the long-term effects of maternal dietary interventions on the development of key neuroendocrine pathways involved in energy balance and mood regulation in offspring. Using triple-label immunofluorescence and quantitative image analysis, we examined whether maternal exposure to a low-protein diet and/or dietary supplementation with phytoestrogens (genistein) during critical developmental periods (gestation and lactation) could cause lasting changes in neurochemical markers in both developing and adult offspring, even after switching to a standard diet following weaning, and in the absence of ongoing dietary exposure.

The study focused on tracking the development of offspring's feeding behavior into adulthood, analyzing anxiety-like behavior through two different apparatuses (Open Field and Elevated Plus Maze) to understand how these behaviors relate to the brain by measuring pro-opiomelanocortin (POMC), serotonin (5-HT), and the serotonin receptor subtype 5-HT_{2C} (5-HT_{2CR}) in the arcuate nucleus of the hypothalamus (ARC), as well as 5-HT-positive neurons in the dorsal raphe nucleus (DRN). These two brain regions are essential for regulating appetite and emotional behavior, respectively. Including them as analysis targets allowed for a combined assessment of potential changes in both the production and release of the mediators studied.

By comparing these conditions, we aimed to gain a deeper understanding of how early-life diet influences the development of neural circuits and contributes to a higher risk of metabolic and mood disorders later in life.

3. MATERIALS and METHODS

This study is part of a broader, multidisciplinary research project that examines how altered maternal diets during

pregnancy and breastfeeding affect future generations. Specifically, it investigates how targeted nutritional interventions may cause metabolic, behavioral, and neurological changes in the offspring of exposed mothers, even when these offspring are fed a standard diet after birth.

The experimental work conducted as part of this thesis focused on assessing the presence and quantifying specific receptors involved in regulating feeding behavior and other neurofunctional circuits in the offspring of mothers subjected to experimental diets. My specific contribution centered on analyzing brain tissues from PND90 animals, representing the adult stage after postnatal exposure to a standard diet.

3.1 Animals and timeline

All experimental procedures involving animals were conducted in compliance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes and with national legislation (Italian Legislative Decree No. 182/2010-A, issued on 03/11/2010). The Italian Ministry of Health approved the study under authorization number 267/2022-PR (protocol E669C.73, dated 22/04/2022).

In this study, female Sprague-Dawley rats (initial body weight $\sim$ 250 g) were used. The animals were obtained from Envigo Srl and housed in polycarbonate cages (45 × 27 × 20 cm) under standard laboratory conditions: controlled temperature ($22 \pm 1^\circ\text{C}$), a 12-hour light/dark cycle, and ad libitum access to food (Mucedola Srl, Settimo Milanese, Italy) and water. Upon arrival, the rats were randomly divided into four experimental groups and assigned specific diets:

- Control group (CON): standard chow
- Genistein group (GEN): standard chow supplemented with Genistin (GEN, genistein glucoside, 60mg/kg)
- Low Protein group (LOW): protein-restricted diet with 8% of calories from proteins.
- Combined group (LOW+GEN): low-protein diet supplemented with genistein

Body weight and food intake were measured weekly during the dietary treatment period. After six weeks of dietary exposure, mating began and lasted for one week (Figure 6).

Parturition occurred approximately three weeks later. At birth (Postnatal Day 0, PND0), offspring were weighed and sexed. Standard diet and water were provided freely throughout the postnatal period. Body weight and food intake were monitored weekly until the animals were sacrificed.

On PND14, maternal milk samples were collected for biochemical analysis by Dr. Sonia Tassone at DISAFA, University of Turin.

The pups underwent behavioral testing before sacrifice. Specifically, anxiety- and depression-like behaviors were assessed using two well-established assays: the Open Field (OF) test and the Elevated Plus Maze (EPM).

Offspring were sacrificed at two key developmental stages: PND1 (neonatal stage) and PND90 (adulthood). Animals were euthanized via intracardiac perfusion, and brains were collected and processed for immunohistochemical analysis to evaluate the presence and distribution of specific neurobiological markers. (R.D. Cone, 2005; C. Blouet, G.J. Schwartz, 2010).

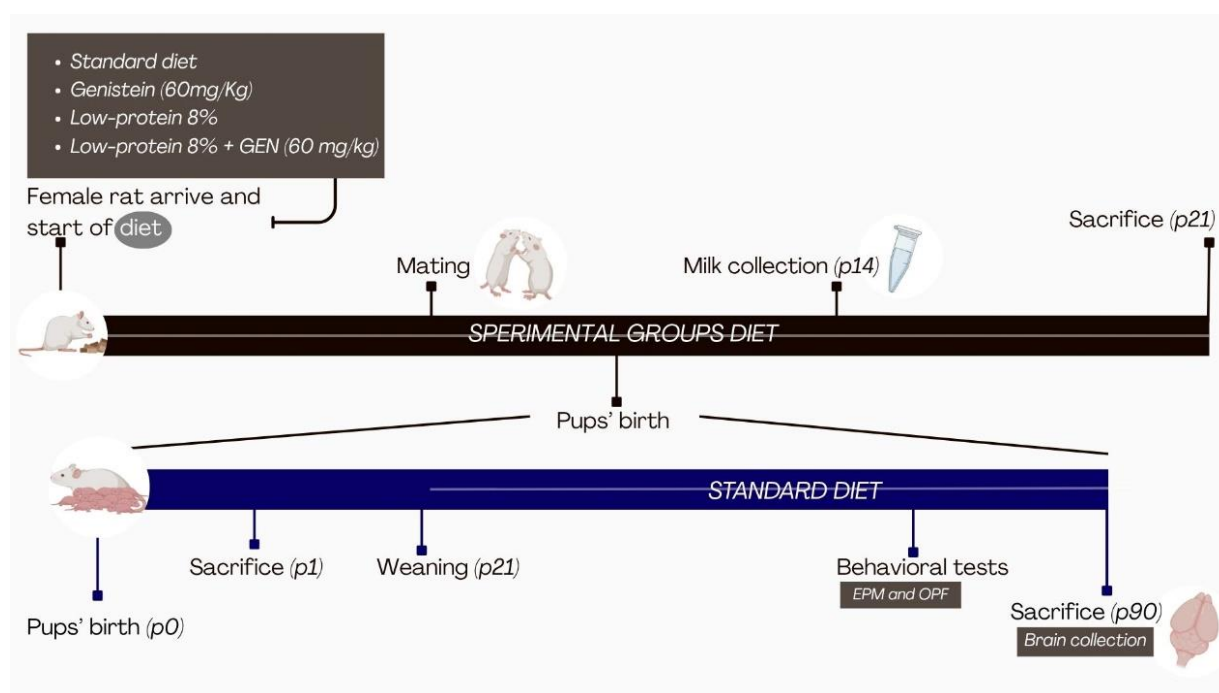


Figure 6: Schematic representation of the experimental design.

The upper part of the timeline illustrates the maternal protocol, including arrival and dietary intervention, mating, birth of the offspring (PND0), milk collection (PND14) and maternal sacrifice.

The lower section outlines the offspring timeline, from birth through early postnatal sacrifice (PND1), weaning (PND21), behavioral tests, and final sacrifice in adulthood (PND90).

3.2 Experimental Diets

Female rats were randomly assigned to four experimental groups based on the type of diet administered (Table 2). The dietary interventions were designed to mimic different nutritional states and assess their respective physiological and metabolic effects.

The groups and the diets were organized as follows:

- **Control Group (CON):** received a standard, nutritionally complete diet (4RF21 *Mucedola s.r.l*) in accordance with established guidelines for laboratory rodents, ensuring adequate intake of macronutrients and micronutrients (National Research Council (US) Subcommittee on Laboratory Animal Nutrition, 1999)
- **Genistein Group (GEN):** received a standard diet (4RF21, *Mucedola s.r.l*) supplemented with Genistin (60 mg/kg of genistein glucosidate, genistein 7-O-glucoside), an isoflavone classified as a phytoestrogen. Genistein is predominantly found in soy-based foods such as tofu, tempeh, and soy milk, and is a notable component of vegetarian and vegan diets. It exhibits antioxidant properties and binds to estrogen receptors with much lower affinity compared to endogenous estrogens, potentially modulating hormonal activity (H.B. Patisaul, H. B., W. Jefferson, W, 2010).
- **Low-Protein Group (LOW):** received a low-protein diet (NFM8, *Mucedola s.r.l*) characterized by a markedly reduced protein content (<8% of total energy intake, compared to ~20% in standard rodent chow). This diet limits the availability of essential amino acids, which may influence protein metabolism, cellular biosynthesis, and nitrogen balance (Y. Fukuda, Y. Taketani, 2021).
- **Low-Protein + Genistein Group (LOWGEN):** received the same low-protein diet (NFM8, *Mucedola s.r.l*), supplemented with genistein (60 mg/kg of genistein glucosidate, genistein 7-O-glucoside). This group was included to assess potential synergistic or interactive effects of protein restriction combined with phytoestrogen exposure on metabolic regulation and endocrine homeostasis.

COMPONENT	U.M.	CON	GEN	LOW	LOWGEN
<i>Crude Protein</i>	%	18.50	18.50	8	8
<i>Lysine</i>	%	0.97	0.97	0.38	0.38
<i>Methionine</i>	%	0.47	0.47	0.18	0.18
<i>Tryptophan</i>	%	0.28	0.28	0.10	0.10
<i>Histidine</i>	%	0.50	0.50	0.19	0.19
<i>Threonine</i>	%	0.72	0.72	0.28	0.28
<i>Isoleucine</i>	%	0.84	0.84	0.32	0.32
<i>Leucine</i>	%	1.57	1.57	0.72	0.72
<i>Phenylalanine</i>	%	0.89	0.89	0.39	0.39
<i>Valine</i>	%	0.96	0.96	0.38	0.38
<i>Crude Fat</i>	%	3.00	3.00	6.35	6.35
<i>Crude Fiber</i>	%	6.00	6.00	6.1	6.1
<i>Ash</i>	%	7.00	7.00	6.73	6.73
<i>Nitrogen-Free Extract-NFE</i>	%	53.5	53.5	60.82	60.82
<i>Moisture</i>	%	12.00	12.00	12.00	12.00
<i>Genistein</i>	mg/kg	0	60	0	60
<i>Metabolisable Energy</i>	kCal/kg	2667	≈ 2667	3320	≈ 3320
<i>Gross Energy</i>	kCal/kg	3876	≈ 3876	3989	≈ 3989

Table 2: Composition of the diets administered to the four experimental groups.

The table highlights the main differences in macronutrient content and genistein supplementation among the group: genistein (GEN), low-protein diet (LOW), low-protein diet supplemented with genistein (LOWGEN), and control group (CON).

3.2.1 Food intake and weight

Body weight was measured weekly using a precision electronic balance (Model: Kern-440-47N; resolution: 0.1 g) from the start of the dietary intervention until the day of sacrifice.

Food intake was measured weekly by subtracting the leftover food in each cage from the amount provided. The total food consumed per cage was then divided by the number of animals in that cage to estimate the average intake per animal. To find the average daily intake per animal, this weekly total was further divided by the number of days between measurements (Table 3).

PARAMETER CALCULATED	FORMULAS
<i>Food eaten</i>	Tot amount – residual food
<i>Food consumption per animal</i>	Food eaten / animals' number in the cage
<i>Food consumption daily (per animal)</i>	Food consumption per animal / N of days

Table 3: Animal Feeding Parameters

The table presents the calculated parameters of food intake per animal. It includes the formulas used to determine total food consumed, food consumption per animal, and daily consumption per animal.

Following weaning, all offspring were subjected to the same weekly monitoring protocol for both body weight and standard food intake.

3.3 Mating Protocol

After approximately six weeks of dietary treatment, the mating phase began. Male rats were introduced into the cages during the dark phase of the light/dark cycle, which is when rodents are most active. Animals were kept on an inverted 12:12 hour light/dark cycle to align experimental procedures with their nocturnal activity patterns.

Each male was placed in a cage with two females from the same experimental group. Successful mating was confirmed by the presence of a vaginal plug, a transient coagulum that forms after copulation and serves as a reliable physiological marker of mating. The detection of the plug is commonly used to determine embryonic day 0.5 (E 0.5) in developmental studies (W. Hamilton, H.G. Wolfe, 1938; S.L. Byers et al., 2012). Once the vaginal plug was observed, males were immediately separated and housed individually to prevent further interaction or mating attempts.

3.4 Maternal Milk Collection

Milk samples were collected from lactating mothers on postnatal day 14 (PND14), which corresponds to the mid-lactation stage. This phase is marked by peak milk production and consistent nutritional content, vital for the optimal growth and neurological development of pups.

This time point was chosen to ensure representativeness of milk constituents during a physiologically critical phase of lactation.

The collection protocol followed established methodologies for rodents. Mothers were initially separated from their litters and gently restrained. To induce a robust and consistent milk ejection response, they received an intraperitoneal injection of oxytocin (300 μ L), which triggers the contraction of myoepithelial cells in the mammary gland (E.J. DePeters, R.C. Hovey, 2009). Milk was manually expressed through gentle and rhythmic massage of the mammary glands, using capillary tubes or manual collection techniques to obtain sufficient volumes for subsequent analysis (HA Paul, MC Hallam, RA Reimer, 2016). Collected samples were immediately placed on ice and then stored at -80 °C until analysis. Samples were subsequently sent to Dr. Sonia Tassone at DISAFA, University of Turin, for detailed profiling of macronutrients, hormones, and bioactive factors.

3.4.1 Maternal Milk Analysis

Milk composition was assessed for crude protein and crude fat.

Crude protein was calculated from the nitrogen content ($N \times 6.38$), which was determined using the same dried samples via elemental analysis.

Crude fat content was measured using a miniaturized version of the Röse-Gottlieb method applied to 100 μL of milk.

All measurements were performed in duplicate or triplicate. The miniaturized analytical methods applied in this protocol were specifically optimized for small sample volumes and have been validated for accuracy and reproducibility, yielding results comparable to standard large-volume techniques (S. Görs et al., 2009).

3.5 Birth and development of puppies

Approximately 300 pups were included in the study. From birth (postnatal day 0, PND0), pups were weighed, sexed, and assigned to their respective experimental groups. At this stage, several parameters were recorded to evaluate the birth rate and initial body weight in both the experimental group and the female sex. Specifically for each experimental group (Table 4):

PARAMETERS RECORDED
<i>Total number of pups per litter</i>
<i>Total number of male pups per litter</i>
<i>Percentage of male pups at PND0</i>
<i>Total number of female pups per litter</i>
<i>Percentage of female pups at PND0</i>
<i>Percentage of dams that lost the entire litter</i>
<i>Percentage of dead pups at PND0</i>
<i>Body weight of male pups at PND0</i>
<i>Body weight of female pups at PND0</i>

Table 4: Reproductive and neonatal parameters recorded at PND0.

At weaning (PND21), animals were housed in groups of three per cage, separated by sex and experimental condition. Food and water were provided *ad libitum*, with food intake standardized to 150 g per cage based on average consumption. Between PND75 and PND85, behavioral assessments were conducted to evaluate anxiety-like behaviors. Specifically, animals underwent two validated behavioral paradigms: the Open Field (OF) test and the Elevated Plus Maze (EPM).

Body weight and food intake were monitored weekly until the animals were sacrificed. Specifically for each experimental group (Table 5):

PARAMETERS RECORDED WEEKLY
<i>Body weight of male pups</i>
<i>Body weight of female pups</i>
<i>Food eaten - male pups</i>
<i>Food eaten - female pups</i>

Table 5: Weekly recorded parameters for male and female pups.

At PND90, animals were deeply anesthetized and sacrificed via transcardial perfusion. Brains were subsequently collected for immunohistochemical analysis.

3.6 Behavioral tests

Before the sacrifice, all pups in the experimental groups underwent behavioral testing to evaluate anxiety- and depression-like behaviors. Before each testing session, vaginal smears were performed to verify that the subjects were in the proestrus phase of the estrous cycle.

Two behavioral paradigms were employed: the Open Field (OF) test and the Elevated Plus Maze (EPM). Each rat's behavior was recorded using a camera mounted above the apparatus, while the experimenter remained outside the testing room to minimize external interference. The recorded videos were later analyzed offline using EthoVision XT 8 software (LP. Noldus, AJ Spink, RA Tegelenbosch, 2001) by a trained researcher. Although each test lasted 10 minutes, behavioral data analyses focused on the first 5 minutes, as prior studies have demonstrated that rodents exhibit the most pronounced avoidance responses within the initial minutes of exposure to a novel environment (AA Walf, CA Frye, 2007). Additionally, the apparatus was thoroughly cleaned before and after each trial to remove feces and olfactory cues left by previous subjects.

3.6.1 Open Field

The Open Field (OF) test is commonly used to assess anxiety-like behavior in rodents. Important metrics include the total distance traveled (in cm) and the percentage of time spent in the central versus peripheral zones of the arena during the test session (ML. Seibenhener, MC. Wooten, 2015). The apparatus consisted of a square arena measuring 80 × 80 cm, with a designated central zone of 40 × 40 cm, surrounded by a peripheral zone and enclosed by opaque walls approximately 40 cm high (Figure 7). Each animal was gently placed in the same corner of the arena by the tail and allowed to freely explore for 10 minutes.

Rodents naturally exhibit thigmotaxis, a tendency to stay close to the walls or edges, so increased time spent in the central area or quicker entry into it is usually seen as a sign of reduced anxiety (L.Prut, C. Belzung, 2003). Behavioral measures assessed included handling frequency, grooming, time spent in the center, time in the periphery, and rearing. Entry into the central area was defined as placing all four paws inside the central zone (Y.Shu-Chuan, S. Kun-Ruey, 2024). Although grooming is normal in rodents, excessive or localized grooming may suggest higher stress levels (D. Vuralli et al., 2019). Rearing (standing on the hind legs) was recorded as an indicator of exploratory behavior and is therefore inversely related to anxiety levels (ML Seibenhener, MC , Wooten, 2015).

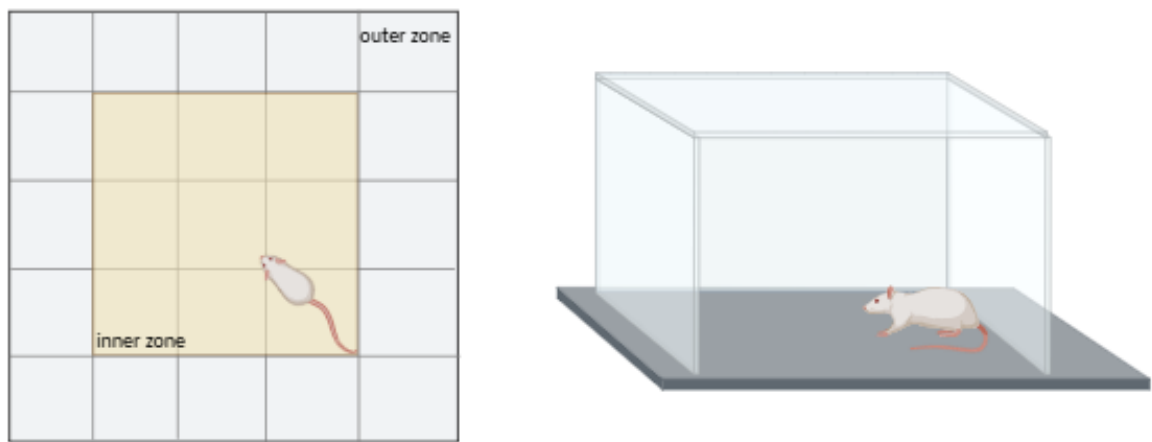


Figure 7: Representation of the Open Field (OF) test apparatus and square arena

3.6.2 Elevated plus maze

The Elevated Plus Maze (EPM) is a widely validated test for assessing anxiety-related behavior in rodents (M. Komada,K. Takao, T. Miyakawa, 2008). Its popularity stems from its simplicity, rapid execution, and cost-effectiveness (M. Komada,K. Takao, T. Miyakawa, 2008). The design is based on Montgomery's early findings that rodents spend less time in the exposed, open arms of a maze than in the enclosed arms, reflecting their aversion to open spaces (S. Pellow et al., 1985).

The EPM apparatus consisted of a plus-shaped maze elevated 50 cm above the floor, with two open arms measuring 50×10 cm and two closed arms of the same size (50×10 cm) but with opaque walls 40 cm high. The arms extended perpendicularly from a central square platform measuring 10×10 cm (Figure 8). Once placed in the maze, the animal was allowed to explore freely. Behavioral measures included total distance traveled, the number of entries into open and closed arms, time spent in each arm type, and latency to first entry into open or closed arms (M. Komada,K. Takao, T. Miyakawa, 2008). Rodents usually avoid open arms because they are more

vulnerable to predators in exposed areas. Therefore, less exploration of the open arms (fewer entries, shorter time spent, and longer latency) is seen as a sign of increased anxiety-like behavior. On the other hand, total distance traveled and entries into closed arms indicate overall locomotor activity.

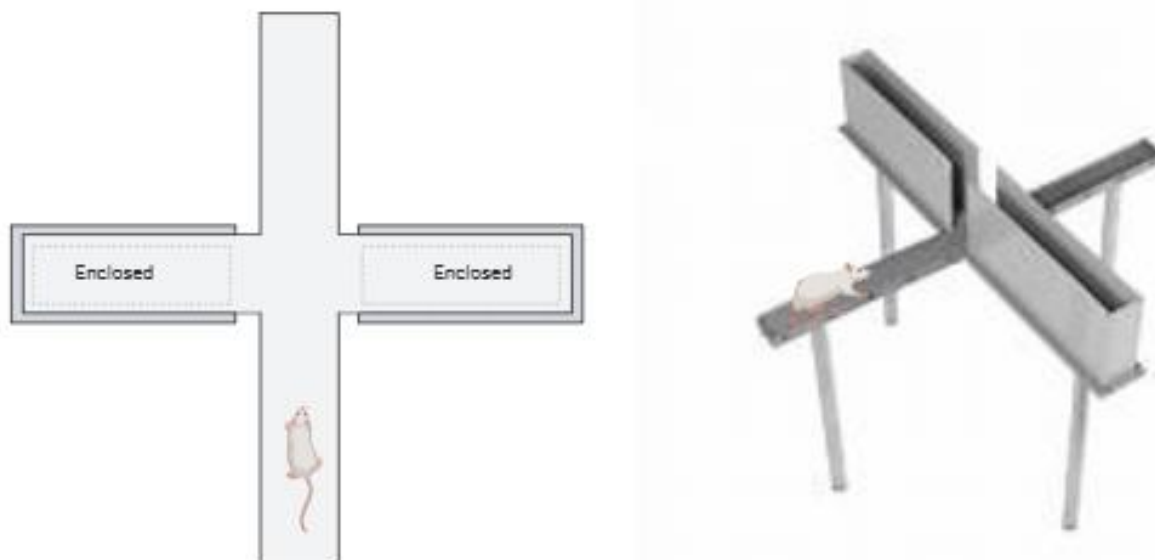


Figure 8: Representation of the Elevated plus maze apparatus and square arena

3.7 Sacrifices

At postnatal day 90 (PND90), animals underwent transcardial perfusion to enable tissue fixation for subsequent histological and immunohistochemical analyses. Female animals were perfused specifically during the proestrus phase, as determined by vaginal smear cytology.

Anesthesia was induced by exposing the subject to high levels of carbon dioxide. The depth of anesthesia was confirmed by monitoring respiratory rate, absence of spontaneous movement, reduced muscle tone, and loss of hind limb withdrawal reflex when gentle pressure was applied to the plantar surface. Once adequate anesthesia was confirmed, a rapid thoracotomy was performed to access the heart. The right atrium was incised to allow blood drainage, and a needle connected to a peristaltic pump was inserted into the left ventricle to start transcardial perfusion. Animals were first perfused with 0.9% saline solution (NaCl) to clear the vasculature, followed by a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3).

Brains were then carefully extracted and post-fixed in 4% paraformaldehyde for 24 hours. After washing in phosphate-buffered saline (PBS, 0.01 M), the brains were immersed in 30% sucrose at 4 °C overnight until they sank. The following day, tissues were snap-frozen in isopentane pre-cooled on dry ice (−30°C to −40°C) and stored at −80°C for long-term preservation. This

procedure ensures rapid and uniform freezing, minimizing the formation of ice crystals that could compromise tissue morphology. (M. Marraudino et al., 2021).

3.8 Cryostat Sectioning

Twenty-four hours before cryosectioning, brains were equilibrated at -20°C to allow gradual thermal stabilization. Each brain was then embedded in an optimal cutting temperature (OCT) compound (Killik®) to ensure uniform coronal sectioning (H. Yang et al., 2021). Coronal brain sections were cut at a thickness of $40\text{ }\mu\text{m}$ using a cryostat (Leica CM1900). Three serial sections (labeled A, B, and C) were collected in parallel.

Initially, the sections were immersed in phosphate-buffered saline (PBS) and then transferred to 12-well plates. In each well, PBS was replaced with a cryoprotective antifreeze solution made of 30% glycerol, 30% ethylene glycol, 30% deionized water, 10% sodium dihydrogen phosphate (0.234 M), and 1.75% sodium hydroxide (x). Plates were stored at -20°C until immunohistochemical processing.

This collection method ensures that sections within the same series are spaced $120\text{ }\mu\text{m}$ apart, which is ideal for consistent anatomical comparisons across series.

The anatomical range of the collected sections extended from the front part of the corpus callosum to the cerebellum. On average, four 12-well plates were needed to collect the entire set of serial sections from each adult rat brain.

3.9 Fluorescence Immunohistochemistry

Immunofluorescence is a highly sensitive immunohistochemical technique that uses fluorophore-conjugated antibodies to detect specific antigens within biological tissues. In indirect immunofluorescence, a primary antibody binds first to the target antigen, followed by a secondary antibody labeled with a fluorophore that recognizes the primary antibody. This two-step method improves both signal specificity and amplification (J.A. Ramos-Vara 2005). This approach is particularly valuable in neuroscience research, as it allows for the precise localization and semi-quantitative analysis of neurotransmitters, neuropeptides, and their corresponding receptors within defined anatomical regions (AN. Van den Pol, 1997; K.D. Micheva, S.J. Smith, 2007).

In the present study, we employed triple-label immunofluorescence to quantify pro-opiomelanocortin (POMC), serotonin (5-HT), and the serotonin receptor subtype 5-HT_{2C}

(5-HT₂CR) in brain sections of rat offspring exposed to maternal dietary interventions. Our primary focus was on quantifying POMC-, 5-HT-, and 5-HT₂CR positive cells within the arcuate nucleus (ARC), as well as 5-HT positive neurons in the dorsal raphe nucleus (DRN).

Anatomical localization was performed using the *Rat Brain in Stereotaxic Coordinates* atlas by Paxinos and Watson (G.Paxinos, C. Watson, 2013). Coronal sections were selected at the following Bregma levels for analysis (Table 6).

ANATOMICAL LOCALIZATION	LEVEL
<i>Arcuate nucleus</i>	bregma -2,56 mm
<i>Raphe nucleus</i>	bregma -7,04 mm

Table 6: Anatomical localization of brain regions and corresponding bregma levels.

3.9.1 Immunofluorescence Protocol

All steps were performed on free-floating sections in 350 µL volumes per well, with gentle agitation. Below is a schematic of the protocol used, divided into two parts.

FIRST PART:

1. Three washes in PBS containing sodium azide (PBS-NaN₃) for 5 minutes each to remove residual antifreeze and prevent microbial growth (Hoffman et al., 2008).
2. Heat-induced antigen retrieval in citrate buffer at 90°C for 20 minutes to unmask epitopes (Shi et al., 1991).
3. Three washes in PBS with 0.5% Triton X-100 (PBS-TX) to permeabilize cellular membranes.
4. Blocking in PBS-TX with 1% BSA and 1% normal donkey serum (NDS) for 1 hour to reduce non-specific binding.
5. Primary antibody incubation was performed overnight at 4 °C in blocking solution with the following antibodies (Figure 6):
 - *Anti-5HT* (Goat, 1:150)
 - *Anti-5HT-R2C* (Mouse, 1:500)
 - *Anti-POMC* (Rabbit, 1:2000)

SECOND PART:

6. Three washes in PBS-TX.
7. Secondary antibody incubation was carried out for 2 hours at room temperature in blocking solution in the dark (Figure 6):
 - *Anti-Goat-Fluor 647* (1:250)
 - *Anti-Mouse-Fluor 488* (1:250)

- *Anti-Rabbit-Fluor 546* (1:250)
 - *DAPI* (1:1000) for nuclear staining
8. A final PBS- NaN_3 wash (5 min), followed by rapid mounting of sections on gelatin-coated glass slides using PBS and the day after closed up with mowiol solution.
 9. Slides were stored at 4°C in the dark until imaging with confocal microscopy.

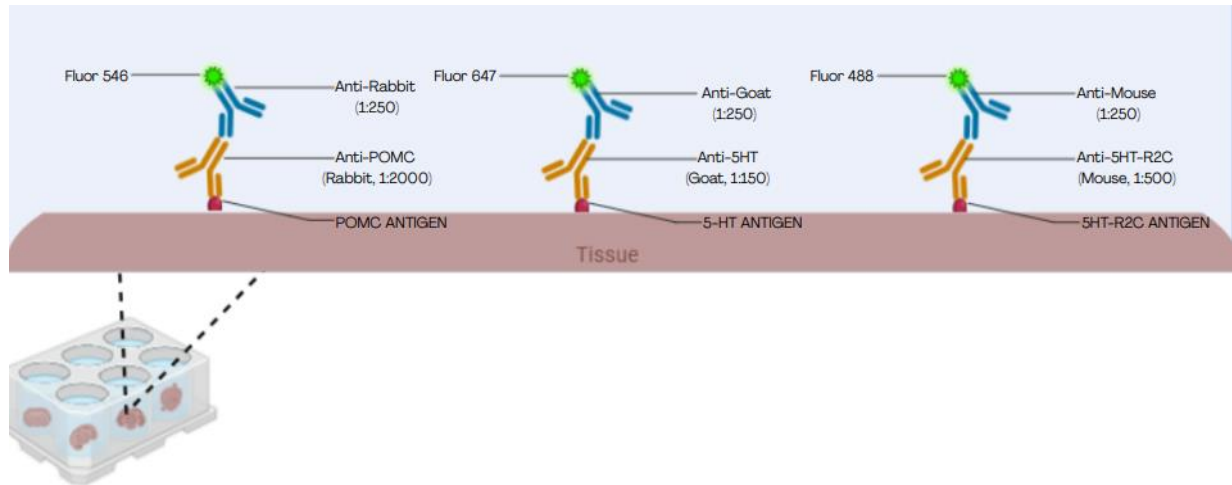


Figure 6 : Binding interactions during the immunofluorescence staining process

The primary antibodies specifically bind to their corresponding target antigens (e.g., POMC, 5-HT, 5-HT₂CR) within the tissue section. Subsequently, fluorophore-conjugated secondary antibodies recognize and bind to the Fc region of the primary antibodies. Each secondary antibody is species-specific and carries a distinct fluorescent dye, enabling the simultaneous visualization of multiple targets under a fluorescence microscope. This indirect labeling approach amplifies the signal and allows precise localization and quantification of the proteins of interest.

3.10 Image Processing and Quantitative Analysis

As the experiment was based on indirect immunofluorescence, image acquisition was performed using a fluorescence microscope, a specialized instrument designed to detect the fluorescent signals emitted by fluorophores bound to secondary antibodies. Unlike conventional bright-field light microscopes, which rely on transmitted white light to generate contrast, fluorescence microscopes utilize high-intensity excitation light sources (such as mercury vapor lamps, LEDs, or lasers) in combination with excitation and emission filters that enable the selective visualization of specific fluorophores. This optical configuration provides enhanced sensitivity and specificity, allowing the precise localization of target antigens within cells or tissues. The superior contrast achieved by detecting fluorescence against a dark background makes this technique particularly valuable in cellular and molecular biology (J.W. Lichtman, J.A. Conchello, 2005).

3.10.1 Arcuate nucleus

Brain sections containing the arcuate nucleus were acquired using ZEISS LSM 980 Airyscan 2 confocal microscope at 20× magnification. The anatomical localization of the arcuate nucleus was determined based on stereotaxic coordinates from the rat brain atlas (bregma -2,56 mm) (G. Paxinos, C. Watson, 2013).

Multidimensional image stacks were opened using FIJI software (J. Schindelin et al., 2012), and a Z-projection was performed for each fluorescence channel. For each image, cellular quantification was performed according to the following protocol (Table 7):

ANATOMICAL LOCALIZATION	COUNTER
<i>Arcuate nucleus</i>	POMC-positive cells
	5-HT2C receptor-positive cells co-expressing POMC
	5-HT2C receptor-positive cells
	5-HT2C receptor-positive cells co-expressing POMC and targeting by 5-HT
	5-HT target cells expressing 5-HT2C recept
	5-HT target cells
	5-HT expression in fractional area (%)

Table 7: Parameters used for cell counting in the Arcuate nucleus

In the final step, a semi-quantitative analysis was conducted to evaluate serotonin expression by measuring the fractional area occupied by the fluorescence signal within defined regions of interest. This approach, based on the relative intensity and distribution of the signal, followed established criteria for immunofluorescence-based quantification (C.R. Taylor, R.M. Levenson, 2006).

3.10.2 Raphe nucleus

Brain sections containing the raphe nuclei were acquired using ZEISS Axio Imager 2 microscope at 40× magnification. The anatomical localization of the raphe region was determined based on stereotaxic coordinates from the rat brain atlas (bregma -7,04 mm). G. Paxinos, C. Watson, 2013).

Multidimensional image stacks were imported into FIJI software, and a Z-projection was performed for each fluorescence channel using the “Average” method to generate representative images. Each image was manually divided into left and right hemispheres, allowing for independent analysis of both sides.

In each projected image, and for each hemisphere, serotonin-positive (5-HT+) cells were manually counted to assess regional distribution and possible asymmetries in expression (Table 8).

ANATOMICAL LOCALIZATION	COUNTER
<i>Raphe nuclei</i>	All 5-HT-immunoreactive cells were manually counted

Table 8: Parameters used for cell counting in the Raphe nuclei

3.11 Statistical Analysis

All statistical analyses were conducted using SPSS software version 28.0 (IBM Corp., Armonk, NY, USA). Data from behavioral tests and immunohistochemical quantifications (POMC, 5-HT, and 5-HT receptor-positive cell counts) were analyzed using two-way analysis of variance (ANOVA), with sex and treatment as independent factors. When a significant main effect or interaction was detected, post hoc comparisons were conducted: Tukey's HSD test was used for 5-HT and receptor-related variables, while the Least Significant Difference (LSD) test was applied to behavioral data.

Statistical significance was defined based on p-values as follows: $p < 0.05$ (*, significant), $p < 0.01$ (**, highly significant), and $p < 0.001$ (***, very highly significant). All results are reported as mean $\pm$ standard error of the mean (SEM).

4. RESULTS

4.1 Maternal milk analysis

Milk composition was evaluated across the four experimental groups (Control, Genistein, Low Protein, and Low Protein + Genistein), focusing on crude protein (CP) and crude fat (FAT) content (Table 9).

The control group (CON) showed an average protein content of 10.45 g/mL (± 0.42). Genistein supplementation slightly increased CP levels (10.64 g/mL ± 0.50), but the difference compared to control was not statistically significant ($p = 0.7796$). In contrast, the LOW group exhibited a significant reduction in protein content (7.27 g/mL ± 0.38) compared to the control rats ($p = 0.0006$). The combination of a low-protein diet with GEN (LOWGEN) resulted in intermediate CP levels (8.06 g/mL ± 0.29), which were still significantly lower than control ($p = 0.0011$).

Regarding fat content, the control group had 9.2 g/mL (± 0.18) of crude fat. The GEN group showed a significant increase in fat content (10.72 g/mL ± 0.43 ; $p = 0.05$) compared to CON. While the LOW group (7.13 g/mL ± 0.67) and LOWGEN group (7.71 g/mL ± 0.50) presented significantly reduced fat levels ($p = 0.0497$ and $p = 0.0166$, respectively) in comparison to control rats.

Overall, maternal protein restriction had a significant impact on milk quality, lowering both CP and FAT concentrations. Although genistein alone did not significantly affect protein levels, it appeared to enhance fat content. Under protein-restricted conditions, genistein partially improved milk composition, though not to the levels observed in the control group.

VALUE	U.M.	CON	GEN	LOW	LOWGEN
Crude protein	g/ml	10,45 $\pm 0,42$	10,64 $\pm 0,50$	7,27 $\pm 0,38$	8,06 $\pm 0,30$
<i>t-test (p-value)</i>				<i>p=0,001</i>	<i>p=0,001</i>
Crude fat	g/ml	9,2 $\pm 0,18$	10,72 $\pm 0,48$	7,12 $\pm 0,68$	7,712 $\pm 0,50$
<i>t-test (p-value)</i>			<i>p=0,05</i>	<i>p=0,049</i>	<i>p=0,017</i>

Table 9: Crude protein (CP) and crude fat (FAT) content in maternal milk from the four experimental groups: Control (CON), Genistein (GEN), Low Protein (LOW), and Low Protein plus Genistein (LOWGEN). Statistical significance (*p-values*) refers to comparisons with the control group, which is evaluated with a *t-test*. The data are represented as mean $\pm$ SEM.

4.2 Birth of puppies

The average number of pups per litter varied among the experimental groups (Table 3). The control group had the highest average (13.56), followed by the LOWGEN group (13), then LOW (12), and finally GEN (10.67).

Looking at sex distribution, the number of male pups per litter was highest in the CON group (4.44), with slightly lower values observed in GEN (5), LOWGEN (4), and LOW (3). This pattern was reflected in the percentage of male pups at birth: GEN had the highest proportions (51.54% and 32.44%), whereas these percentages dropped in the CON and LOWGEN (32.44% and 30.77%, respectively) and LOW (25%) groups.

On the female side, the highest numbers were observed in the LOW and LOWGEN groups (both with 9 female pups per litter on average), while the GEN and CON groups showed lower averages (5.17 and 8.56, respectively). Accordingly, the highest percentages of female pups were seen in the LOW (75%) compared to the CON (67.56%) group, with slightly lower values in the GEN (48.45%).

No dams from any group lost their entire litter. However, pup mortality rate at birth (PND0) varied: the highest percentages were observed in the CON (4.09%) and GEN (4.69%) groups, while the LOW (2.04%) and LOWGEN (1.09%) groups had notably lower rates of neonatal death.

As for body weight at birth, male pups in the GEN group showed the highest average weight (7.19 g), followed by those in LOWGEN (6.64 g), CON (6.23 g), and LOW (5.68 g). A similar trend was observed among female pups, with GEN again leading (6.46 g), followed by CON (5.89 g), LOW (5.49 g), and LOWGEN (5.19 g). These findings suggest that genistein supplementation may positively influence neonatal weight, especially in males, while maternal protein restriction tends to reduce birth weight, particularly in the absence of supplementation.

PARAMETERS RECORDED	CON	GEN	LOW	LOWGEN
<i>Total number of pups per litter</i>	13,56	10,67	12	13
<i>Total number of male pups per litter</i>	4,44	5,5	3	4
<i>Percentage of male pups at PND0</i>	32,44%	51,54%	25%	30,77%
<i>Total number of female pups per litter</i>	8,56	5,17	9	9
<i>Percentage of female pups at PND0</i>	67,56%	48,45%	75%	69,23%
<i>Percentage of dams that lost the entire litter</i>	0	0	0	0
<i>Percentage of dead pups at PND0</i>	4,09%	4,69%	2,04%	1,09%
<i>Body weight of male pups at PND0</i>	6,23	7,19	5,68	6,64
<i>Body weight of female pups at PND0</i>	5,89	6,46	5,49	5,19

Table 3: Litter size, sex distribution, pup mortality at birth (PND0), and average body weight of male and female pups in the four experimental groups: Control (CON), Genistein (GEN), Low Protein (LOW), and Low Protein plus Genistein (LOWGEN). Values are presented as means or percentages.

4.3 Food Feeding

Regarding food intake, control groups—both male and female—exhibit relatively stable consumption over time (Figure 7B; D).

In males treated with genistein (GEN M), food intake is initially slightly lower than in controls, although the difference is not statistically significant. However, starting from PND65, a more pronounced increase is observed, reaching approximately 500 grams, with a significant difference compared to controls at PND94 ($p=0.031$; Figure 7B).

Females treated with genistein (GEN F) showed a similar pattern, with a significantly lower intake observed early on (PND 37, $p=0.029$; PND 51, $p=0.018$; PND 58, $p=0.017$; Figure 7D), and then gradually aligning with the female controls.

Body weight trends (Figure 7A; B) partially mirror the observations in food intake. In GEN males, a significant and early weight increase is observed compared to controls (PND 30 and PND 37, $p < 0.001$), followed by a temporary stabilization between PND 44 and PND 65, and a renewed increase in the later stages (from PND72 to PND108, $p < 0.001$). Similarly, GEN females showed a consistent and significantly higher weight gain than controls at most timepoints (PND30, $p=0,011$; PND37, $p< 0.001$; PND44, $p=0,03$; PND52, $p=0.001$; PND59, $p=0,033$; PND72, $p=0,008$; PND80, $p=0,002$; PND94; PND101; PND108 $p < 0.001$).

These findings suggest a potential obesogenic effect of genistein, particularly in the long term, possibly through mechanisms involving metabolic dysregulation triggered by early-life exposure to this phytoestrogen. Moreover, the observed sex-specific differences may be linked to differing hormonal profiles and a greater sensitivity to estrogenic stimulation in females.

In the LOWPROTEIN group, both males and females exhibited a significantly lower body weight compared to controls from PND 30 through PND 108 ($p < 0.001$; Figure 7A; B). No evidence of catch-up growth was observed, suggesting a long-lasting effect of early protein restriction on somatic development.

In males, a slight but significant reduction in food intake was present at PND 37 ($p = 0.001$; Figure 7B), with subsequent values remaining similar to controls, but did not result in a corresponding increase in body weight. In females, overall food intake was comparable to that of control females. A slight upward trend between PND 44 and PND 51 was observed, although this does not reach statistical significance. From PND 87 onward, intake values gradually realigned with those of the control group, remaining comparable until the end of the study period.

These findings suggest that maternal protein restriction resulted in consistently reduced growth, likely not solely due to lower caloric intake but possibly involving changes in nutrient use or hormonal regulation. Sex differences are minimal in this group and will be further discussed.

In the LOWGEN group, both males and females displayed a significantly lower body weight than controls across all timepoints (PND 30–108; $p < 0.001$; Figure 7A; C), with no signs of catch-up growth. This persistent growth deficit points to a long-term effect of combined perinatal protein restriction and early GEN exposure.

In males, food intake is significantly lower than that of controls at PND 37 ($p < 0.001$; Figure 7B), but subsequently showed a progressive normalization, remaining comparable to male control values across later timepoints. In females, however, food intake appeared more unstable and affected. An initial significant reduction is observed at PND 37 (PND37 $p < 0.001$; Figure 7D), followed by partial recovery. A second significant drop occurs at PND 58 ($p < 0.034$; Figure 7D), after which intake temporarily aligns with controls between PND 65 and PND 80. A further positive fluctuation was observed between PND 80 ($p = 0.034$; Figure 7D) before values returned to stable, control-like levels from PND 101 onward.

These results suggest that combined maternal exposure to genistein and protein restriction leads to a profound and lasting metabolic impact, not compensated by increased food intake.

Overall, LOWGEN females show greater susceptibility to disruptions in food intake, both in the number of affected timepoints and in the irregularity of the trend. This may indicate a heightened vulnerability to metabolic and/or hormonal dysregulation triggered by early-life genistein exposure under protein-restricted conditions.

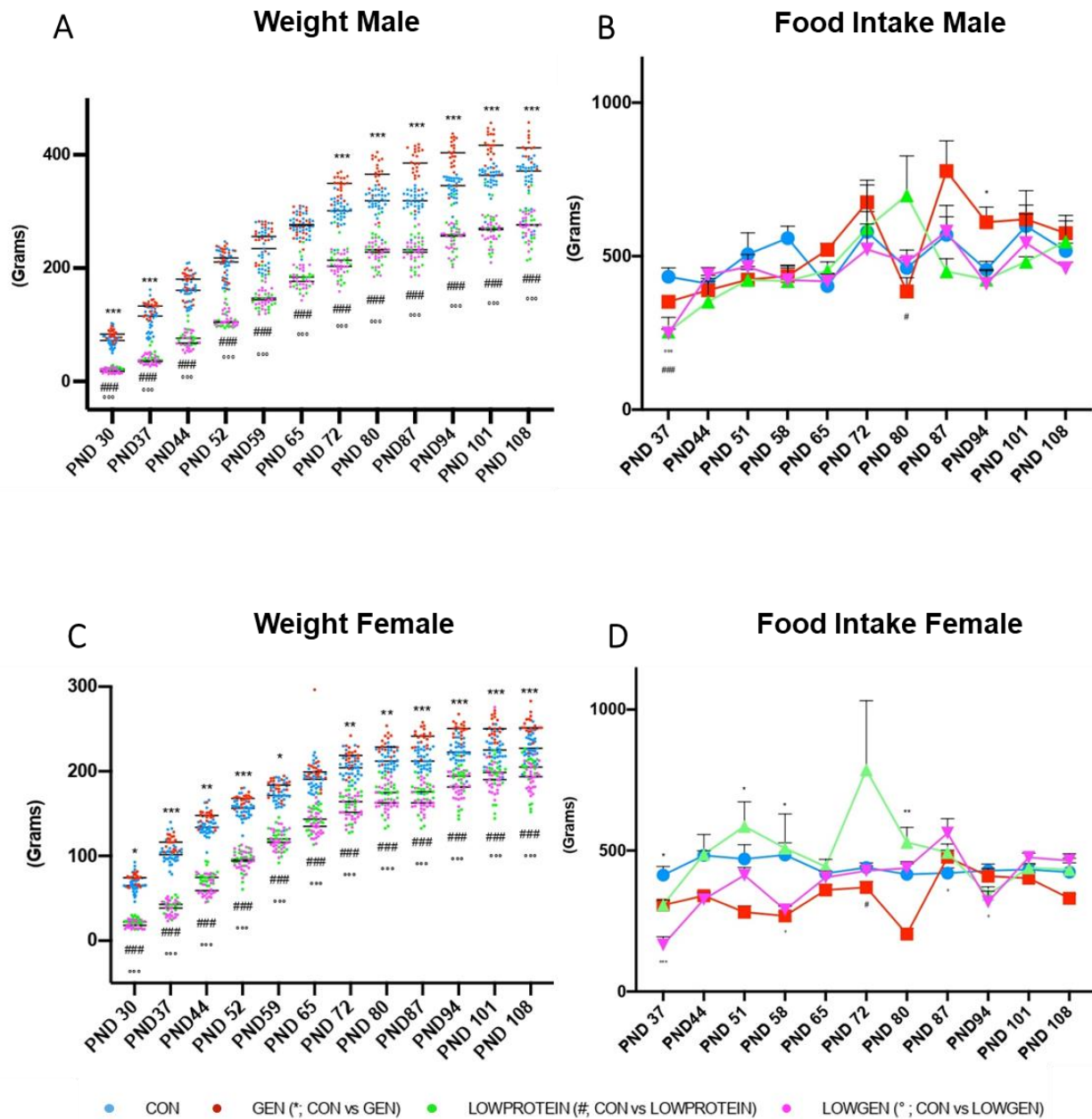


Figure 7: Body weight and food intake progression

A) Body weight (g) of male rats; B) Body weight (g) of female rats; C) Cumulative food intake (g) of male rats; D) Cumulative food intake (g) of female rats.

Line graphs represent mean values across postnatal days (PND 30–108) for each experimental group: Control (blue), Genistein (orange), Low Protein (green), and Low Protein + Genistein (pink). Data are presented as Mean $\pm$ SEM.

Statistical comparisons were performed against sex-matched controls.

Asterisks (*) indicate significant differences between the Control and GEN groups;

Number signs (#) indicate differences between the Control and LOWPROTEIN groups;

Dots (°) indicate differences between the Control and LOWGEN groups.

Symbols correspond to significance value (p) as follows: *, #, ° < 0.05; **, ##, °° < 0.01; ***, ###, °°° < 0.001.

4.3 Behavioral tests

4.3.1 Elevated Plus Maze (EPM)

During the 5-minute Elevated Plus Maze (EPM) test, several behavioral parameters were assessed, including open arm entry frequency, cumulative time spent in the open arms, latency to the first entry, and the duration of “high mobility.” These indicators are commonly used to assess anxiety-like behavior and overall motor activity. Reduced exploration of the open arms (i.e., low frequency and duration, high latency) is generally linked to increased anxiety-like behavior, while increased mobility may indicate enhanced motor activity, sometimes interpreted as disinhibition or hyperactivity.

In the control group, no significant differences between sexes were observed.

In the **GEN** *male* group, a significant effect was observed in the cumulative duration spent in the open arms ($p < 0.001$; Figure 8E) compared to control males. In *females*, the effect was even more pronounced, with significant differences not only in the cumulative duration ($p=0.001$; Figure 8E), but also in the frequency in open arms ($p=0.002$; Figure 9D) with respect to the female control group, as also shown by the activity traces in Figures 9A-B. Moreover, the high mobile duration was also significant ($p<0.001$; Figure 9C) in GEN compared to control females. These findings, which associate significant differences with increased locomotor activity, suggest the presence of hyperactive-like behavior.

Additionally, significant sex differences were found for both frequency in open arms ($p=0.009$; Figure 9D) and high mobile duration ($p=<0.001$; Figure 9C), highlighting that the behavioral alterations differ between sexes. Specifically, in females, the behavioral profile appears to be driven by a marked hyperactivity, which is not equally evident in males.

In the **LOW** *male* group, significant effects were again detected for both the frequency in open arms ($p=0.003$; Figure 9D) and the cumulative duration in open arms ($p=0.006$; Figure 9E) when compared to the control group. In *females*, however, no significant differences were observed compared to control females. Nevertheless, a significant sex difference emerged in the cumulative duration ($p=0.05$; Figure 9E), which may reflect a greater disinterest in females compared to the alterations observed in males.

In the **LOWGEN** *male* group, a significant increase in the latency to the first entry into open arms was found ($p=0.006$ vs. control males; Figure 9F), which may indicate an initial state of fear or disinterest. In *females*, compared to the control group, both the frequency in open arms

($p = 0.009$; Figure 9D) and the cumulative duration ($p = 0.007$; Figure 9E) were significantly increased. In this group, sex differences were particularly marked, with significant effects in the cumulative duration ($p=0.049$; Figure 9E), the frequency in open arms ($p=0.022$; Figure 9D), and the latency to the first entry ($p=0.008$; Figure 9F).

These findings suggest the presence of a clear sexual dimorphism in behavioral alterations: males showed an initial avoidance, possibly related to fear or disinterest, while females exhibited a different response profile, in which the combination of a low-protein diet and genistein supplementation produced opposite behavioral outcomes.

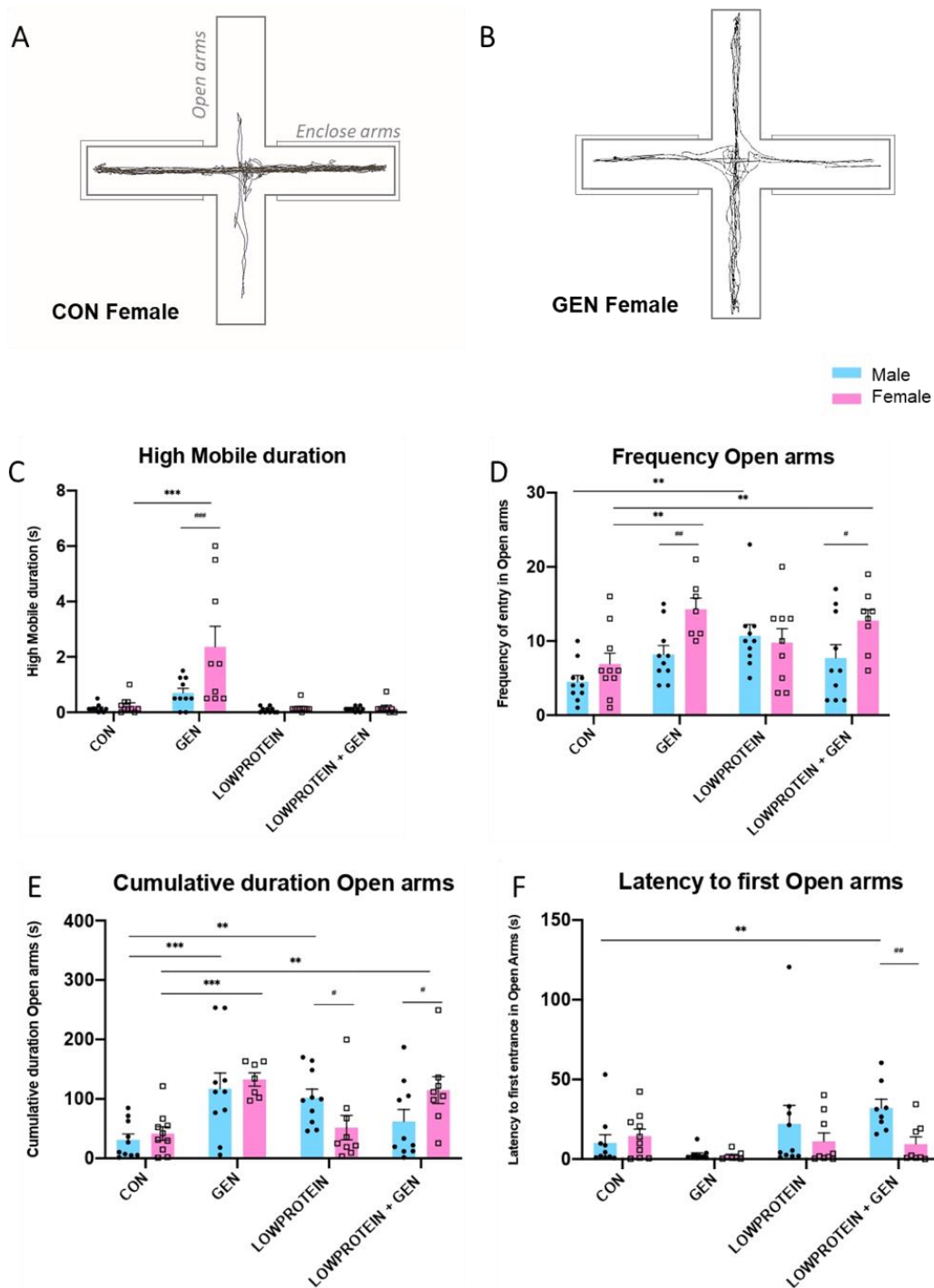


Figure 8: Behavioral outcomes in the Elevated Plus Maze (EPM) test.

A) Representative movement track of a control female mouse. B) Representative movement track of a GEN-treated female mouse. C–F) Quantitative analysis of behavioral parameters: C) Duration of high mobility, D) Frequency of entries into Open Arms, E) Cumulative duration spent in the Open Arms, F) Latency to the first in Open Arms

Bar graphs compare male (blue) and female (pink) subjects across experimental groups: CON, GEN, LOWPROTEIN, and LOWGEN. Data are expressed as mean $\pm$ SEM. Asterisks indicate statistical significance (p) of comparisons between control and treatment groups (* < 0.05 ; ** < 0.01 ; *** < 0.001), while number signs (#) denote sex differences within each group (# < 0.05 ; ## < 0.01 ; ### < 0.001).

4.3.2 Open Field (OF)

This test was used to evaluate anxiety-like behavior in rodents. The following parameters were assessed: time spent in the center (s), latency to the first center entry (i.e., the time taken by the mouse to enter the center for the first time), frequency of center entries, and high mobility duration.

In the control group, none of the parameters analyzed were statistically significant in the comparison between the sexes; the females tested were in the estrus phase.

While *males* in the **GEN** group do not show any significant parameters, as outlined by the EPM, *females* born to mothers treated with phytoestrogens also do not exhibit notable differences in the OF assessments but showed a significant increase in center frequency ($p = 0.006$; Figure 9D) and total time spent in the center ($p < 0.001$; Figure 9E), along with a non-significant trend toward decreased latency to the first center ($p = 0.058$; Figure 9F) compared to control females (Figure 9A; B). These effects, combined with a significant increase in high mobility duration ($p < 0.001$; Figure 9C), suggest a possible hyperactive phenotype rather than an anxiolytic effect. Moreover, these differences, especially in the analysis of the total time spent in the center ($p=0.017$; Figure 9E) and the high level of mobility ($p<.001$; Figure 9A), led to the development of sexual dimorphism between males and females of this group, with an anxiogenic phenotype in male rats.

In **LOW** *males*, center frequency, cumulative duration, and high mobility duration (Figure 9C; D; E) were equal to or lower than those of controls, with no significant differences. However, latency to the first center was notably increased (Figure 9F; $p = 0.05$), suggesting heightened anxiety or hesitation to explore open space relative to control males. At the same time, the **LOW** *females* did not show significant behavioral changes in the analyzed OF parameters.

Finally, in the **LOWGEN** group, although the *male* animals showed only a delay in the first center, it was significantly longer (Figure 9F; $p = 0.039$), which may indicate increased anxiety or decreased exploratory drive, *females* presented a significant increase in center frequency ($p < 0.001$; Figure 9D) and cumulative center duration ($p = 0.003$; Figure 9E), along with a significant reduction in latency to the first center ($p = 0.039$; Figure 9F). Despite these changes, high mobility duration remained low and similar between sexes. These results may indicate an anxiolytic effect in females not associated with hyperactivity, and a potential loss of exploratory motivation in males, possibly suggesting a depression-like phenotype.

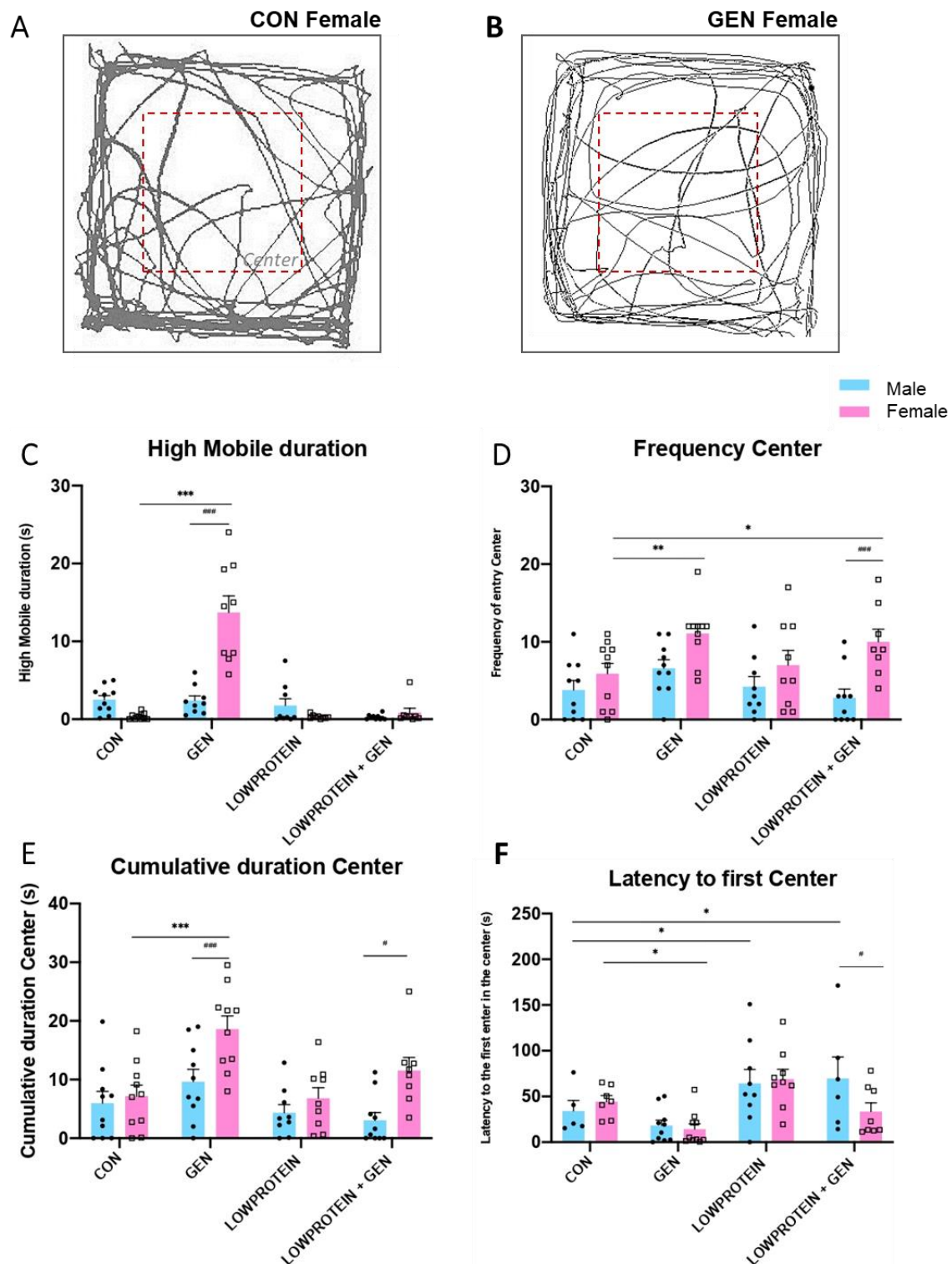


Figure 9: Behavioral outcomes in the Open Field (OF) test.

A) Representative movement track of a control female mouse. B) Representative movement track of a GEN-treated female mouse.

C–F) Quantitative analysis of behavioral parameters: C) Duration of high mobility, D) Frequency of entries into the center zone, E) Cumulative duration spent in the center, F) Latency to the first center entry.

Bar graphs compare male (blue) and female (pink) subjects across experimental groups: CON, GEN, LOWPROTEIN, and LOWGEN. Data are expressed as mean $\pm$ SEM.

Asterisks indicate statistical significance (p) of comparisons between control and treatment groups (* < 0.05; ** < 0.01; *** < 0.001), while number signs (#) denote sex differences within each group (# < 0.05; ## < 0.01; ### < 0.001).

4.4 Fluorescence Immunohistochemistry

4.4.1 Raphe nuclei

The analysis of serotonin-positive (5-HT+) cells in the Raphe (Figure 10) was conducted to evaluate potential alterations in baseline serotonin production, and to later correlate these data with cell counts in the arcuate nucleus, aiming to identify possible alterations in neurotransmitter release.

In the control group, no significant sex differences were observed. In the **GEN** *males*, a significant increase was detected ($p < 0.001$), and the same result was confirmed in *females* ($p = 0.05$) compared to the control groups.

In the **LOW** protein, no significant values were found in either males or females. Conversely, in the **LOWGEN** group, a significant increase was observed in both *males* ($p = 0.024$) and *females* ($p = 0.011$) in comparison to the control groups.

These results suggest that the observed differences may be influenced by the presence of phytoestrogens in the maternal diet and reflect a differential modulation of serotonergic neuronal activity. Understanding this will require a detailed analysis of data from the arcuate nucleus of the hypothalamus (ARH), given the strong functional interconnection between these two brain regions.

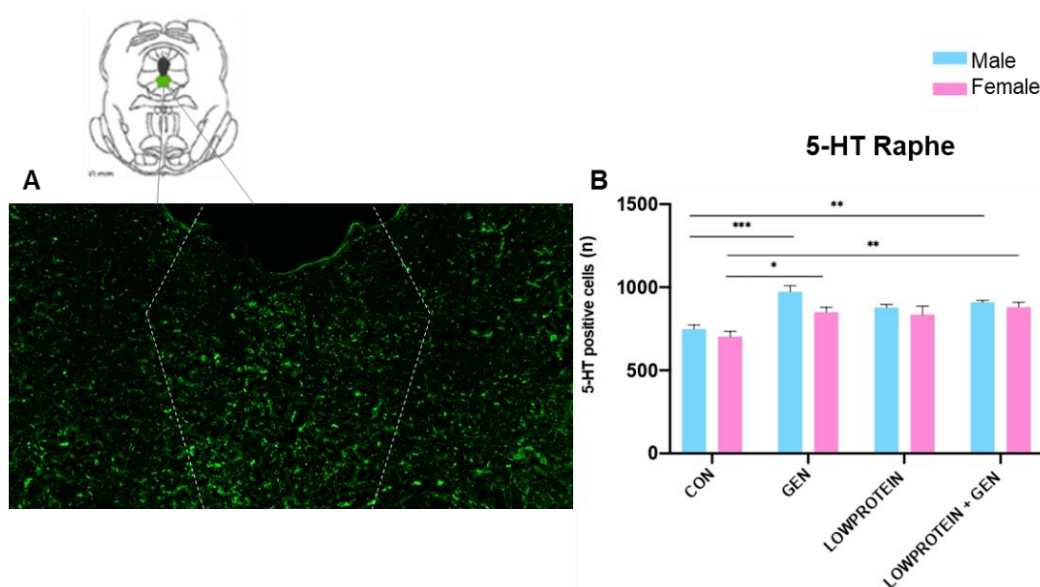


Figure 10: Serotonin-positive cells in the raphe nuclei.

A) Representative 40x confocal image of the Dorsal Raphe nuclei with Axio Imager 2 microscope expression of the 5-HT system (in green)

B) Bar graphs showing the number of raphe nuclei 5-HT-positive cells in male (blue) and female (pink) rats. Data are expressed as mean $\pm$ SEM.

Asterisks indicate statistical significance of comparisons between control and treatment groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

4.4.2 Arcuate nucleus

Expression analyses of the 5-HT system (5-HTR2C and 5-HT) were conducted in the arcuate nucleus (Figure 11) of the hypothalamus to better understand whether potential changes in the serotonergic system, as observed in Raphe after maternal diet modulation, could influence hunger and satiety regulation in offspring across different experimental groups by affecting the POMC system.

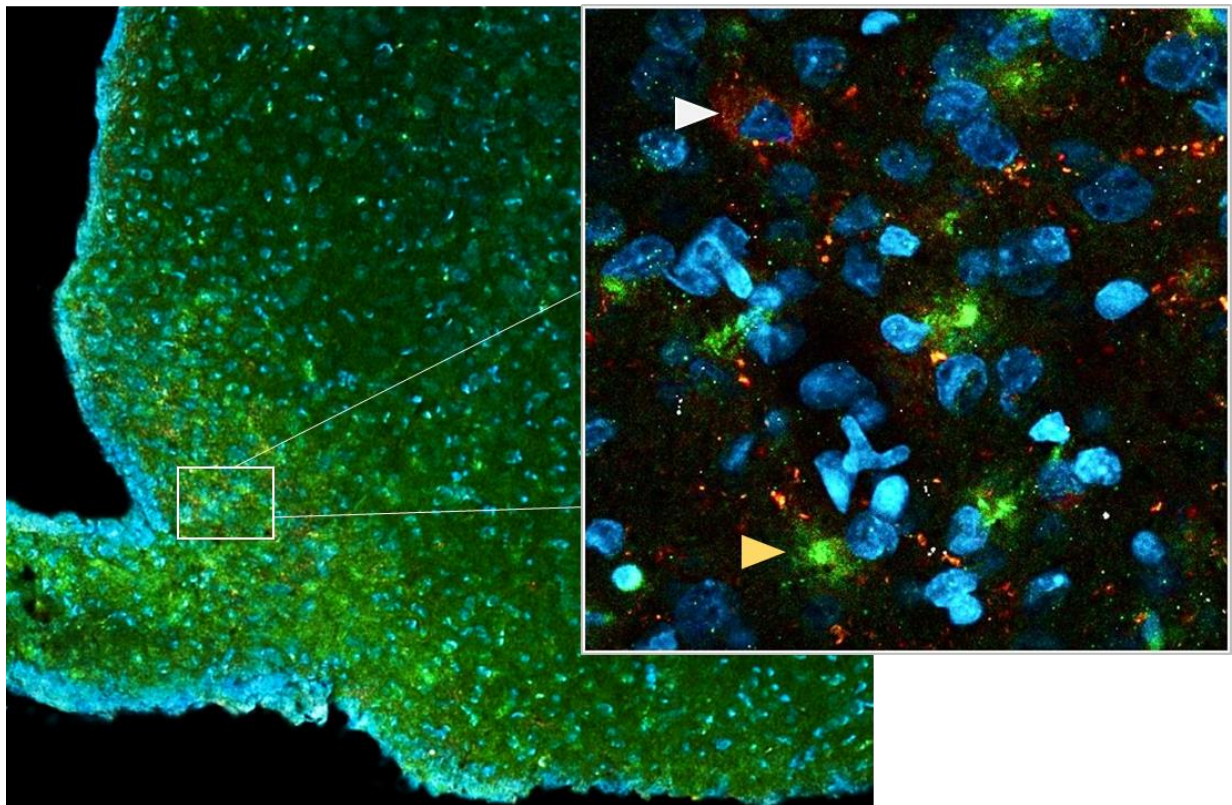


Figure 11: Immunofluorescence imaging of the arcuate nucleus.

Representative 20x confocal image of the arcuate nucleus with magnified inset showing cellular colocalization patterns. Green: 5-HT (serotonin); Red: POMC; White: 5-HT receptors; Blue: DAPI.

The white arrowhead indicates a cell coexpressing 5-HT, POMC, and 5-HT receptors. The yellow arrowhead indicates a serotonergic cell (5-HT only).

In the control group, none of the analyzed parameters showed statistically significant differences between sexes.

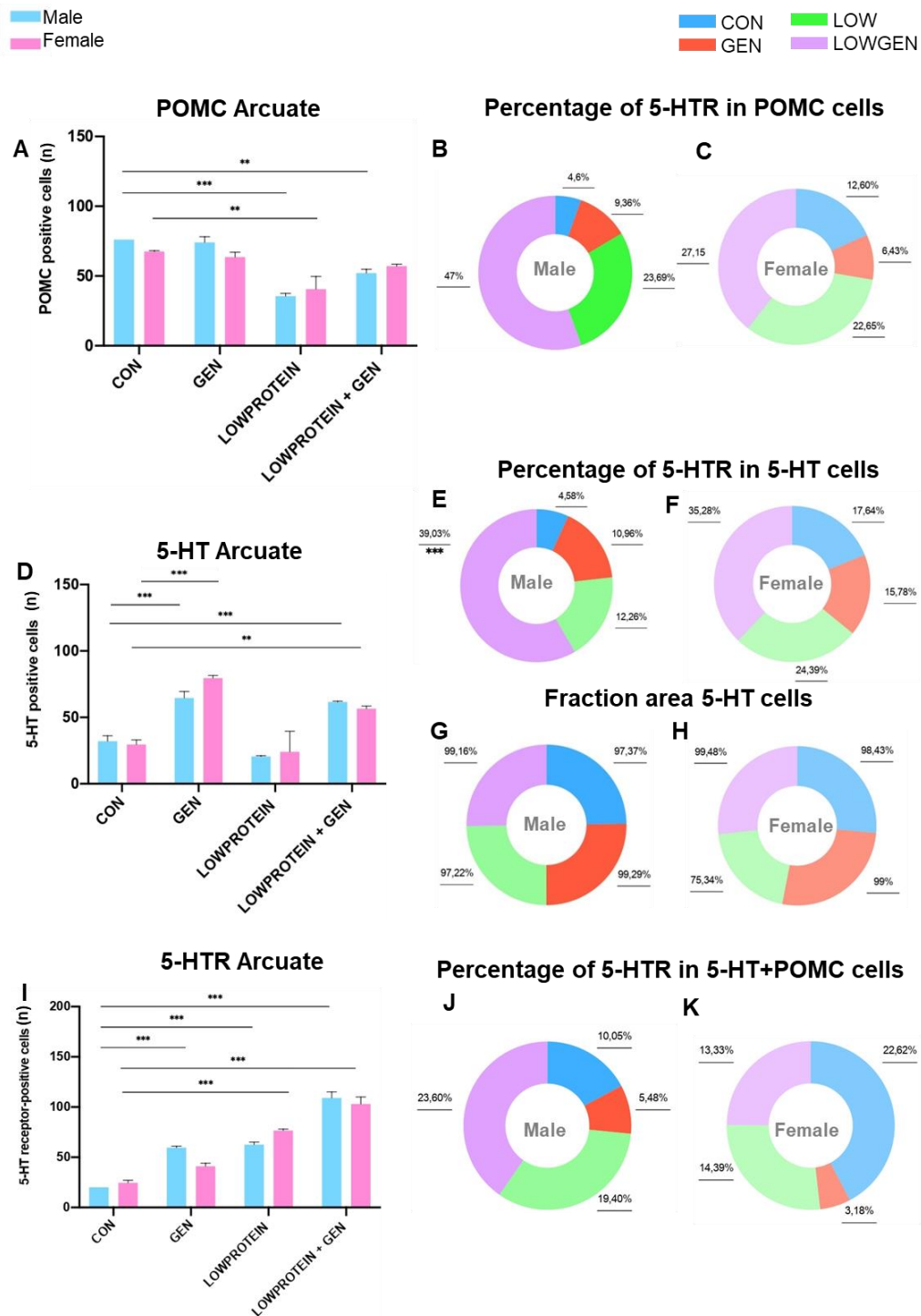
While it did not show significant changes in POMC expression in *males* born to mothers with a diet rich in phytoestrogens, these animals, consistent with the increase in 5-HT cells in the Raphe nucleus, exhibited a significant rise in both 5-HT+ expression ($p = 0.010$; Figure 12B) and serotonin receptor expression ($p = 0.001$; Figure 12C) in ARC nucleus. In contrast, **GEN** *females* showed a significant increase only in 5-HT+ expression ($p < 0.001$; Figure 12C), with no significant changes in receptor levels. Although no statistically significant sex-specific differences were detected, these findings may point to a differential modulation of the serotonergic system that warrants further investigation.

Unlike the other groups, **LOW** *males* exhibited a significant decrease in POMC-positive neurons ($p < 0.001$; Figure 12A), highlighting how a maternal diet low in protein causes a substantial change in adult offspring's metabolic regulation and food intake. Although serotonin levels did not show significant changes, the expression of the serotonin receptor (5HTR) was significantly increased ($p < 0.001$; Figure 12C). A similar condition was observed in *females*, with a significant decrease in POMC-positive neurons ($p = 0.002$; Figure 12A) and a considerable increase in 5HTR expression ($p < 0.001$; Figure 12C). The significant reduction in both sexes in POMC+ neurons, along with the increased expression of serotonin receptors (5HTR) despite unchanged serotonin levels, reflects a central compensatory mechanism aimed at reducing anorexigenic signaling and enhancing serotonergic sensitivity.

In **LOWGEN** *males*, a significant reduction in POMC expression was again observed ($p = 0.005$; Figure 12A), together with a significant increase in 5-HT+ ($p = 0.018$; Figure 12B) and serotonin receptor expression ($p < 0.001$; Figure 12C). Notably, this group was the only one to show a significant increase in the proportion of 5HTR expression specifically within serotonergic neurons ($p = 0.003$; Figure 12E).

These alterations, particularly the increased serotonergic sensitivity, could have functional implications for appetite regulation and energy homeostasis, suggesting a possible early-life programming of neuroendocrine circuits involved in metabolic control.

In **LOWGEN** *females*, there was a significant increase in both 5-HT+ ($p = 0.030$; Figure 12B) and 5HTR expression ($p < 0.001$; Figure 12C), while POMC expression stayed the same. Although no statistically significant sex-specific differences were found, these results may suggest a common serotonergic response to the combined effects of early-life low-protein diet and maternal phytoestrogen exposure.



5. DISCUSSION

It is widely accepted that a healthy, balanced diet comprising both macro- and micronutrients is one of the key foundations for maintaining a proper lifestyle and preventing many chronic diseases (World Health Organization, 2020) including oncological (World Cancer Research Fund International, 2025). and psychological disorders (B. Bizzozero-Peroni, 2025)

Pregnancy is recognized as a highly delicate and critical stage, during which proper nutritional management is essential not only to meet maternal needs but also to ensure optimal fetal development (NE. Marshall et al., 2022). In particular, it has been shown that gestation, along with the first two years of life, forms a crucial window—known as the first 1,000 days—during which nutritional exposures can have lasting effects on a child's health (Tadeja Gracner et al., 2024). Numerous studies confirm that the intrauterine period is a critical window for metabolic programming: nutritional exposures during pregnancy and lactation can permanently influence offspring health, either increasing or decreasing the risk of chronic diseases later in life (CH. Fall, 2011; M. Carolan-Olah, M. Duarte-Gardea, J. Lechuga, 2015). Another important aspect in this context is breastfeeding, which is widely regarded as the gold standard for infant nutrition. Human milk not only supplies essential nutrients but also contains bioactive and immunomodulatory components that foster microbiota development, support the immune system, and help decrease the risk of future metabolic diseases (H Szyller, 2024).

This thesis examines the impact of specific diets during pregnancy and lactation on offspring from both metabolic and behavioral perspectives. It focuses on three experimental groups: a low-protein diet (LOW), a diet enriched with phytoestrogen genistein (GEN), and a combined diet (LOWGEN). These dietary conditions mimic potential scenarios of altered protein intake, such as those seen in faulty vegan diets, where essential amino acids may be deficient and require proper supplementation, particularly during pregnancy.

The global increase in soy use—driven by exportation and the widespread adoption of various protein-rich products—has led to a significant rise in exposure to genistein, an isoflavone with a chemical structure very similar to 17 β -estradiol. This structural affinity allows genistein to mimic estrogenic action, significantly influencing hormonal regulation, glucose and lipid metabolism, as well as behavior (RWL. Godschalk et al., 2022; JL. Bolton, SD Bilbo, 2014; ER. Ball et al., 2010). Although genistein is generally considered safe, its potential role as an endocrine-disrupting compound remains debated (AA. Suen et al., 2021). Experimental studies have shown conflicting effects: on one side, metabolic and microbiota-modulating benefits (L. Zhou et al., 2018; M. Cheng et al., 2022) when exposure happens during pregnancy and lactation;

on the other side, possible long-term hormonal and epigenetic effects, including changes in female reproductive cycles, lasting alterations in neonatal development, and oxidative DNA damage in male offspring (M. Marraudino et al., 2021; RWL. Godschalk et al., 2022). These findings suggest that exposure to genistein during critical developmental periods should be approached with caution. In this context, we questioned how such increasingly common diets—although not recommended during pregnancy and lactation—might affect offspring during the intrauterine and lactation periods, even without subsequent direct exposure.

Maternal diet rich in phytoestrogens

Mothers receiving a genistein-enriched diet exhibited a significant increase in milk lipid content. Although direct studies examining the impact of GEN on milk lipid composition are currently lacking, the established role of genistein as an activator of nuclear peroxisome proliferator-activated receptors (PPARs)—key regulators of lipid metabolism, fatty acid synthesis, and adipocyte differentiation—provides a plausible mechanistic basis for this observation (S. Kim et al., 2004; O. Mazei et al., 2003). Additionally, GEN's affinity for estrogen receptor β (ER β), which is known to influence mammary gland differentiation, may further contribute to the increase in milk lipids (E. Padilla-Banks, W.N. Jefferson, R.R. Newbold, 2006).

This lipid-rich milk created a hypercaloric nutritional environment during the critical neonatal window (I. Petersohn et al., 2024) in offspring, correlating with increased early postnatal body weight. Early changes in milk composition are known to imprint lasting metabolic programming, potentially reshaping the development of hypothalamic circuits responsible for energy homeostasis and behavior (Li X et al., 2025). In our model, the initial lipid excess seems to act as a trigger that promotes weight gain in both sexes. Although it is not enough to explain all the observed changes, it likely helps establish a modified metabolic set-point.

When examining dietary intake, male offspring displayed continued weight gain during the postnatal period and into adulthood—despite having a caloric intake comparable to that of controls—suggesting changes in metabolic efficiency or energy balance. Among female offspring, weight increase was consistent across all developmental stages, pointing toward an obesogenic programming effect in females, in agreement with some existing studies (M. Marraudino et al., 2021; RS. Strakovsky et al., 2013). This occurred even in the presence of reduced food intake, indicating that GEN-induced metabolic programming involves not just caloric availability but also how the organism utilizes and “interprets” nutrients, likely through alterations to hypothalamic circuits.

Behavioral tests used, EPM and OF—widely used to assess anxiety-like and exploratory behaviors (P. Knight et al., 2021) revealed sexually dimorphic effects in the offspring of mothers fed a genistein-enriched diet. Male offspring displayed a predominantly anxiolytic-like behavior, with significant differences observed only in the EPM, consistent with previous studies on males directly treated with GEN (M. Marraudino et al., 2024) or exposed during the perinatal period (A. Rodriguez-Gomez et al., 2014).

In contrast, female offspring showed a significant increase in the overall time and frequency of exploration in the EPM open arms and the OF center zone, a decreased latency to first entry into the OF center, and, most notably, an increase in overall locomotor activity in both tests. These findings indicate a primarily hyperactive behavioral pattern rather than a specific anxiolytic effect.

Previous studies indicate that perinatal GEN exposure may induce persistent epigenetic and neuroendocrine modifications, thereby altering the neural circuits involved in regulating arousal and motor activity (RS. Rosenfeld et al., 2019). Genistein readily crosses the placental barrier, reaching the fetal circulation (DR. Doerge et al., 2001) whereas only limited amounts are transferred through maternal milk (DR. Doerge et al., 2006). These observations support the hypothesis that the behavioral effects in the offspring are predominantly mediated by in utero exposure, consistent with the concept of maternal programming.

To complement the behavioral test data, immunohistochemical analyses were conducted. These analyses focused on the raphe nucleus and the arcuate nucleus of the hypothalamus, critical regions involved in regulating anxiety, depression, feeding, and sexual behaviors (J.G. Hensler et al., 2013).

Specifically, in the raphe nucleus, offspring born to mothers exposed to phytoestrogens exhibited an increased number of 5-HT positive cells in both sexes, without evidence of sexual dimorphism, in contrast to previous reports in the literature (M. Marraudino et al., 2014; D. Giulian et al., 1973). This discrepancy may be explained by the fact that most existing studies focus primarily on the neonatal or perinatal period. In contrast, the present study aims to assess the effects of maternal-fetal exposure.

In the arcuate nucleus, although both sexes showed an increase in 5-HT target cells, males also displayed a significant increase in serotonin receptor (5-HTR) expression, suggesting increased sensitization of serotonergic circuits. In females, the increase was limited to serotonin levels alone, indicating distinct post-receptor regulation or compensatory mechanisms.

Overall, these findings suggest that maternal dietary genistein induces sexually dimorphic neurochemical programming in the offspring.

Maternal low-protein diet

The protein content of maternal milk from dams fed a low-protein diet was significantly reduced. This finding is consistent with previous reports, which document not only a decrease in milk protein concentration following low-protein diets (CJ. Bautista et al., 2019; Adhikari et al., 2022), but also impaired mammary gland development and reduced prolactin levels (VL. Moretto et al., 2011), both of which are key factors for milk synthesis and secretion.

Regarding the lipid content of maternal milk, current evidence remains limited (CJ. Bautista et al., 2019). Some studies suggest that a low-protein diet may lead to a reduction in the fraction of polyunsaturated fatty acids (PC. Bello-Medina et al., 2024), nutrients essential for fetal and neonatal neural development (LV. Mennitti et al., 2015). In the present study, although the fatty acid profile was not analyzed in detail, a significant reduction in total milk lipid content was observed.

Analysis of body weight and food intake from PND30 to PND108 revealed, in agreement with the literature (S. Mohammed et al., 2023), a significant reduction in both parameters in offspring born to mothers fed a low-protein diet. It has been proposed that maternal protein restriction during gestation may activate the placental amino acid response pathway, thereby altering nutrient transfer to the fetus and subsequently affecting its growth potential (S. Strakovsky et al., 2010). This activation may induce early metabolic programming, which, on one hand, optimizes energy utilization efficiency, but on the other, reduces postnatal growth potential with lasting effects on the individual's physiology.

The low-protein diet induced behavioral alterations exclusively in males, suggesting greater susceptibility compared to females. This vulnerability may be related to physiological differences in body composition: males generally have a higher proportion of lean mass and lower fat mass than females (MA. Bredella, 2017), making them more dependent on adequate protein intake to maintain muscular homeostasis. In murine models, males under food restriction lose more lean mass and exhibit distinct metabolic adaptations compared to females (GO. De Souza ET AL., 2022), supporting the hypothesis of greater “somatic” vulnerability to nutritional deprivation.

Males showed contrasting results between EPM and OF tests: in the EPM, there was an increase in cumulative duration and frequency in the open arms, consistent with previous reports (HR.

Lieberman et al., 2005; SS: Almeida et al., 2003), whereas in the OF, latency to first entry into the center was increased.

This apparent discrepancy likely reflects the different behavioral demands of the tasks. In the EPM, exploration of open arms may represent adaptive behavior to increase foraging opportunities, whereas in the OF, where the arena is obvious, latency to enter the center reflects more direct avoidance behavior. Previous studies indicate that behavioral responses can vary depending on experimental conditions, including environmental stressors such as temperature changes (HR. Lieberman et al., 2005) or visual distractors (LA Newman et al., 2019) in association with protein restriction.

This alteration, together with evidence of decreased dopaminergic function in other brain regions (P. Kehoe et al., 2001), suggests a widespread effect of maternal protein malnutrition. As right prefrontal DA laterally regulates stress responses, such deficits may explain the atypical responses observed in this model, which do not exhibit the physiological DA increase in response to stress. Similar findings, observed in both animal models and human studies (e.g., blunted cortisol responses), highlight the crucial role of DA in the vmPFC for modulating behavioral and neuroendocrine stress responses. These results, along with findings by Newman et al. (2019), also point to a significant role for the PFC and noradrenaline (NE) in attention deficits observed in malnourished animals (LA. Newman et al., 2019). Changes in DA and NE levels in the right vmPFC may therefore contribute, at least in part, to the cognitive inflexibility reported in this model (JA. McGaughy et al., 2014; LA Newman et al., 2019).

Overall, these data, together with previous studies on the effects of protein restriction on the developing and adult brain (WC. Stern et al., 1975; J.C. Cheng et al., 1992; J.C. Cheng et al., 1995; GJ. Blatt et al., 1994; DJ. Mokler et al., 1999; Mokler et al., 2003; Mokler et al., 2007; R. Soto-Moyano et al., 1999; R. Soto-Moyano et al., 2005; JA. McGaughy et al., 2014; LA. Newman et al., 2019), suggest an actual brain reprogramming. This adaptation may have functional value, allowing the animal to prioritize foraging behavior in a resource-limited environment, which is reflected in the observed cognitive inflexibility.

In this group, immunohistochemical analyses revealed a significant reduction of POMC cells within the ARC in both sexes. These neurons are essential for the production of anorexigenic neuropeptides, such as α -MSH, which, through the activation of melanocortin receptors (MC4R), play a crucial role in regulating appetite and energy metabolism (SG. Bouret, 2022; RD. Cone, 2005; KG. Mountjoy et al., 1992). Their reduction therefore suggests a weakening of the anorexigenic signal and a diminished ability to exert homeostatic control over food intake.

Under physiological conditions, activation of MC4R receptors located on GABAergic neurons in the dorsal raphe nucleus exerts an inhibitory influence on serotonergic neurons (S. Yamagata et al., 2025). A reduction in the POMC/ α -MSH signal would therefore be expected to result in disinhibition of the raphe and an increase in serotonergic activity. However, our results did not show an increase in 5-HT-positive cells in the raphe, suggesting that a low-protein diet is insufficient to enhance serotonergic circuits.

A possible explanation lies in the fact that serotonin synthesis strictly depends on the availability of tryptophan, an essential amino acid that must be obtained exclusively through the diet (JD. Fernstrom, 2013). Maternal protein restriction may therefore result in reduced availability of the substrate required for 5-HT synthesis, limiting the ability of serotonergic neurons to respond to the loss of inhibition caused by the reduction of POMC.

In parallel, an increased expression of the serotonergic receptor 5-HT_{2C} was observed in the ARC. This receptor enhances the excitability of POMC neurons (DD. Lam et al., 2007) and its upregulation, in the presence of reduced POMC cells, may represent a compensatory mechanism aimed at partially preserving the anorexigenic tone and metabolic regulation (Y. Xu et al., 2008; MS. Martin-Gronert et al., 2016). Beyond metabolism, 5-HT_{2C} receptors are also known to modulate dopaminergic tone, mood, and stress response (L. Valencia-Torres et al., 2016; LH. Tecott et al., 1995) thereby occupying a key position for understanding how maternal protein restriction may affect not only metabolism but also the offspring's behavior.

In summary, our findings suggest that a maternal low-protein diet during gestation and lactation compromises the POMC/ α -MSH circuit without producing an increase in serotonin in the dorsal raphe, most likely due to the reduced availability of tryptophan or other adaptive mechanisms. The upregulation of 5-HT_{2C} receptors appears to act as a partial compensatory attempt, which, however, does not seem sufficient to restore proper metabolic and behavioral balance.

Maternal low-protein diet rich in phytoestrogens

The interaction between a maternal low-protein diet and genistein supplementation (LOWGEN experimental group) produced distinctive effects on the offspring's metabolism, behavior, and neurochemistry.

Although genistein is known for its role in modulating lipid metabolism and mammary gland function, maternal milk in the LOWGEN group was low in both protein and lipids. This suggests that protein restriction has a dominant effect, capable of overshadowing the potential benefits of

genistein, aligning with the limited availability of metabolic substrates needed for milk synthesis (MR. Grigor et al., 1987; F. Bravi et al., 2016).

Furthermore, offspring exhibited reduced growth in both sexes, with caloric intake equal to or lower than that of controls, particularly in females. These findings suggest that the metabolic imprint of protein restriction persists despite the known effects of genistein, in line with observations from prenatal protein malnutrition models (S. Mohammed et al., 2023).

Behaviorally, the LOWGEN profile was sexually dimorphic. In males, there was an increased latency to enter the less safe areas in both the EPM and OF, indicative of anxiety-like behavior. This contrasts with the generally anxiolytic behavior observed in GEN males, suggesting that protein deficiency can override the estrogenic modulation exerted by genistein. In females, however, tests showed increased time spent and frequency in the open arms of the EPM, as well as increased center zone frequency in the OF, indicative of an anxiolytic profile similar to that of GEN females, but without hyperactivity. This finding supports the hypothesis of greater female sensitivity to the estrogenic effects of genistein (M. Marraudino et al., 2019) which in this case appears to dominate over protein deficiency at the behavioral level.

At the neurochemical level, an increase in serotonin-positive (5-HT+) cells in the raphe nuclei was observed, consistent with GEN and aligned with genistein's role in modulating serotonergic circuits. In the arcuate nucleus, both sexes showed an increase in serotonin and 5-HT receptor expression, but with significant dimorphism: in males, this was associated with a reduction in POMC neurons, similar to the LOW group, and an increased percentage of 5-HT receptors in serotonergic cells. This suggests that protein deprivation remains the dominant influence on POMC signaling in males, compromising the anorexigenic pathway, while the receptor increase may serve as a compensatory mechanism to modulate serotonergic activity. In females, the absence of POMC reduction indicates relative protection from protein restriction, consistent with the anxiolytic behavioral profile observed.

Overall, these findings suggest that in the LOWGEN group, protein restriction predominates in regulating metabolism (characterized by nutrient-poor milk, reduced body weight, and POMC reduction in males). In contrast, genistein continues to exert an influence over serotonergic circuits and behavior, particularly in females. This model thus reveals a functional dissociation: protein deficiency “determines” metabolic fate, while genistein finely modulates neurobehavioral circuits, with sexually dimorphic effects.

6. CONCLUSIONS

These results reveal a greater metabolic impact of a maternal diet rich in phytoestrogens in females and a sex-specific modulation of the serotonergic system, highlighting that maternal-fetal exposure to genistein can have sex-dependent effects on both metabolism and behavior. While mothers subjected to a low-protein diet during gestation and lactation compromise the POMC/ α -MSH circuit, likely due to reduced tryptophan availability or the activation of additional adaptive mechanisms, with long-term repercussions on both metabolism and offspring behavior.

The combination of a maternal low-protein diet with genistein enrichment protein deficiency primarily drives metabolism. In contrast, genistein influences serotonergic circuits and behavior with sex-specific effects, highlighting a functional dissociation between metabolic regulation and neurobehavioral modulation.

Overall, this data suggests that a maternal diet low in high-quality protein may increase the risk of low birth weight, impaired growth, and a higher likelihood of developing metabolic and cognitive disorders in adulthood. Males seem especially vulnerable, showing profiles linked to cognitive rigidity and attention deficits.

Conversely, high consumption of phytoestrogens through soy-based foods may have a potentially obesogenic role, especially in females, and may alter serotonergic circuits with sex-specific behavioral effects: anxiolytic behavior in males reduces risk perception, while females exhibit increased hyperactivity. When these phytoestrogens are consumed without adequate protein intake, they do not appear to compensate for protein deprivation; however, they may persistently modulate serotonergic circuits and behavior, with sex-specific responses. Although partial protection is observed in some female parameters, the serotonergic system remains altered, and the long-term implications for estrogen susceptibility warrant further investigation.

These observations underscore the importance of ensuring adequate protein intake during pregnancy and lactation and of carefully evaluating phytoestrogen consumption, critical periods during which maternal nutritional choices can have lasting consequences on offspring health.

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