

Article

Bovine Milk Whey Intake in Offspring Rats Shows Restorative Metabolic Effects Against Negative Fetal Programming Induced by Maternal Obesity

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Abstract

Maternal obesity has been associated with offspring fetal programming, increasing the risk of developing chronic diseases in adulthood, including type 2 diabetes, hypertension, and behavioral disorders such as anxiety and depression. Milk whey (MW) peptides, administered as a supplement, reduce food intake and body weight in overweight rodents. The aim of this work was to study the possible metabolic and behavioral restorative effects of a bovine MW supplement against the negative fetal programming of juvenile offspring. Dam Wistar rats were fed during gestation and lactation with a standard (SD) or a hypercaloric diet (HD); after weaning, the male offspring were fed SD or SD and MW. HD dams showed increases in blood cholesterol, body weight, and calorie intake, while administering MW in offspring from HD mothers reduced these alterations. Serum corticosterone, LDL cholesterol, fasting blood glucose, and glucose intolerance were significantly higher in offspring from HD dams; however, MW did not affect these changes. Offspring from HD dams showed signs of anxiety that were not reversed by MW, as well as a reduction in hippocampal BDNF that was partially restored with MW. Maternal HD consumption during pregnancy and lactation elicited negative developmental programming of adult offspring metabolism, which was partially restored by bovine MW supplementation.

Keywords: milk whey; fetal programming; juvenile offspring; rat; anxiety; hippocampal BDNF



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1. Introduction

Obesity is a condition characterized by an excessive accumulation of fat in the body, usually caused by an imbalance between calorie intake and energy expenditure, resulting in a positive energy balance [1]. This condition reduces quality of life and is a risk factor for developing several chronic diseases, such as type 2 diabetes, heart disease, high blood pressure, liver disease, respiratory disorders, certain types of cancer, and musculoskeletal problems, as well as neurological disorders such as anxiety, depression, and learning and memory deficiencies [2,3].

Obesity is a global public health problem that affects populations of all ages. Recent reports estimated that, in 2022, 2.5 billion adults aged 18 and over were overweight, including more than 890 million people with obesity. This means that 43% of adults over 18 years of age (43% of men and 44% of women) were overweight. In addition, more than 390 million children and adolescents aged 5 to 19 were overweight, including 160 million

who were obese [1]. Furthermore, in 2024, it was reported that 35 million children under the age of 5 were overweight. Children, adolescents, and pregnant women are the most vulnerable to obesity-related diseases [4].

Maternal obesity has been associated with an increased risk of gestational diabetes, high blood pressure, and premature birth [5,6]. Regarding the offspring, there is a greater risk of high birth weight and developing obesity in childhood and adulthood, as well as metabolic diseases, such as type 2 diabetes, and cardiovascular disorders related to the fetal programming of the offspring [7,8]. In addition, neuropsychiatric developmental disorders such as anxiety and depression [9–11] as well as impaired cognitive function [12,13] have been reported in offspring. It has also been reported that a high-calorie diet and maternal obesity can reduce the expression of brain-derived neurotrophic factor (BDNF) in offspring, which contributes to long-term neurobehavioral and metabolic changes [14,15]. Similarly, maternal exposure to high-fat diets has been linked to disruptions in the programming of the hypothalamic–pituitary–adrenal axis, leading to elevated corticosterone levels and promoting an enhanced stress response in offspring [16,17].

Fetal programming refers to the processes by which the maternal uterus environment during pregnancy determines the development and health of the fetus, including its susceptibility to diseases in adulthood [8,18]. During pregnancy, the fetus is exposed to various signals and factors from the mother, such as diet, nutritional status, stress, exposure to toxins, and other environmental factors, which can produce permanent alterations to the development and functioning of the organs and systems of the fetus [19]. In other words, the intrauterine environment of an obese woman can establish an “overfed” fetal state and contribute to the development of certain obesity-related diseases [8], which is considered a predictive response of the fetus to the environment in which it is likely to be born, allowing it to adapt to gain a competitive advantage after birth [20].

The programming of the expansion of adipose tissue cells during the neonatal period favors the adipose tissue capacity to accumulate the excess energy produced by the altered maternal environment [21]; therefore, the commitment and differentiation of mesenchymal stem cells (MSCs) to mature adipocytes determine an altered adipogenic state that is considered to be the primary mechanism in the programming of cardiometabolic alterations in later life in response to maternal obesity or diabetes. It has been shown that maternal obesity programs the MSC secretome, particularly toward reducing the expression of proteins related to redox balance and increasing the expression of proteins associated with lipid synthesis and proinflammatory adipokines, thus predisposing offspring to the development of obesity and metabolic dysfunction [22].

The molecular mechanisms and adipose tissue signals involved in the altered adipogenic state in MSCs are currently under investigation. In humans, Boyle et al. [23] investigated the potential of umbilical cord-derived MSCs from infants born to obese mothers for adipogenesis. These cells exhibited changes in glycogen synthase kinase 3 β (GSK-3 β) activity and reduced nuclear β -catenin content, suggesting that reductions in the activity of the Wnt signaling pathway may be one of the mechanisms for the increased adipogenesis shown by infants of obese mothers. In a recent review, Gyllenhammer and Boyle [24] proposed the use of umbilical cord MSCs as a model for determining the early life causes of obesity.

Therefore, the prevention and treatment of obesity are fundamental to improving quality of life and reducing the risk of diseases related to this condition in offspring. This involves adopting a healthy lifestyle that includes a balanced diet rich in fruits, vegetables, and whole grains, as well as regular physical activity. In recent years, the supplementation of natural alternatives has been shown to have a great impact on the treatment of obesity. Among the options, a series of peptides from animal and plant sources have demonstrated

biological activities producing beneficial effects on health by suppressing appetite, interfering with lipid synthesis, or decreasing their accumulation in adipocytes [25,26]. Adjusting the quality and quantity of proteins or even specific amino acids can be considered a potential therapeutic intervention impacting body composition, metabolic syndrome parameters, and appetite regulation in obese patients. The effects observed vary according to age groups, the amount of protein consumed, its quality (i.e., its digestibility and essential amino acid composition), and the duration of supplementation [27].

The most studied food proteins are those from eggs, fish, beef, algae, and milk whey (MW). The latter has had a great impact in recent years due to its properties for the treatment of obesity [26]. In 2020, Nonogaki and Kaji [28], fed male mice of the C57BL6J strain a high-fat diet and subsequently supplemented it with MW for 13 days, which resulted in a decrease in body weight, food intake, and blood glucose. MW protein is characterized as a “fast” digesting protein that activates postprandial protein synthesis and has beneficial effects on preserving lean body mass in older people. In addition, MW protein shows the greatest satiating properties compared to other protein sources [29]. MW protein and dietary protein sources have also been shown to be equally effective in improving physical performance and cardiometabolic health in obese individuals [27].

A hormone and paracrine factor that shows important increases during the infantile and juvenile stages is the insulin growth factor I (IGF-1), which stimulates bone growth and has anabolic and adipogenic effects. In prepubertal boys, short-term whey protein supplementation produced an insulinotropic effect without changes in IGF-1 [30], while in young rats, the administration of a whey protein hydrolysate increased IGF-1 serum levels and promoted bone growth [31]. Furthermore, whey protein was more efficient in promoting bone growth and bone mineralization than soy protein in prepubertal male rats [32]. With regard to adipogenesis, Hu et al. [33] reported that IGF-1 promotes the migration, proliferation and adipogenesis of adipose stem cells by upregulating Axin-2 and peroxisome proliferation-activated receptor γ (PPAR- γ) and by attenuating Wnt/ β -catenin. IGF-1 likely contributes substantially to the metabolic effects of MW in both humans and experimental animals.

It has been shown that bioactive peptides from MW play an important role in appetite suppression by increasing the secretion of the intestinal hormone cholecystokinin (CCK), which plays a relevant role as a satiety signal in the brain and in the release of two incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [34–36]. The main function of these incretins is to enhance the insulin response to a meal to ensure adequate blood glucose control, helping to maintain a balance in terms of energy and appetite; in addition, the effects of GLP-1 and GIP combine to enhance appetite suppression and slow gastric emptying, promoting satiety [35].

Jakubowicz et al. [37] reported that type 2 diabetic subjects who ingested a whey protein supplement followed by a high glycemic index meal showed a significantly lower increase in glucose plasma levels and higher insulin levels when compared to a control group. In addition, the total and intact plasma levels of GLP-1 also increased, suggesting whey protein enhances the insulinotropic effect of GLP-1. A similar increase in plasma GLP-1 levels after whey protein intake was reported in a study conducted on healthy women [38], as well as after the ingestion of a whey protein hydrolysate rich in calcium by healthy volunteers, which is one of the strongest physiological GLP-1 responses reported in humans [39].

The acute increase in plasma levels of GLP-1 is observed within minutes of MW ingestion. Whey proteins are rapidly digested, and the released amino acids isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, and valine stimulate the enteroendocrine L cells in the distal intestine and ileum to release the incretin hormone

GLP-1 into circulation, producing satiating effects [40]. This hormone and the released amino acids also stimulate pancreatic beta cell insulin secretion.

Interestingly, it has been shown that, beyond glycemic control, GLP-1 modulates the proliferation of human MSCs and inhibits adipogenesis by activating the Wnt/ β -catenin, bone morphogenetic protein 2/Smad, PI3K/Akt, and protein kinase A signaling pathways [41]. Furthermore, GLP-1 showed an in vitro inhibition of adipogenesis and lipogenic genes, as well as increasing the lipolytic genes present in human adipose tissue, suggesting that these changes are related to the improved metabolism observed after GLP-1 treatment in obese subjects or those with type 2 diabetes [42].

After ingesting a meal, the products of digestion—fatty acids, peptides, and amino acids—stimulate the release of GLP-1 by the enteroendocrine L-cells present in the epithelia of the duodenum and distal intestine in the ileum and colon. Vagal terminals that are in proximity to L cells express GLP-1R and are stimulated in a paracrine way by local GLP-1. These vagal afferents convey the information to the area postrema and the nucleus tractus solitarius (NTS) situated in the brain stem. GLP-1 is also produced and released by a small population of NTS neurons [43], which project to brain regions like the arcuate nucleus, containing the anorexigenic neurons, which induce satiety by stimulating pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons. This same area is also where the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons reside [44]. The neuroendocrine effects of GLP-1 are highly relevant due to its multiple functions and the current use of GLP-1 receptor agonists as medications to reduce food intake in obese patients.

In addition to their positive effects on body weight and glucose metabolism, recent findings suggest that bioactive peptides derived from whey proteins influence the gut–brain axis and serotonergic signaling pathways and may exert anxiolytic and stress-modulating effects in both animal models and humans. These peptides have been associated with a reduction in anxiety-like behaviors and lower levels of stress hormones, highlighting their potential neuroprotective role beyond metabolic regulation [45–47].

In summary, in the rat model of metabolic programming caused by maternal obesity, the main alterations that the offspring present during adult life are increased body weight and adipose tissue depots, hyperglycemia, insulin resistance, hypertension, depression, anxiety, and learning and memory difficulties. The evidence suggests that MW proteins provide benefits for body weight, food intake, and metabolic and behavioral parameters in obese subjects. Therefore, the aim of this work is to study the potential post-weaning metabolic and behavioral restorative effects of bovine MW protein isolate in juvenile offspring that have undergone metabolic programming during gestation and lactation due to a hypercaloric diet (HD) consumed by the dam rat during these stages.

2. Materials and Methods

2.1. Animals

Adult male and female Wistar rats (260–300 g) were selected for use and kept in individual cages under standard conditions in a temperature-regulated animal facility (22 ± 1 °C), with a 12:12 h lighting cycle (light–dark), and water and commercial food rodent diet 5001 (LabDiet, St. Louis, MO, USA) available ad libitum. All the animal procedures contributing to this work comply with the ethical standards of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the United States of America (National Research Council, 2010, National Institutes of Health, Bethesda, MD, USA; Animal Welfare Assurance #A5036-01) and the Norma Oficial Mexicana (NOM-062-ZOO-1999, SAGARPA) and have been approved by the institutional Ethics Committee of

Research on Animal Studies of the Escuela Nacional de Ciencias Biológicas, IPN (CEI-ENCB, approval number ZOO-005-2025).

2.2. Maternal Diet Composition

After three weeks of acclimation, female rats were mated with males at a 1:1 ratio for 5 days. During the mating period, pregnancy was confirmed by the presence of a vaginal plug and subsequent monitoring of gestational progression. The pregnant females were housed individually in maternity cages and randomly assigned to one of two groups ($n = 8$ per group): (a) a control diet group (SD) and (b) a hypercaloric diet group (HD). The rats in the SD group were fed ad libitum with a standard commercial rodent diet, 5001 (LabDiet, St. Louis, MO, USA). During the periods of diet adaptation, gestation, and lactation, the dam rats in the HD group received a hypercaloric diet prepared in our laboratory with ingredients acquired from MP Biomedicals (Irvine, CA, USA). The macronutrient composition and energy content of both diets is shown in Table 1. Maternal body weight and food intake were monitored weekly throughout the experimental period, including a 3-week acclimatization period, a 3-week dietary adaptation period before mating, a 1-week mating period, the gestation period (3 weeks), the lactation period (3 weeks), and one week after weaning. Dams were maintained on their respective diets from the 3-week pre-mating period until the end of lactation (Figure 1).

Table 1. Macronutrient composition of the standard commercial diet (SD) and the hypercaloric diet (HD).

	SD (%)	SD (kcal/g)	HD (%)	HD (kcal/g)
Protein	24.1	0.92	19.8	0.76
Carbohydrate total	48.7	1.87	49.4	1.89
Carbohydrate from sucrose	3.2	0.12	34.0	1.31
Fat	6.4	0.57	21.2	1.85
Fiber	12.8	0	5	0
Mineral mix	6.9	0	3.5	0
Vitamins	1.1	0	1.1	0
Total	100	3.3	100	4.5

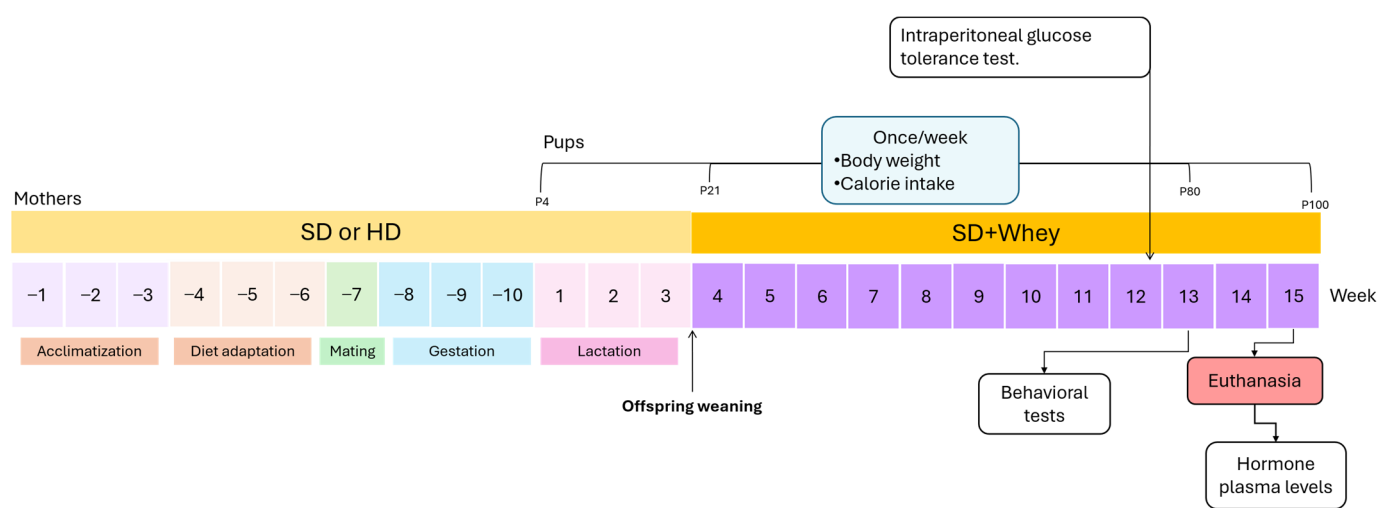


Figure 1. Experimental protocol.

2.3. Offspring Experimental Protocol

On postnatal day (PN) 2, the number of pups was adjusted to 4 males and 4 females per litter. After the lactation period, on PN 21, the offspring were separated from their

mothers and housed in individual cages. From that day onwards (the day of weaning), the study continued only with the male offspring.

Both groups of pup rats, from dam rats fed SD or HD, were divided into 2 subgroups: the SD-SD and HD-SD groups received the standard rodent diet, while the SD-MW and HD-MW groups were fed the standard diet and received bovine MW protein (Hilmar 9010 Bovine Whey Protein Isolate, Hilmar Ingredients, Hilmar, CA, USA) for 12 weeks, which was administered dissolved in tap water (4 g/rat/day) as a supplement (nutritional composition and amino acid profile are shown in Table 2), following the protocol from previous reports [28,48]. Body weight, food intake, and whey consumption (from the SD-MW and HD-MW groups) were recorded individually once a week throughout the experiment. The area under the curve of body weight was also calculated.

Table 2. Nutritional composition and amino acid profile of the Hilmar 9010 Bovine Whey Protein Isolate used for supplementation.

Component	Content
Protein (as is), %	87.5
Protein (dry basis), %	92.0
Fat, %	1.5
Lactose, %	1.0
Ash, %	2.5
Moisture, %	4.8
Energy (kcal/100 g)	375
Cholesterol (mg/100 g)	5
Amino acid profile (g/100 g product)	
Alanine	4.8
Arginine	2.0
Aspartic acid	10.0
Cystine/cysteine	2.3
Glutamic acid	16.3
Glycine	1.5
Histidine *	1.5
Hydroxyproline	<0.1
Isoleucine *†	6.3
Leucine *†	9.7
Lysine *	9.2
Methionine *	2.1
Phenylalanine *	2.8
Proline	5.7
Serine	4.3
Threonine *	6.5
Tryptophan *	1.9
Tyrosine	2.7
Valine *†	5.1

* Essential amino acid. † Branched-chain amino acid (BCAA).

During these weeks, MW protein intake was also measured in the animals that received the supplement. At the end of the experiment, behavioral tests were conducted, and hormone plasma levels and metabolic parameters were determined (Figure 1).

All behavioral tests were conducted by the same experimenter, who was blinded to the experimental groups to minimize observer bias and inter-observer variability.

2.3.1. Motor Activity Test in Open Field

In week 13, the rats were subjected to the open-field test to evaluate motor activity [49], for which a $60 \times 60 \times 65$ cm black box was used, with 16 delineated 15×15 cm quadrants on the floor. The rats were evaluated individually; each rat was placed in the center of the arena and allowed to explore for 5 min. The following variables were recorded: (1) ambulatory movement time (time the rat takes to move through the quadrants); (2) non-ambulatory movement time (time in which the rat grooms itself, scratches, licks itself, or performs any activity that does not involve displacement movements on the ground); (3) immobility time (time the rat remains immobile, calculated by subtracting the time of ambulatory and non-ambulatory movements from the total time spent in the arena); (4) time in the outer quadrants; (5) time in the central quadrants; (6) average speed; and (7) rearing events (number of times the rat stands up on its two hind legs). The variables were recorded using digital recording equipment (Webcam Logitech HD C270, Mexico City, Mexico) installed above the arena. All tests were performed in an isolated room illuminated with conventional white light; at the end of each test the arena was cleaned with 70% ethyl alcohol. The videos were evaluated using the Open Field software (Version 1.0; CINVESTAV, Mexico City, Mexico) and Cowlog software (Version 3.0.2; University of Helsinki, Helsinki, Finland) [50].

2.3.2. Anxiety Test (Elevated Plus Maze)

In week 13, the anxiety level of the animals was assessed. The elevated plus maze is formed by two opposite open arms (10×50 cm) and two opposite closed arms ($10 \times 50 \times 40$ cm) that are connected at a central square (10×10 cm) in a cross shape. These arms are elevated 50 cm from the floor. The walls and floor of the apparatus are black. The test consists of placing each rat in the central square, releasing it, and letting it explore the maze for 5 min. The following variables were recorded: (1) time in closed arms, (2) time in open arms, and (3) total number of crossings. The retention rate in the open arms was calculated, which is the time the rat spends in the open arms divided by the sum of the time it spends in all the arms; as well as the entry rate in the open arms, which is the number of entries into the open arms divided by the sum of all entries into the arms [51]. The animal was considered to have entered a maze arm when all four of its paws passed beyond its boundary. All tests were conducted in an isolated room with moderate lighting, and variables were recorded using digital recording equipment installed above the maze. At the end of each test, the maze was cleaned with 70% ethyl alcohol. The videos were evaluated using the Cowlog software.

2.3.3. Learning and Memory Test (Barnes Maze)

The Barnes maze is a behavioral test used to assess spatial learning and memory in laboratory animals [52]. The test was conducted one day after the anxiety test. The Barnes maze is a device consisting of a circular platform (120 cm in diameter) with 18 equidistant holes (9 cm in diameter) around its perimeter, raised 1 m above the ground. Only one “target” hole leads to a dark, hidden escape box, while others are closed. All tests were performed in an isolated room with external visual cues and bright lighting (150 W). Before the test began, the platform was aligned with external visual cues, and the escape box was always placed in the same hole for the same animal, but in different holes for different animals. Each animal was placed in the center of the maze and allowed to explore for a maximum of 5 min. In each trial, the latency and number of errors in finding the escape box were recorded. The habituation phase was carried out on day 1, the acquisition phase between days 2 and 5, and the memory phase on day 8 without the escape box, assessing permanence in the target quadrant. Variables were recorded using

digital recording equipment (Webcam Logitech HD C270, Mexico City, Mexico) installed above the platform. At the end of each test, the platform and the escape box were cleaned with 70% ethyl alcohol. The videos were evaluated using the Cowlog software.

2.3.4. Intraperitoneal Glucose Tolerance Test

At the end of week 12 of treatment, the offspring rats were subjected to a 12 h overnight fast. Then, a drop of blood was obtained by tail puncture, and the baseline glucose level of each rat was measured with a glucometer Accu-Chek Performa (Roche Diabetes Care GmbH, Mannheim, Germany). This first measurement corresponds to the baseline blood glucose value at time zero; subsequently, a glucose tolerance test was performed. For this purpose, a dose of 2.5 g/kg of body weight of glucose was administered intraperitoneally using a 30% glucose solution. Blood glucose levels were recorded at 15, 30, 60, and 120 min after glucose administration. The values obtained were used to calculate the glucose tolerance curve. The area under the glycemic curve (AUC) was also calculated.

2.3.5. Determination of Serum Concentrations of Hormones and Metabolites

At the end of week 15, the rats were euthanized by an overdose of anesthesia with sodium pentobarbital (90 mg/kg, i.p.) and a rapid decapitation, and trunk blood was collected for biochemical analyses. Blood samples were centrifuged, and the serum was stored frozen at $-20\text{ }^{\circ}\text{C}$ until further analysis. Serum concentrations of insulin and leptin were determined using commercial ELISA kits following the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA; EZRMI-13K, RRID: AB_2783856 and L3410, RRID: AB_260402), corticosterone was quantified using the commercial ELISA kit from Enzo Life Sciences (Farmingdale, NY, USA; ADI-900-097, RRID: AB_2307314), and plasma glucagon-like peptide-1 (GLP-1) was determined using the commercial kit (Millipore Sigma, Burlington, MA, USA; Cat. Nos. EZGLP1T-36K). Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric methods using commercial assay kits (Randox Laboratories Ltd., Crumlin, County Antrim, UK). Serum low-density lipoprotein cholesterol (LDL-C) concentrations were measured using an enzymatic colorimetric assay kit (Spinreact S.A., Sant Esteve de Bas, Girona, Spain). All analyses were performed according to the manufacturers' instructions.

2.3.6. Determination of Brain-Derived Neurotrophic Factor (BDNF) in the Hippocampus

Following euthanasia, the brains were removed, and the hippocampi were dissected and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. For BDNF determination, 50 mg of hippocampal tissue was homogenized in ice-cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4) containing protease inhibitors at a ratio of 1:10 (*w/v*). The homogenate was centrifuged at 12,000 rpm for 15 min at $4\text{ }^{\circ}\text{C}$, and the supernatant was collected for BDNF quantification using a commercial rat ELISA kit (RAB1138-1KT, Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. This kit measures mature BDNF using antibodies generated against the mature domain and generally shows very low cross-reactivity with the precursor protein (proBDNF). Total protein concentration was determined by the Bradford method, and BDNF levels were normalized to total protein content and expressed as pg/mg protein.

2.4. Statistical Analysis

For variables measured over time, one-way or two-way repeated measures analysis of variance (ANOVA) was performed, while variables determined only once were analyzed using two-way ANOVA. When tests for normality and/or equality of variances failed, a Kruskal–Wallis ANOVA on ranks was used. In all cases, a Tukey post hoc analysis was applied to determine the difference between groups. SigmaPlot (version 14.0; Systat

Software Inc., San José, CA, USA) (RRID:SCR_003210) was used to perform the statistical analyses and GraphPad Software (version 8.0; GraphPad Software LLC, San Diego, CA, USA) (RRID:SCR_002798) was used to create the graphs. The significance level was set at $p < 0.05$ for all statistical tests.

3. Results

3.1. Maternal Body Weight and Caloric Intake

Figure 2A shows the maternal body weight during the experimental period. Dams fed the hypercaloric diet (HD) exhibited, starting from week 5, significantly greater body weight than those receiving the standard diet (SD). Accordingly, the area under the curve (AUC) analysis demonstrated a higher cumulative body weight in the HD group (Figure 2B). Similarly, HD dams displayed increased calorie intake from week 4 until the end of the lactation period (Figure 2C), resulting in a significantly greater calorie intake AUC compared with the SD group (Figure 2D).

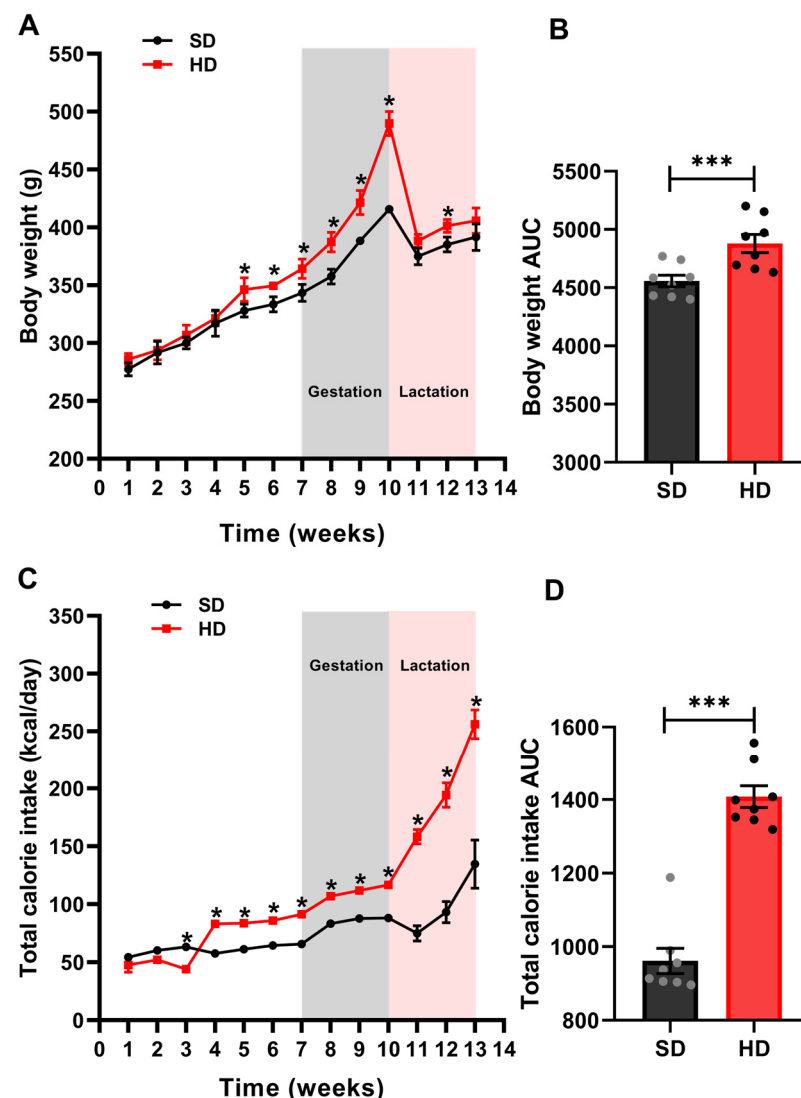


Figure 2. Maternal body weight progression (A), area under the curve for maternal body weight (B), total calorie intake (C), and area under the curve for calorie intake (D) of mothers fed with a balanced rodent diet (SD) or hypercaloric diet (HD) before and during gestation and lactation. Data are means $\pm$ SEM ($n = 8$). Two-way repeated measures ANOVA (A,C) and Student's t -test (B,D) were conducted. * is the difference between HD mothers and SD mothers (A,C) ($p < 0.05$). In (B,D), *** $p < 0.001$.

3.2. Body Weight Gain in Offspring

Figure 3A shows that the body weight gain of the offspring from control mothers that were fed with the standard diet and were supplemented with whey from weaning to postnatal week 15 (SD-MW) was similar to that of the offspring that were fed only with the standard diet (SD-SD). In contrast, the offspring of mothers who were perinatally fed with the hypercaloric diet and from weaning onwards with the standard diet (HD-SD) were heavier throughout the study, with this difference being significant between week 4 and week 11, and there was a significant difference in the area under the curve of body weight gain compared to all other groups (Figure 3B). Interestingly, offspring whose mothers were fed the hypercaloric diet during gestation and lactation and were supplemented with milk whey at weaning (HD-MW) have similar weights in the first 4 weeks compared to those of the HD-SD group, but after week 5, a smaller weight gain is shown in the HD-MW group, reaching values similar to those of the SD-SD group from week 8 onwards and even being significantly lower in the last 2 weeks of the study (Figure 3A).

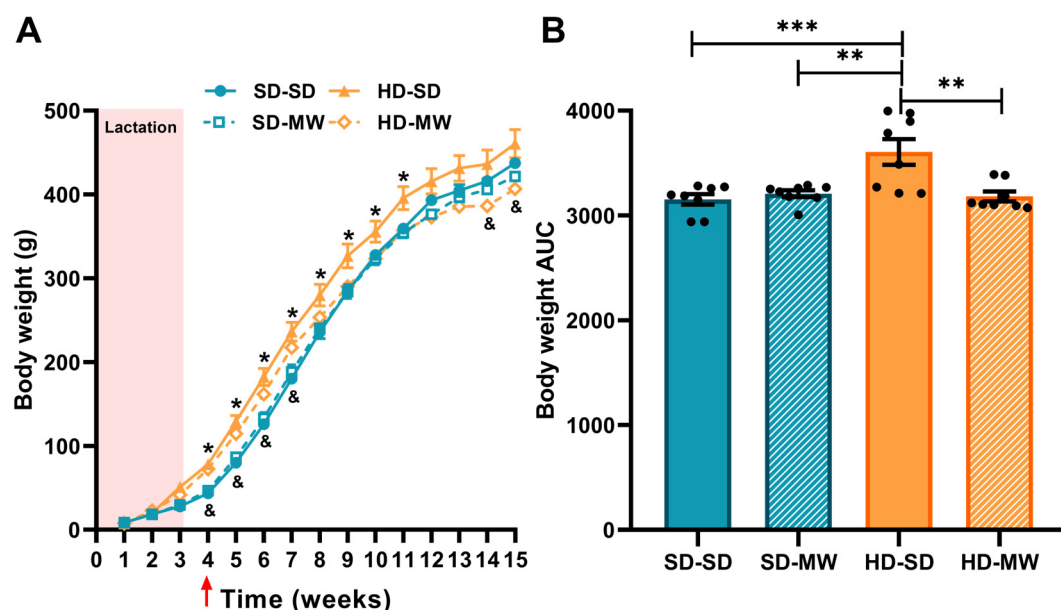


Figure 3. Body weight (A) and area under the body weight curve (B) of pups from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM ($n = 8$). The arrow indicates the start of milk whey administration. Two-way repeated measures ANOVA (A) and one-way ANOVA (B) were conducted, followed by Tukey's post hoc test. * is the difference between HD-SD group and SD-SD group; & is the difference between HD-MW group and SD-SD group ($p < 0.05$). In (B), ** $p < 0.01$, *** $p < 0.001$.

3.3. Total Calorie Intake and Milk Whey Protein Intake in Offspring

Total calorie intake comprises the sum of calories ingested from the diet plus calories provided by MW. From week 4 to week 7, the offspring of the HD-SD and HD-MW groups showed a lower total caloric intake compared to the SD-SD group, while an increase was observed in the SD-MW group from week 4 to week 6 (Figure 4A). A similar pattern in diet caloric consumption was observed in the HD-SD and HD-MW groups during the initial weeks of the experiment (Figure 4B). However, from week 8 onwards, a significant reduction was observed only in the HD-MW group compared to the SD-SD group. Interestingly, this reduction was also present in the SD-MW group. Finally, the HD-MW group showed

a lower MW intake compared to the SD-MW group (Figure 4C). However, from week 10 onwards, caloric intake from MW was similar between these two groups.

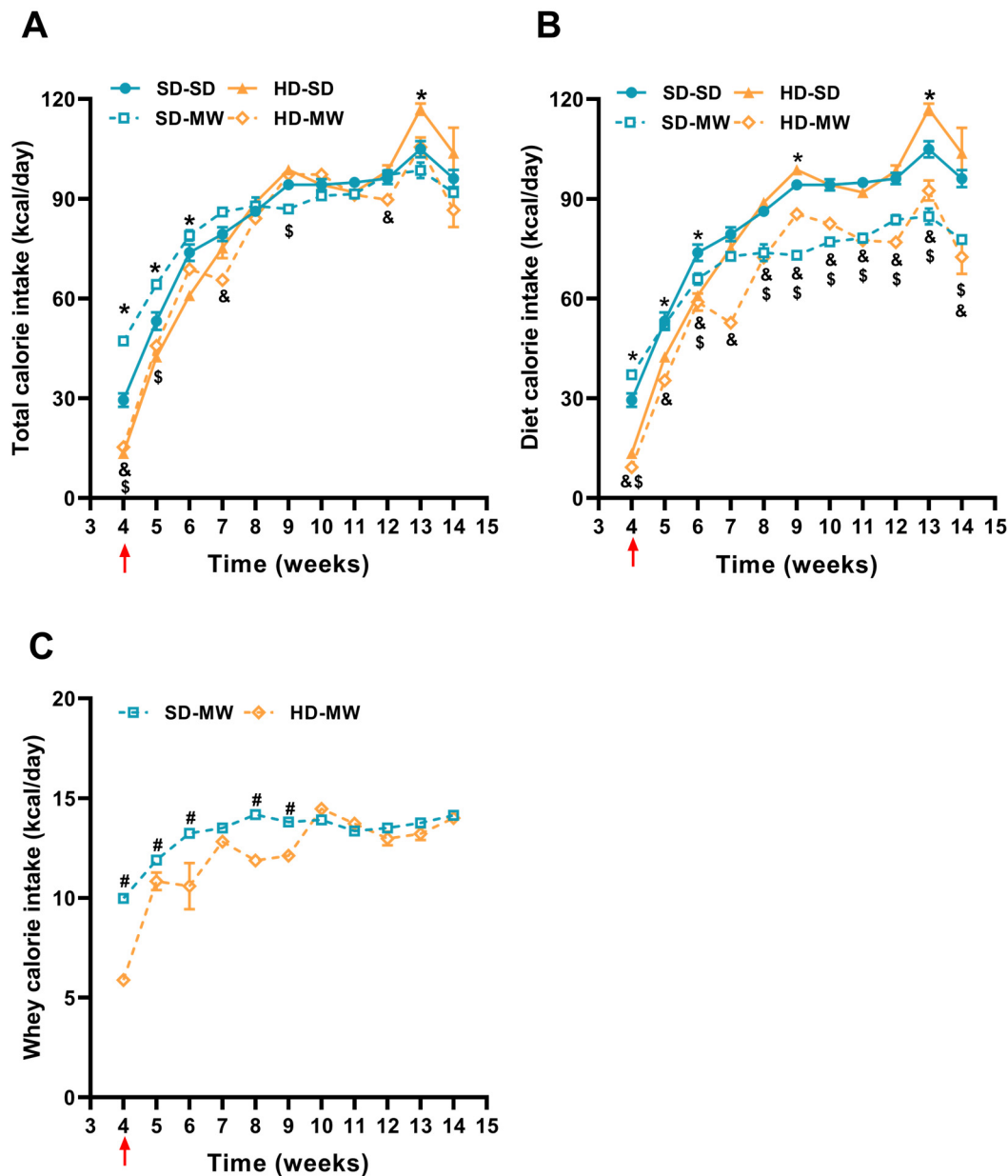


Figure 4. Total calorie intake (kcal) (A), diet calorie intake (B), and whey calorie intake (C) of pups from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM ($n = 8$). The arrows indicate the start of milk whey administration. Two-way repeated measures ANOVA was conducted, followed by Tukey's post hoc test. * is the difference between HD-SD group and SD-SD group; & is the difference between HD-MW group and SD-SD group; \$ is the difference between SD-MW group and SD-SD group; # is the difference between SD-MW group and HD-MW group ($p < 0.05$).

3.4. Glucose Homeostasis

Figure 5A shows that at the end of week 12, the offspring of the SD-SD and SD-MW groups had similar fasting blood glucose levels, while in the HD-SD and HD-MW groups, higher fasting blood glucose levels were shown. Regarding serum insulin concentration

(Figure 5B), animals in the SD-MW group showed a significant decrease compared to the values recorded for the other groups. Rats whose mothers received the hypercaloric diet showed no changes compared to the SD-SD control group, while the HD-MW group showed a non-significant increase in serum insulin compared to the HD-SD group. Concerning serum GLP-1 levels, no significant differences were found between the study groups (Figure 5C). Furthermore, the glucose tolerance test shows that, 15 min after the administration of a glucose solution, the blood glucose levels of the HD-MW group showed an increase compared to the other three groups, being significant only with respect to the SD-SD group. At minute 30, the glycemia of the HD-SD showed the highest values, while the SD-MW and HD-MW groups did not show the same decrease observed in the control animals of the SD-SD group. Finally, blood glucose levels in all groups decreased to similar values at 120 min (Figure 5D). The area under the glucose curve during the glucose tolerance test was significantly higher in the offspring of mothers fed HD compared to those of mothers in the SD-SD group. This increase was not attenuated by MW supplementation in the HD-MW group (Figure 5E).

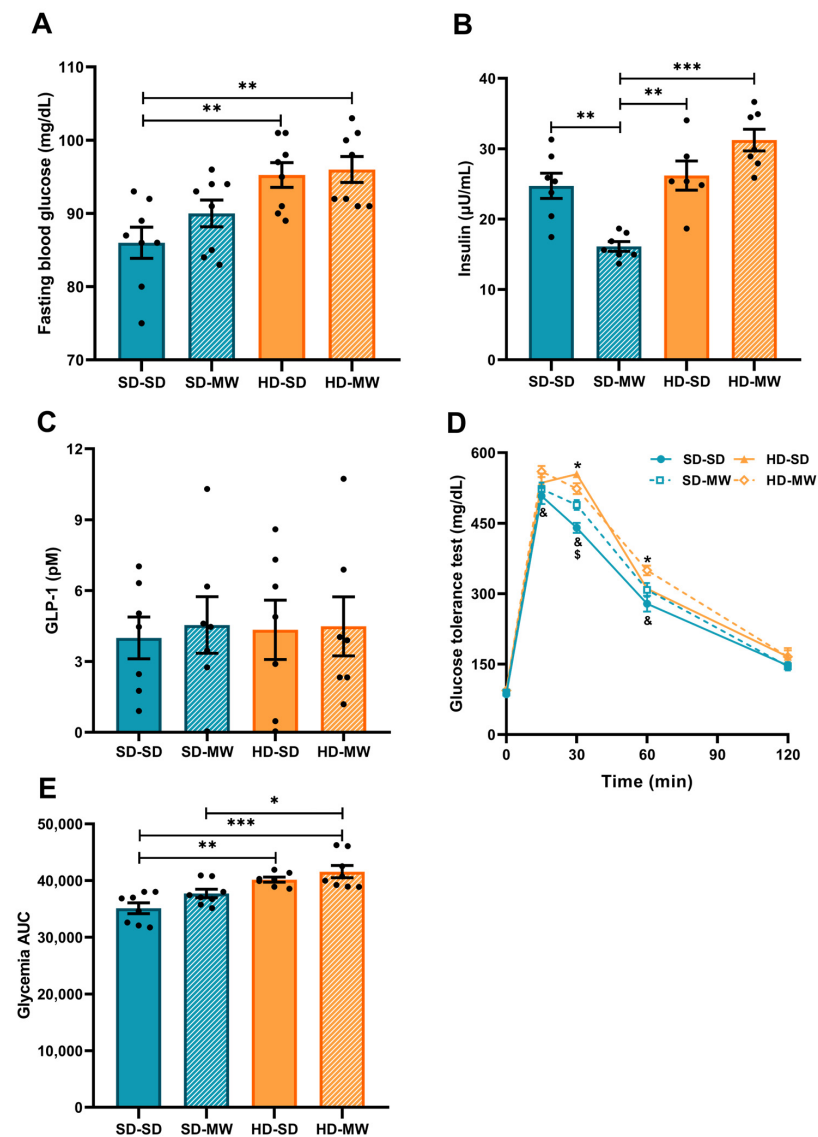


Figure 5. Fasting blood glucose (A), serum insulin concentrations (B), serum GLP-1 concentrations (C), glucose tolerance test (D), and area under the glucose tolerance test curve (E) of pups from control

mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM ($n = 7-8$). Kruskal–Wallis ANOVA on ranks (A), one-way ANOVA (B–D), and two-way repeated measures ANOVA (E) were conducted, followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In (D), * difference between HD-SD group and SD-SD group; & difference between HD-MW group and SD-SD group; \$ difference between SD-MW group and SD-SD group ($p < 0.05$).

3.5. Serum Cholesterol, LDL Cholesterol, Triglyceride and Leptin Levels

The offspring from mothers fed with HD (HD-SD group) showed no difference with respect to the control groups (SD-SD and SD-MW) in serum cholesterol levels; interestingly, the group from mothers fed with HD and supplemented with MW (HD-MW) showed lower cholesterol serum concentrations with respect to the SD-SD and HD-SD groups (Figure 6A). In contrast, in the HD-SD group, an increase in serum LDL cholesterol levels was observed compared to controls, which was partially reduced with MW supplementation (Figure 6B). Regarding serum triglyceride (Figure 6C) and leptin (Figure 6D) levels, no significant differences were found between the study groups. However, for leptin, a trend ($p = 0.057$) toward an increase was observed in all study groups compared to the control group (Figure 6D).

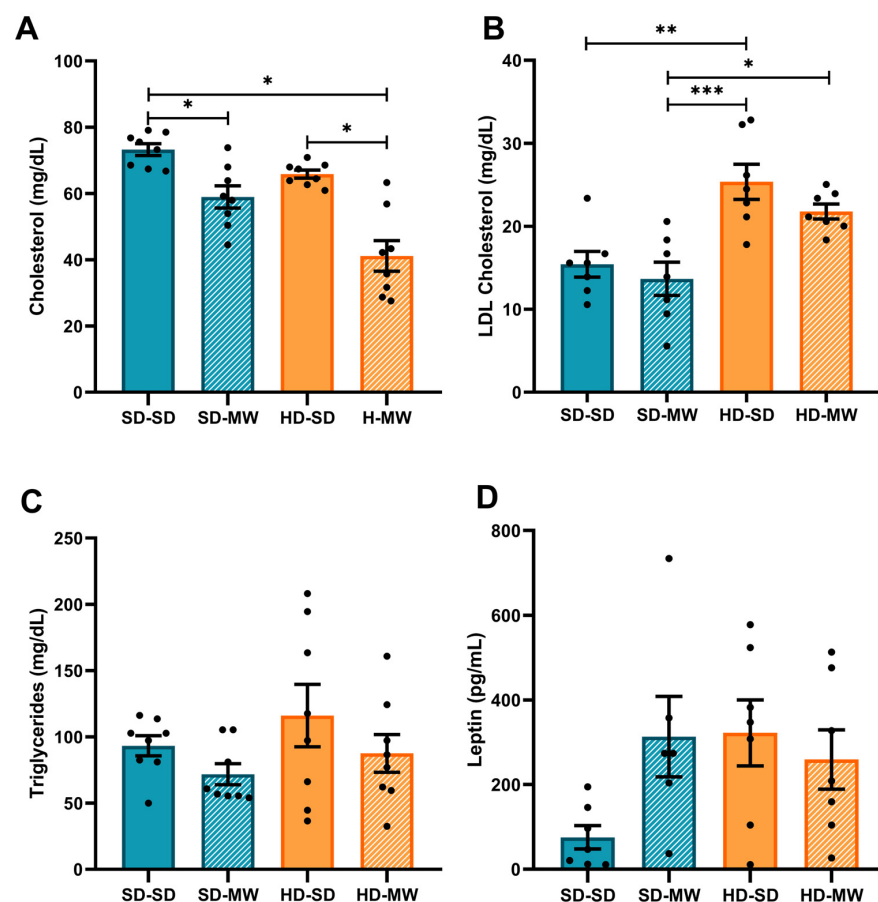


Figure 6. Serum concentration of cholesterol (A), LDL cholesterol (B), triglyceride (C), and leptin (D) of pups from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout

the experiment. Data are means $\pm$ SEM ($n = 7-8$). Kruskal–Wallis ANOVA on ranks (A,C,D) and one-way ANOVA (B) were conducted, followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. Behavioral Tests: Motor Activity in the Open-Field Test

Figure 7 shows the open-field test results. A significant difference was found in the time spent on ambulatory movements in the HD-MW group, which was greater compared to the SD-SD and SD-MW groups (Figure 7A). Significant increases were also found in the average speed in the SD-MW group compared to the other groups (Figure 7D). Similarly, an increase in distance traveled was also found in the SD-MW group, but this difference was only significant compared to the HD-MW group (Figure 7E). Regarding the time spent on non-ambulatory movements and immobility, there were no significant differences between groups (Figure 7B,C). Anxiety behavior is also assessed in the open-field test by measuring behaviors such as standing on hind legs (rearing) or time spent in the central squares of the arena. A lower frequency of these behaviors usually indicates greater anxiety. However, no significant differences were found between the groups in the time spent in the inner quadrants or in the number of rearing behaviors (Figure 7F,G).

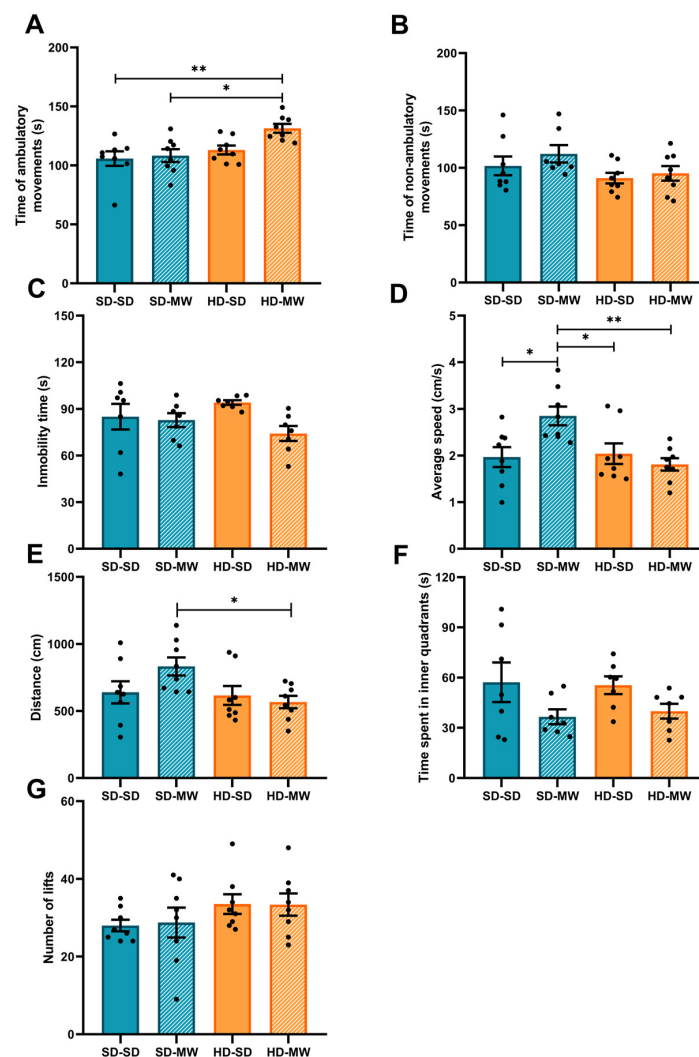


Figure 7. Time of ambulatory movements (A), non-ambulatory movements (B), and immobility (C), along with the average speed (D), distance (E), time in inner quadrants (F), and number of lifts (G) in the open-field test performed on rats from control mothers fed only a balanced rodent diet (SD-SD),

a balanced diet and milk whey supplementation in the water at weaning (SD-MW), hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM, ($n = 8$). One-way ANOVA (A,D,E,G) and Kruskal–Wallis ANOVA on ranks were conducted (B,C,F), followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$.

3.7. Anxiety Test (Elevated Plus Maze)

In this test, higher open-arm retention and open-arm entry rates generally indicate lower anxiety. The HD-SD and HD-MW groups showed significant differences, with a lower open-arm retention rate compared to the control groups (Figure 8A). In contrast, no significant differences were found in the open-arm entry rate, with a trend toward its decrease in the HD-SD and HD-MW groups (Figure 8B). Therefore, it can be suggested that the anxiety level of these groups was higher than that of the SD-SD and SD-MW groups. In rats, corticosterone is a key stress hormone that, when chronically elevated, causes anxiety-like behavior. In this study, serum corticosterone concentration was measured in the blood samples obtained the day of euthanasia, that is, after the anxiety test, and were significantly higher in the SD group that received whey protein and in the groups of pups whose mothers received a high-calorie diet, compared to the SD-SD group. Offspring whose mothers were fed HD (HD-SD and HD-MW groups) showed the highest levels of serum corticosterone compared to the groups of mothers who were fed SD (SD-SD and SD-MW), and MW supplementation did not prevent this increase (Figure 8C). It is worth noting that the SD-MW group also showed a significant increase compared to the SD-SD group, although not as high as those observed in the groups from mothers fed HD (Figure 8C).

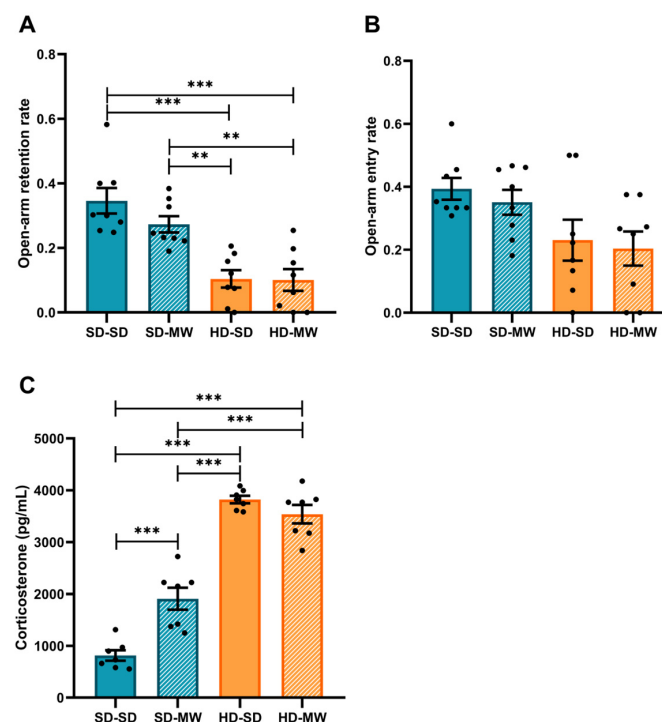


Figure 8. Open-arm retention rate (A) and open-arm entry rate (B) in the elevated plus maze test and serum concentrations of corticosterone (C) of rats from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM, ($n = 8$). One-way ANOVA was conducted, followed by Tukey's post hoc test. ** $p < 0.01$, *** $p < 0.001$.

3.8. Learning and Memory Test (Barnes Maze) and BDNF

In the learning phase, which was carried out over 4 consecutive days with one trial per day, the two-way repeated measures ANOVA detected the main effects of both factors (trial and group) but not a significant interaction between them regarding the time to locate and enter the escape box (Figure 9A,B). The latencies for locating and entering the escape box decreased across the trials, becoming significant in trials 3 and 4 compared to trial 1. Therefore, it can be suggested that learning capacity was present in all study groups. Better performance was observed in the offspring of mothers fed with HD, although significant differences were only found with respect to the SD-MW group, but not with respect to the SD-SD control group (Figure 9A,B). There was also a decrease in the number of errors in finding the escape box throughout the trials in all groups, with a significance change from trial 2 to 4 compared to trial 1; however, the two-way repeated measures ANOVA did not detect a main effect of the group factor or a significant interaction between the trial and group factors (Figure 9C).

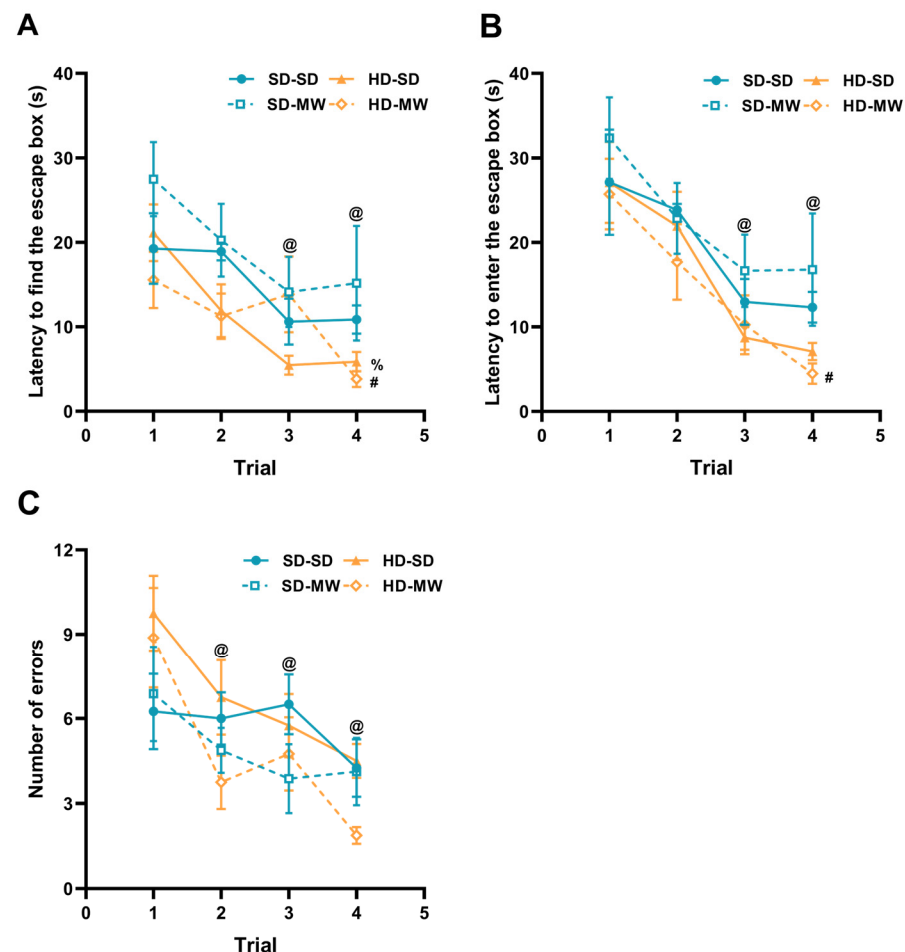


Figure 9. Latency to find the escape box (A), latency to enter the escape box, (B) and number of errors (C) in the Barnes maze test (learning phase) performed on rats from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM ($n = 8$). Two-way repeated measures ANOVA was conducted, followed by Tukey's post hoc test. @ is the differences vs. trial 1; # is the difference between SD-MW group and HD-MW group; % is the difference between SD-MW group and HD-SD group ($p < 0.05$).

In the memory phase, which was performed on the fifth day of the test, the time required by the rats to find the target hole and the time spent in the target quadrant were longer in the HD-MW compared to the HD-SD group (Figure 10A,B); additionally, a significant increase in the time spent in the target quadrant was observed in the HD-MW group compared to the SD-MW group (Figure 10B), while no significant differences were found in the number of errors between the groups (Figure 10C). Finally, mature BDNF levels were significantly lower in the HD-SD group than in the SD-SD group (Figure 10D).

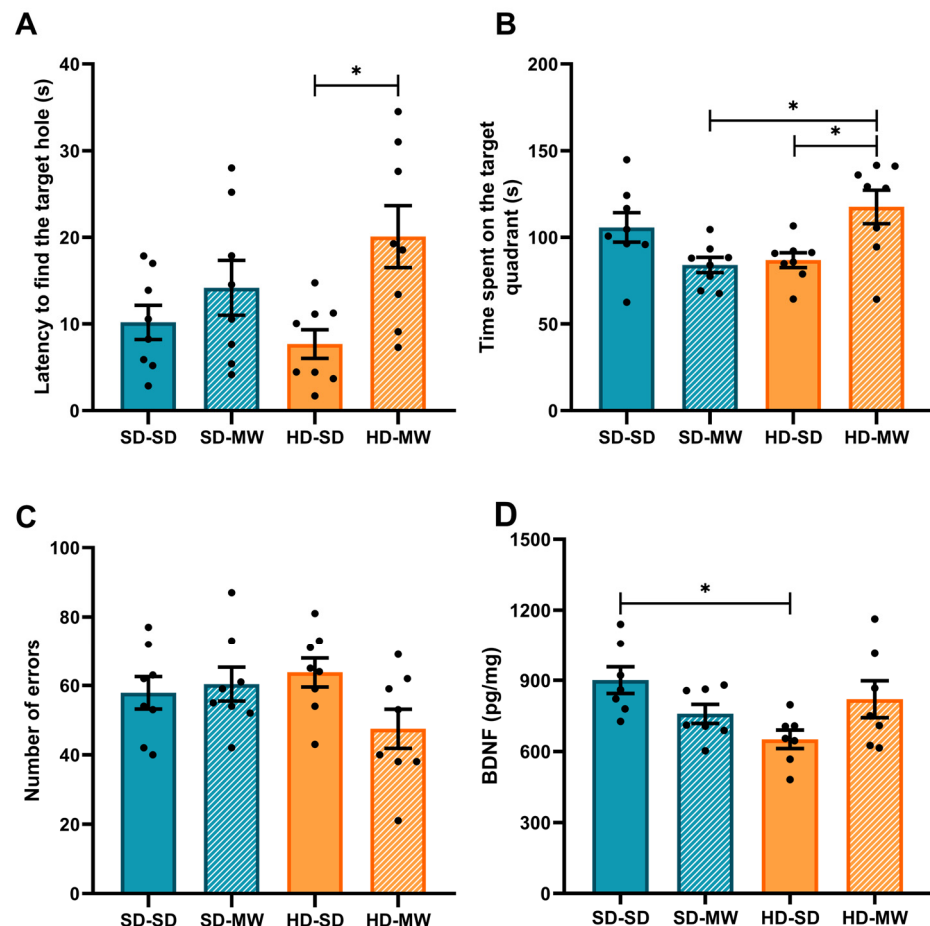


Figure 10. Latency in finding the target hole (A), time spent in the target quadrant (B), and number of errors until the target hole was found (C) in the Barnes maze test (memory phase) and mature brain-derived neurotrophic factor (BDNF) in the hippocampus (D) in rats from control mothers fed only a balanced rodent diet (SD-SD), a balanced diet and milk whey supplementation in the water at weaning (SD-MW), a hypercaloric diet during gestation and lactation and a standard diet at offspring weaning (HD-SD), or a hypercaloric diet during gestation and lactation and a standard diet plus milk whey supplementation at weaning (HD-MW) throughout the experiment. Data are means $\pm$ SEM ($n = 10$). One-way ANOVA was conducted, followed by Tukey's post hoc test. * $p < 0.05$.

4. Discussion

In this study, we characterized the metabolic and behavioral restorative effects of bovine MW supplement against the negative fetal programming of juvenile male offspring. The main findings revealed that MW in offspring from HD mothers decreased body weight, calorie intake, and blood cholesterol. Serum levels of LDL cholesterol and corticosterone, as well as fasting blood glucose and glucose intolerance, were significantly higher in the offspring of HD mothers; however, MW did not affect these increases. The greater increase in serum corticosterone levels shown in the HD-SD and HD-MW groups suggests a basal activation of the hypothalamic–pituitary–adrenal (HPA) axis. The offspring of mothers fed

with HD also showed signs of anxiety that were not reversed by MW, and a reduction in hippocampal BDNF that was partially restored by MW.

Obesity is a highly relevant metabolic risk factor, characterized by an excessive accumulation of adipose tissue, which leads to significant systemic alterations in health [1]. In the context of maternal obesity, being overweight plays a crucial role, since several studies have documented that this condition in the mother during gestation and lactation programs the metabolism of the offspring to a greater risk of developing obesity, insulin resistance, dyslipidemia, and neurobehavioral disorders in later stages of life [17,53–56].

Different strategies have been developed to prevent the negative effects of metabolic programming in offspring, focusing on improving the mother's eating habits during pregnancy and lactation. These nutritional interventions have been shown to be effective in reducing the risk of metabolic disorders in offspring, such as dyslipidemia, insulin resistance, and obesity [55,57]. However, in many cases, programming has already occurred, and the alterations on health are manifested even when the offspring is fed with a normocaloric diet after weaning [56]. In this context, it is important to investigate post-weaning intervention strategies that may counteract the negative programming effects of a maternal high-calorie diet. The literature suggests that functional nutrients, such as bioactive MW peptides, can modulate fat and carbohydrate metabolism, reduce fat accumulation, and improve insulin sensitivity when administered post-weaning, even in pre-programmed organisms [13,58,59].

In this study, we found a clear effect of maternal diet on the programming of offspring metabolism. The offspring of dam rats fed a high-calorie diet (HD-SD) showed a significant increase in body weight gain from weaning onwards compared to the other groups. This is consistent with previous studies that have reported that maternal obesity leads to metabolic programming, with lasting effects on body fat accumulation [53,56]. Interestingly, when animals in the metabolically programmed group received a MW protein supplement at weaning (HD-MW), their body weight showed a smaller increase than in the HD-SD group and reached values similar to those from rats fed with the standard diet (SD-SD and SD-MW). This finding suggests that MW protein intervention after weaning may attenuate the programmed effects of maternal obesity on offspring body weight. The absence of differences between the SD-SD and SD-MW groups also suggests that MW does not significantly alter body weight in animals without metabolic programming, underscoring a modulating, rather than reducing, role on body weight. Taken together, these results are consistent with studies that have shown that MW protein improves energy metabolism and reduces body weight and calorie intake [13,58,59].

The perinatal period is a critical window that plays an important role in fetal adipocyte development. Reduced Wnt signaling pathway activation has been proposed as one mechanism for the greater adipogenesis shown by infants from obese mothers. Umbilical cord-derived MSCs exhibit reduced cytosolic β catenin, which is constitutively targeted for degradation by GSK-3 β -mediated phosphorylation in several serine/threonine residues with a consequent increased adipogenesis [23]. Additionally, the MSC secretome is programmed to an altered profile, reducing the expression of proteins related to redox balance, and increasing those associated with lipid synthesis and proinflammatory adipokines, thus predisposing offspring to the development of obesity and metabolic dysfunction [22]. Future studies should be performed to determine the possible implication of this pathway in the body weight reduction produced by the MW supplement.

The groups that received MW (SD-MW and HD-MW) after weaning showed different patterns of intake for this supplement. In the SD-MW group, MW consumption was significantly higher than in metabolically programmed animals in the first six weeks of treatment, which could be due to greater sensory capacity or preference for the flavor of

MW in non-programmed animals, which reflects adequate acceptance of MW. In humans fed with formula, which implies postnatal protein overfeeding, leucin-stimulated insulin secretion and protein synthesis are stimulated via the mechanistic target of rapamycin complex 1 (mTORC1) and enhanced hepatic IGF-1 secretion. The overactivation of mTORC1 and S6 kinase 1 (S6K1) inhibits the Wnt/ β catenin signaling pathway, thus promoting an increase in the number of preadipocytes. These epigenetic changes accelerate the expansion of adipocyte stem cells during postnatal white adipose tissue development, giving rise to the early protein hypothesis of childhood obesity [60]. In our study, the SD-MW rats did not show increases in body weight during the ingestion of MW protein in comparison to control rats due to a homeostatic reduction in chow ingestion. Previous studies have shown that the voluntary intake of protein-rich solutions can be modulated by specific satiety mechanisms, such as the activation of the gut-brain axis by intestinal peptides. These mechanisms could help regulate food intake in the groups receiving MW, promoting self-regulation of energy intake [61,62].

Fasting blood glucose levels showed statistically significant differences between groups, especially in the HD-SD and HD-MW groups, which presented the highest baseline glucose levels compared to the SD-SD control group. These data suggest that the high-calorie diet fed to the mother caused negative programming of glucose metabolism and increased the risk of glucose disorders in offspring. In addition, insulin levels are higher in the HD-MW group, suggesting that whey protein supplementation after weaning did not significantly reduce fasting glucose or insulin levels compared to the HD-SD group, suggesting a partial or limited efficacy of this intervention on basal glucose homeostasis. Additionally, in the glucose tolerance test, the glycemic profiles after glucose administration showed a typical pattern of rise and progressive return to baseline levels in all groups, although the HD-SD and HD-MW groups showed higher blood glucose levels at 30 and 60 min compared to the control groups. Regarding the area under the curve for this test, the values were also higher in the groups of mothers fed with HD compared to those of mothers fed with SD, but there was no difference caused by MW supplementation. These results suggest that MW supplementation was not sufficient to improve the body's response to hyperglycemia in negatively programmed animals. This is consistent with studies that have shown that maternal obesity can alter insulin signaling and glucose transporter (GLUT4) expression in peripheral tissues, and that postnatal nutritional interventions, such as bioactive MW peptides, can partially modulate this dysfunction, although they do not always completely reverse the phenotype, as observed in this study [63–65].

In a recent study, Ibáñez et al. [66] showed that adipose tissue and metabolic programmed alterations elicited by maternal obesity have a sexual dimorphic profile. Male offspring of obese mothers had greater fat mass, serum leptin, triglycerides, glucose, and insulin resistance than females. Female offspring showed an adipogenic expression profile with a greater capacity for expansion compared to males in retroperitoneal adipose tissue, with newly differentiated adipocytes and lower lipid levels, making female rats less insulin resistant. In male offspring, the retroperitoneal adipose tissue showed downregulation of genes related to the Wnt signaling pathway accompanied by hypertrophy and insulin resistance. Genes related to the PI3K/Akt signaling pathway were also downregulated and may mediate the insulin resistance observed in male rats [66]. A limitation of our study is the exclusive use of male rats; therefore, further research is needed to determine the differential effects of MW supplementation on the metabolism of programmed female rats.

The Wnt signaling pathway has also been proposed as a mechanism for neuroendocrine control of the hypothalamus, a brain structure that regulates energy balance and whose dysfunction has been implicated in metabolic disorders such as type 2 diabetes and obesity. The main genes of the Wnt pathway are expressed in the arcuate nucleus of

mouse hypothalamus, and the inhibition of GSK-3 β activity improved insulin signaling by activating the PI3K/Akt signaling pathway, which was also activated by leptin [67]. The glucose-lowering effects of leptin depended on the activation of the Wnt coreceptor LRP-6. The inhibition of the orexigenic NPY neurons and the inhibition of GSK-3 β caused by leptin also sensitized insulin signaling and contributed to the neuroendocrine control of energy homeostasis [67]. In our study, the tendency toward higher levels of insulin, increased glucose, and glucose intolerance shown by the HD-SD group suggested a state of insulin resistance in the hypothalamus that was not improved by the MW supplement.

The lower total and dietary caloric intake, as well as the lower body weight, shown by the HD-MW rats indicate that GLP-1 should be increased in this group of rats and be responsible for these changes. Surprisingly, GLP-1 serum levels did not differ between groups. A possible explanation is that serum samples were obtained in fasting conditions. As previously described, GLP-1 release and its satiating effect are increased acutely after food ingestion. Nevertheless, the ingestion of a food enriched with 24% whey protein by Sprague Dawley rats for 10 weeks resulted in reductions in food intake and body weight, as well as a significant increase in plasma GLP-1 levels [58], suggesting that higher quantities of whey protein and blood sampling in fed conditions are needed to observe the expected increase in GLP-1 serum levels.

With respect to adipogenesis, the bioconversion of whey proteins by lactic acid bacteria reduce intracellular lipid accumulation in 3T3-L1 preadipocytes and downregulate the adipogenesis-related transcription factor PPAR γ , which sequentially upregulates adipocyte fatty acid binding protein (aP2) and lipoprotein lipase (LPL), genes that control fatty acid accumulation in adipocytes, suggesting that whey-derived bioactive compounds decrease the adipogenic differentiation of 3T3-L1 cells [68].

The lower levels of total serum cholesterol and tendency toward lower LDL cholesterol in the HD-MW group suggest a hypocholesterolemic effect of MW protein supplementation in animals with negative metabolic programming and lipid profile modulating properties, especially in organisms that have been previously exposed to an unfavorable intrauterine and early postnatal environment. This effect is attributed to the presence of bioactive peptides such as lactoferrin, α -lactalbumin, and glycomacropeptide, which can influence the expression of liver genes associated with cholesterol synthesis and utilization, such as HMG-CoA reductase and LDL receptors [69–71].

Fetal exposure to excess maternal fat and obesity alters the neurochemical architecture of the developing brain. This occurs through hormonal, inflammatory, and metabolic changes that affect neurotransmitter pathways and the regulation of appetite, emotions, and cognition [17,54]. Several studies have shown that a high-calorie diet during the early stages of development can alter motor activity, as measured in the open-field test. Some studies report an increase in exploratory and motor activity in offspring [9,72], while others describe a decrease in these behaviors, which could be related to changes in the stress axis or in dopaminergic circuits [73]. Likewise, there are studies in which no significant changes in motor activity are observed [74]. This suggests that the effects of fetal programming depend on the experimental model and factors such as sex, age, and the duration of maternal exposure to the HD. Here, the HD-SD group showed no differences compared to the SD-SD control group, while a slight increase in locomotion was observed in the HD-MW group. The above is probably due to MW protein supplementation, as the SD-MW group showed increased distance covered and average speed, which could be explained by the protein composition of MW, particularly its high α -lactalbumin content, a tryptophan-rich protein that promotes serotonin synthesis in the central nervous system. Diets enriched with α -lactalbumin have been shown to increase serotonin release and modify exploratory behavior, as observed in the open-field test [75,76].

A maternal high-calorie diet is known to alter neurogenesis and serotonergic signaling in the fetal brain, predisposing to anxiety and depressive behaviors [17,77]. The assessment of anxiety behavior using the elevated plus maze in the offspring detected that the HD groups showed a significant reduction in open-arm retention rate and a similar trend as in the open-arm entry rate compared to the SD-SD and SD-MW groups. This indicates a general increase in anxiety-related behavior associated with metabolic programming and evidence that MW supplementation was not effective in counteracting this increase caused by the maternal HD. This is consistent with previous studies that have shown that maternal obesity can affect the development of limbic circuits associated with the control of emotions, especially in regions such as the amygdala and hippocampus, and that these alterations can persist into adulthood in offspring [78,79]. Furthermore, although some whey peptides have been proposed to act as natural anxiolytics, their effectiveness appears to depend on the physiological context and the baseline state of the HPA axis [80].

In relation to our results, the greater increase in serum corticosterone levels shown in the HD-SD and HD-MW groups suggests a basal activation of the HPA axis in animals with negative metabolic programming. This finding is consistent with studies reporting HPA axis hypersensitivity in offspring exposed to nutritionally unfavorable environments during fetal and neonatal development, which could contribute to the development of metabolic disorders, anxiety, and neuroendocrine dysfunction in adulthood [81,82]. Taken together, these results suggest that, although MW showed rehabilitative effects on key metabolic parameters, such as body weight, calorie intake, and lipid profile, its capacity to regulate the neuroendocrine response to programmed stress may be limited and require a more prolonged intervention or a combination with other therapeutic approaches.

Using rodent models, it has been suggested that the metabolic programming caused by HD during pregnancy and lactation may alter the neurobehavioral development of offspring and increase the predisposition to developing anxiety-like behaviors later in life associated with alterations in neurobiological systems related to mood, including changes in the HPA axis, inflammatory processes, and neurotransmitter systems such as serotonin and dopamine [83,84]. Diets enriched with whey protein have been shown to increase tryptophan availability and serotonergic activity in the brain, which may improve motivation and reduce anxiety behavior in animal models and humans [75,85].

Although various studies have indicated that perinatal exposure to a high-fat diet can affect the learning and memory capacity of offspring, especially through alterations in the hippocampus [73,77,86,87], in the present study, we observed a significant reduction in hippocampal BDNF levels in offspring from hypercaloric diet-programmed dams compared to controls. However, this molecular alteration was not reflected in behavioral performance, as no significant differences were observed in learning and memory tests between the programmed HD-SD group and the control group (SD-SD). Interestingly, improved performance was observed in the learning phase of the Barnes test in the HD-MW rats, but only compared to the SD-MW group and not to the SD-SD control group. These findings are consistent with studies in which the effects of metabolic programming are mainly manifested in emotional or motivational behaviors, such as anxiety, depression, or changes in motor activity, without necessarily affecting cognitive performance [88,89].

It has also been reported in developmental programming models that reduced levels of hippocampal BDNF following maternal exposure to a high-fat diet precedes behavioral deficits in hippocampus-dependent tasks [77]. Furthermore, these BDNF deficits were linked to delayed spatial learning in mouse offspring; however, the Barnes maze test demonstrated that offspring of mothers fed a high-fat diet showed impaired acquisition of spatial learning in the juvenile period (PD 21) but not in the adult period (PD 70), indicating that the effects of a high-fat maternal diet on learning may be more prominent in early

life, which is consistent with our results in the HD-SD group. Taken together, the changes induced by a high-calorie maternal diet on the learning and memory capacity may depend on several factors, including the study period, the age and sex of the animals, feeding after weaning, and the sensitivity of the behavioral tests used.

Among the limitations of this study is the exclusive use of a male rat model, which means that possible sex-specific differences in response to programming or intervention were not assessed. Moreover, the duration of MW supplementation was limited to the period immediately following weaning, so it cannot be ruled out that a longer or earlier intervention would have shown additional effects, especially at the neurobehavioral level.

5. Conclusions

A high-calorie maternal diet during pregnancy and lactation caused persistent metabolic and neurobehavioral alterations in the offspring. Although whey supplementation after weaning effectively improved several epigenetically programmed metabolic biomarkers, it did not completely reverse anxiety-like behavior or corticosterone abnormalities. These results suggest that milk whey supplementation may be a valuable nutritional strategy for mitigating the metabolic consequences of developmental programming, while also highlighting that neuroendocrine alterations may require earlier intervention or multimodal therapeutic approaches.

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Data Availability Statement: The raw and processed data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
CCK	Cholecystokinin
GIP	Glucose-dependent insulintropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter 4
GSK-3 β	Glycogen synthase kinase 3 β
HD-SD	Group which received the standard rodent diet at the weaning from mothers fed a high-calorie diet during gestation and lactation
HD	Hypercaloric diet

HD-MW	Group which was fed the standard diet and received the MW protein from mothers fed a high-calorie diet during gestation and lactation
HPA	Hypothalamic–pituitary–adrenal
IGF-1	Insulin growth factor I
LDL	Low-density lipoprotein
MSCs	Mesenchymal stem cells
NPY	Neuropeptide Y
PN	Postnatal day
PPAR- γ	Peroxisome proliferation-activated receptor γ
SD	Standard diet
SD-MW	Group which was fed the standard diet and received the MW protein from mothers fed a standard diet during gestation and lactation
SD-SD	Group which received the standard rodent diet from mothers fed a standard diet during gestation and lactation

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