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Early protein malnutrition negatively impacts physical growth and neurological reflexes and evokes anxiety and depressive-like behaviors

Laura M. Belluscio, Bruno G. Berardino, Nadina M. Ferroni, Julieta M. Ceruti, Eduardo T. Cánepa *

Laboratorio de Biología Molecular, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pabellón II, 1428 Ciudad de Buenos Aires, Argentina

HIGHLIGHTS

- Malnourished dams exert less maternal care and show an anxious-like behavior.
- Motivation and neuromuscular coordination are diminished in protein malnourished mice.
- Early malnutrition promotes anxiety and depression-related behaviors.
- Perinatal protein malnutrition delays progeny's physical growth and neurodevelopment.
- Male and female mice's neurodevelopment and behavior are affected by malnutrition.

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ABSTRACT

Malnutrition is a worldwide problem affecting millions of unborn and young children during the most vulnerable stages of their development. In humans, poor maternal nutrition is a major cause of intrauterine growth restriction which is associated with an increased risk of perinatal mortality and long-term morbidity. In addition, intrauterine growth restriction correlates with neurodevelopmental delays and alterations of brain structure and neurochemistry. While there is no doubt that maternal malnutrition is a principal cause of perturbed development of the fetal brain and that all nutrients have certain influence on brain maturation, proteins appear to be the most critical for the development of neurological functions. In the present study we assessed male and female mouse offspring, born to dams protein restricted during pregnancy and lactation, in physical growth and neurobehavioral development and also in social interaction, motivation, anxiety and depressive behaviors. Moreover, we evaluate the impact of the low protein diet on dams in relation to their maternal care and anxiety-related behavior given that these clearly affect pups development. We observed that maternal protein restriction during pregnancy and lactation delayed the physical growth and neurodevelopment of the offspring in a sex-independent manner. In addition, maternal undernutrition negatively affected offspring's juvenile social play, motivation, exploratory activity and risk assessment behaviors. These findings show that protein restriction during critical periods of development detrimentally program progeny behavior.

1. Introduction

Maternal nutrition plays a critical role in the offspring's physical growth and behavior. Malnutrition is a worldwide problem affecting millions of unborn and young children during the most vulnerable stages of their brain development [1–3].

An inadequate diet during the perinatal period of life can negatively influence the development of the brain resulting in changes in its structure and hence functioning [4,5]. In humans, poor maternal nutrition is a major cause of intrauterine growth restriction which is associated with an increased risk of perinatal mortality and long-term morbidity. In addition, intrauterine growth restriction is associated with neurodevelopmental delays and alterations in brain structure and neurochemistry [6,7]. Epidemiological studies in human offspring have demonstrated negative influences of insufficient perinatal nutrients on cognition and behavior [3].

While there is no doubt that maternal malnutrition is a principal cause of perturbed development of the fetal brain and that all nutrients have certain influence on brain maturation, proteins appear to be the most critical for the development of neurological functions [8]. The nutrient value of proteins in the diet resides essentially in the individual amino acids that have been absorbed by the maternal digestive system.

Abbreviations: LP, low protein; NP, normal protein; PD, postnatal day.

* Corresponding author at: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria Pabellón II Piso 4, 1428 Ciudad de Buenos Aires, Argentina. Tel./fax: +54 11 45763342.
E-mail address: ecanepa@qb.fcen.uba.ar (E.T. Cánepa).

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Various amino acids are precursors of neurotransmitters and peptide hormones or, in many cases, are neurotransmitters themselves [9,10]. Therefore, it is obvious that the broad involvement of amino acids in the functions of the central nervous system go considerably beyond their simple role in protein synthesis.

Experimental studies in rodents have assessed the consequences of malnutrition on brain development and behavior. Protein malnutrition in early stages of life could alter neurogenesis, cell migration, differentiation and plasticity [11–14]. In addition, reported effects of pre- and/or postnatal protein malnutrition on the offspring include changes in exploratory behavior and anxiety, reduced social interaction, altered learning and memory abilities and a different response to aversive stimuli [15–20]. However, alterations in physical growth and neurological reflexes have received less consideration [4,21,22]. In addition, little is known about the gender-dependent effects of maternal protein undernutrition [23].

The above-mentioned findings on anxiety, learning and motivation are indicative of the negative behavioral effects on the offspring following inadequate protein intake in early life. However, most previous studies on the effects of insufficient perinatal protein intake during development have been carried out with a two-third reduction in protein from the control diet. Therefore we investigated the effects of a 55% reduction in perinatal protein on female offspring behavior. This level of protein reduction more accurately reflects the human dietary deficiency in developed countries [16,24].

Both animal and human studies indicate that a combination of prenatal and postnatal malnutrition is more detrimental than a nutritional insult occurring during either period alone [8]. However, most studies also show that prenatal malnutrition results in greater permanent mental deficiencies than postnatal malnutrition. Furthermore, pregestational combined with gestational malnutrition results in more severe effects on behavior than gestational malnutrition alone [25,26]. Considering the high incidence of nutritional insecurity within the low-income population, it is probable that most of the children in this low socio-economic group have sustained nutritional deprivation during pregnancy with many individuals continuing to be malnourished during lactation. For this reason considering protein malnutrition in both periods is more realistic. Also, it is important to consider that brain development in rodents occurs later than in humans, being the first ten days of postnatal development in rats equivalent to the prenatal growth during the third trimester in humans [27,28].

The vulnerability to psychopathologies is determined by the interaction between genes and the environment. Genes are affected by the environment during selective critical developmental periods [29,30]. Gender is another important factor in this equation, but its contribution at a mechanistic level has been largely ignored. The incidence of several neurodevelopmental diseases largely differs between sexes: while autism and schizophrenia are more prominent in males than in females, major depressive disorder (MDD) affects twice as many women as men [31]. However, despite an obvious sex bias in these diseases, little has been examined as to how such early-life experiences may alter perinatal brain development and plasticity. Therefore, understanding the underlying neurobiological mechanisms by which the sex-specific vulnerability arises is of fundamental importance.

In the present study, we assessed the physical growth and neurobehavioral development as well as the social interaction, motivation, anxiety and depressive behaviors of male and female mouse offspring, born to mothers protein-restricted during pregnancy and lactation. In addition, we evaluated the impact of the low-protein diet on dams, in relation to their maternal care and anxiety-related behaviors, given that these behaviors clearly affect the development of pups. We hypothesized that maternal protein restriction during pregnancy and lactation would negatively affect neurodevelopment and subsequent behavior in male and female offspring.

2. Materials and methods

2.1. Animals and diets

CF-1 mice from the colony of the Bioterio Central, Facultad de Ciencias Exactas y Naturales (University of Buenos Aires), were used for all experiments. The diets employed in this study were prepared according to the AIN-93 Final Report [32]. Nutrient composition of normal protein (NP) and low protein (LP) manually prepared-diets are indicated in Table 1. NP and LP diets contain 20% and 9% of casein as the sole source of proteins, respectively.

Female and male mice (F_0) were fed with standard laboratory chow diet (SC) until five days prior to mating when they were fed with NP diet. SC diet is the commercially available nourishment for laboratory rodents. For mating one male was housed with two nulliparous females for ten consecutive days, after which every mouse was individually housed. Females were inspected for the presence of a vaginal plug in order to estimate the day of conception.

Parents were fed either with the NP or the LP diet during the mating period, after which dams were kept on the same diet during pregnancy and lactation. Male and female parents were weighed at 10:00 a.m. every day. Efforts were made to balance the litter size within a range of 8 to 9 pups per dam on PD2 (NP: average = 8.7; LP: average = 8.3). From PD1 to PD13, pups were marked with 1% methyl violet solution on the skin. From PD13 to PD21, they were identified with black ink on the tail. Marks were replaced every three days.

Dams that were subjected to the elevated plus maze and the open field tests after weaning were kept under the same diet until the completion of these tests. Pups were weaned at postnatal day 21 (PD21), from which were fed with SC diet.

Animals were kept in a 12:12 h light:dark cycle with lights on at 6 a.m., and food and water were administered ad libitum. Experiments were performed in accordance with local regulations and the National Institutes of Health (NIH) *Guide of the Care and Use of Laboratory Animals* (NIH publication 80-23/96) and were previously approved by the Ethical Committee (CICUAL) of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Maternal behavior testing

Maternal care was evaluated from PD1 to PD7. Cages were placed in the first column of the rack with the identification card taped to the lid, in order to facilitate a complete view of the inside of the cage, without disturbing the mother's behavior. Every day, dams were observed for 1 h from 9 a.m. to 10 a.m. before daily developmental and weight

Table 1
Dietary composition (weight and calories).

Ingredient	Normal protein (NP)		Low protein (LP)	
	Weight (g/kg)	Calories (kcal/kg)	Weight (g/kg)	Calories (kcal/kg)
Cornstarch	529.50	2118.00	639.50	2558.00
Casein	200.00	800.00	90.00	360.00
Sucrose	100.00	400.00	100.00	400.00
Alpha cellulose	50.00	0.00	50.00	0.00
Soybean oil	70.00	630.00	70.00	630.00
Mineral mixture (AIN-93G-MX)	35.00	0.00	35.00	0.00
Vitamin mixture (AIN-93-VX)	10.00	40.00	10.00	40.00
L-Cystine	3.00	12.00	3.00	12.00
Choline bitartrate	2.50	0.00	2.50	0.00

measures were performed. During this hour mother's activity was recorded by an observer every minute and classified in one of the following categories: arched-back posture over the pups, licking and grooming of the pups, no nursing posture in the nest, sniffing the pups, sniffing the environment in the nest, digging and building the nest, carrying pups to the nest, self grooming in the nest, moving the pups with the muzzle, drinking, eating, rearing, climbing, self-grooming, sniffing environment, sleeping outside the nest, carrying its own tail with the mouth (considered a form of displaced maternal behavior, present mainly in pregnant rats), chewing anything other than food (e.g. cage bars). Data from the seven days was averaged for each mother.

F_0 dams were also subjected to the elevated plus maze and open field tests, these tests were performed as described below for the F_1.

2.3. Developmental landmarks

Pups were weighed every other day. For the assessment of physical growth, the following changes were observed and recorded for each individual: dorsal and ventral fur emergence, ear lobe detachment, opening of the auditory canal and eyes opening. In order to reduce the stress throughout tests, pups were gently manipulated on a heating pad during no more than 3 min each.

Physical parameters were expressed as the postnatal days required for the appearance of these milestones. The anogenital distance was measured on PD1. Mice were placed in a sitting position on a transparent acrylic plate 10 cm above the working table. A picture was taken from below the acrylic plate and the anogenital distance was measured digitally with the Adobe Photoshop CS software. Pixels were converted into distance by taking a photo to a ruler placed on the acrylic plate and using it as a scale.

2.4. Neurological reflexes

Surface righting reflex: Mice were placed on their backs and let them turn over on to ventral surface and place all four paws in contact with the surface. Latency to reach the prone position was recorded with an upper limit of 60 s. This reflex was measured every day from PD2 until

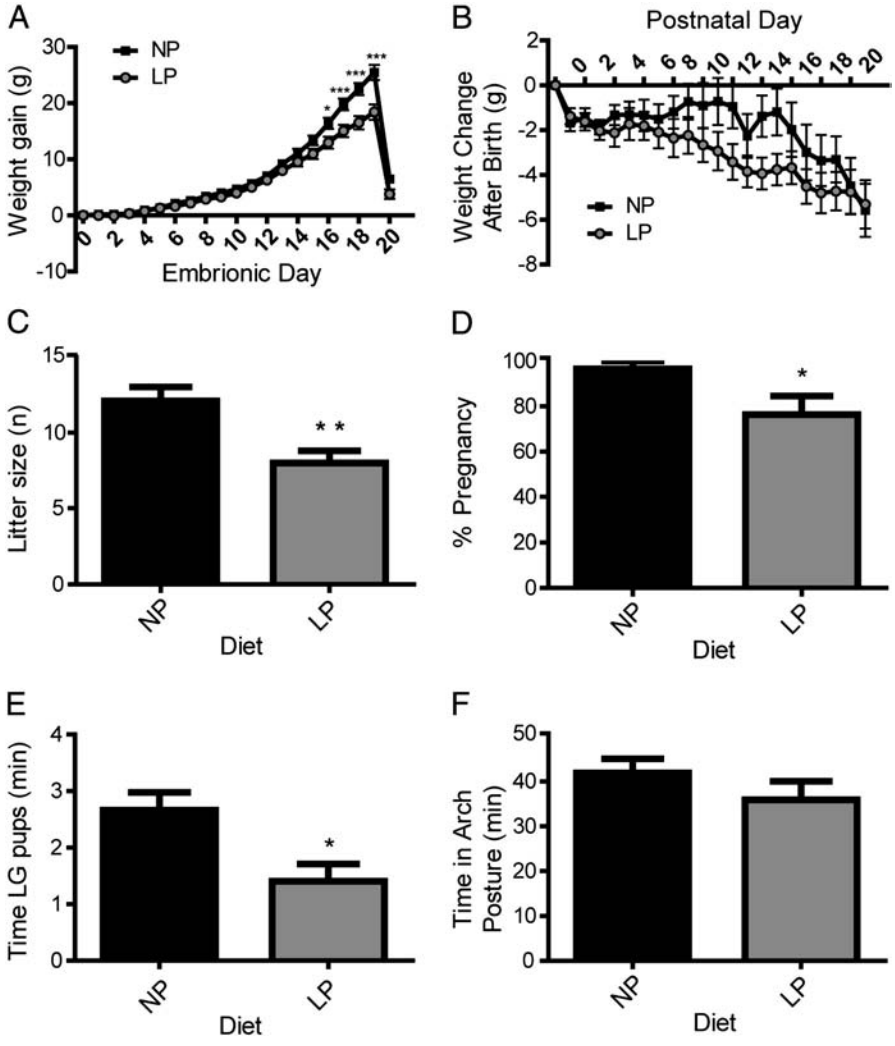

Fig. 1. Maternal weight change, fecundity and behavior. (A) Weight change during pregnancy. For each time point (x) weight change is calculated as: weight at EDx − weight at ED0. Mean ± SEM. Two-way RM ANOVA, Bonferroni post hoc test. $n = 10$–13 for each treatment. $^{*}p < 0.05$ and $^{***}p < 0.001$. (B) Weight change after birth. For each time point (x), weight change is calculated as: weight at PDx − weight at PD0. Mean ± SEM. Two-way RM ANOVA, Bonferroni post hoc test. $n = 10$–13 for each treatment. (C) Litter size of each dam at P0. Mean + SEM Student's *t*-test. $n = 10$–15 for each treatment. $^{**}p < 0.01$. Maternal care measured as time the mother spent licking and grooming the pups. (D) Percent of pregnant dams for each treatment. Each value is taken as the %pregnancy in the assay. Mean + SEM Mann–Whitney. $n = 6$ assays $^{*}p < 0.05$. (E), and in a nursing posture in the nest (F). Mean + SEM Student's *t*-test. $n = 10$–13 of litters analyzed for each treatment. $^{*}p < 0.05$.

they were able to do it in no more than a second for two consecutive days.

Negative geotaxis: Mice were placed facing down on a metallic grid with a 25° inclination and the performance was measured as the time it took them to rotate 90°, considering 60 s as the upper limit. Measures were made on PD5, 7, 9, 11 and 13.

Hang wire test: Pups between PD12 and PD20 were placed on a metal grid which was gently shaken so that mice held on to it. The grid was then turned upside down leaving mice hanging 30 cm above a cage with clean bedding. Latency to fall onto the bedding was recorded, with a 60 s cut-off time.

Startle reflex: This test was performed everyday between PD12 and PD21. Pups were placed over a flat surface and presented with a sound of 80 dB in a 74 dB background, approximately 10 cm above the head. This reflex was considered present when a sudden and transient freezing was detected immediately after the sound. Presence or absence of the startle response was registered.

2.5. Behavioral testing of the F_1

After weaning animals were transferred to a new housing room adjacent to the behavioral testing room. They were kept under the same dark:light cycle described in Section 2.1. During the behavioral testing period mice were left mainly unhandled, except for routine cage changes. Before every test subjects were habituated to the testing room for 40–50 min, with lights set at the desired intensity and a constant

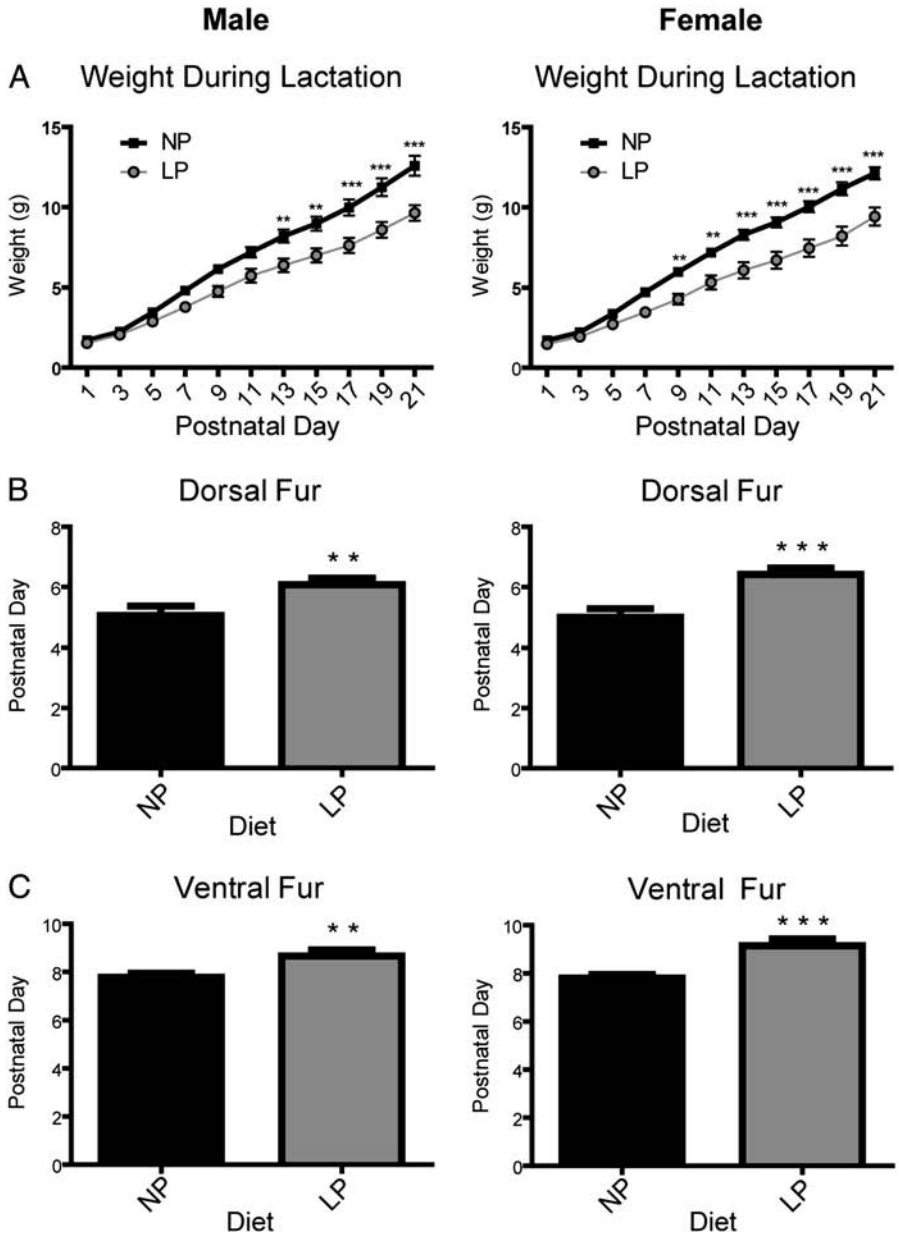

Fig. 2. Pups physical development. Male (left column) and female (right column) pups were analyzed separately. Pups weight during lactation (A). Weight of every pup was measured and an average was calculated per litter for the postnatal days indicated in the x-axis, $n = 10$–12 litters for each treatment. Mean ± SEM. Two-way RM ANOVA, Bonferroni, post hoc test. $^{**}p < 0.01$, $^{***}p < 0.001$. PND of dorsal fur emergence (B), ventral fur emergence (C) eye opening (D) and ears opening (E), was registered for each pup and averaged per litter. Mean + SEM Mann–Whitney or Student's *t*-test. $n = 10$–12 for each treatment and sex except for (E) where $n = 22$–32, $^{**}p < 0.01$, and $^{***}p < 0.001$. Pups weight after weaning (F). A few individuals of every litter were chosen and their weight was measured in the postnatal days indicated in the x axes, $n = 12$–14 individuals. Mean ± SEM. Two-way RM ANOVA, Bonferroni post hoc test. $^{*}p < 0.05$, $^{***}p < 0.001$.

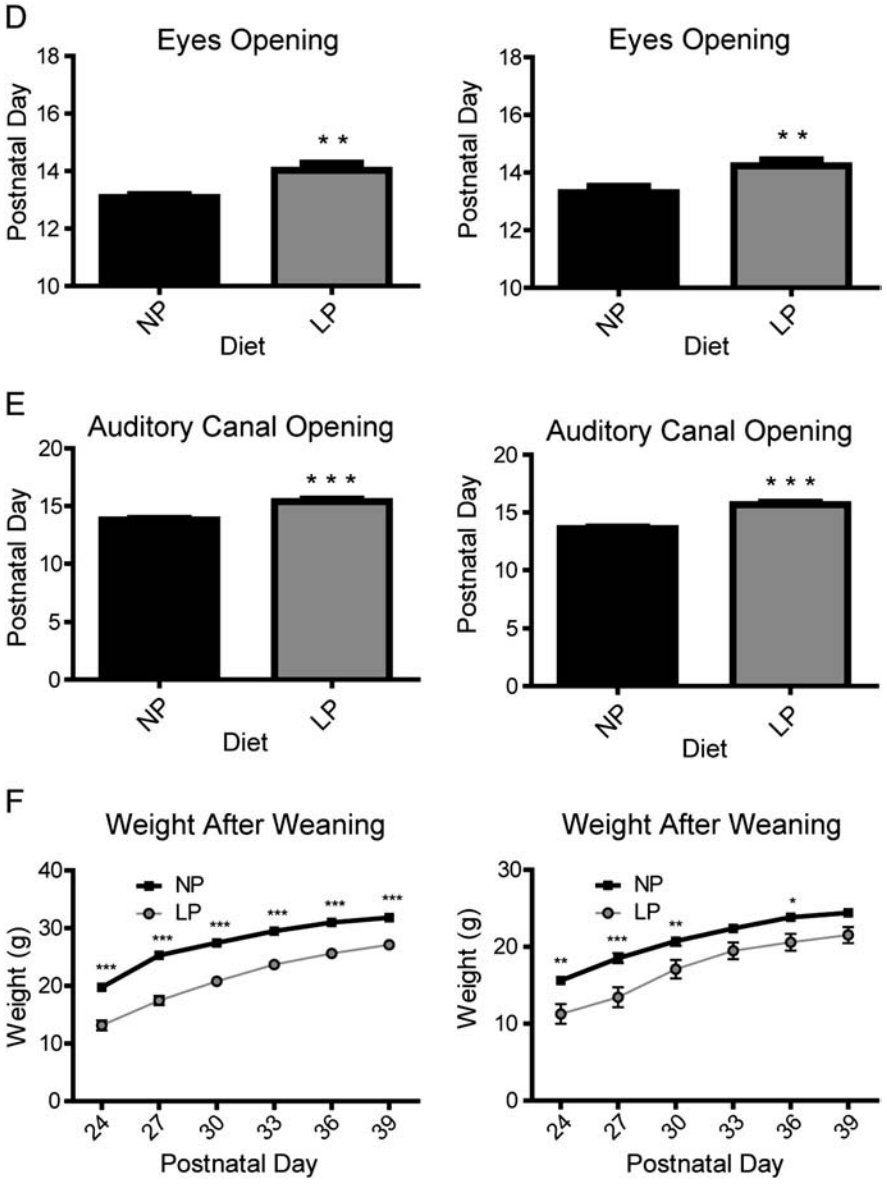

Fig. 2 (*continued*).

background noise of 60 dB. Every test was filmed from above, with the exception of the tail suspension test in which the camera was placed in front of the apparatus. Webcams were connected to a computer placed outside the room and videos were recorded and tracked using the ANY-Maze™ Video Tracking Software (Stoelting). Juvenile play was evaluated in mice between 22 and 26 days of age. The remaining tests were performed observing the following age schedule: social interaction at 5 weeks, elevated plus maze at 6 weeks, open field at 7 weeks, cage escape at 8 weeks and tail suspension at 9 weeks after birth. Not every test was performed in all the animal cohorts (i.e. different experiments), but the age was the same for all the experiments. This sequence was designed in order to minimize interference produced by previous tests, i.e. most stressful tests (tail suspension) were performed least and those more sensitive to stress were performed first.

2.5.1. Juvenile play

Social play behaviors can result in useful strategies later in adult life and prepare the individual for future environmental contingencies. Juvenile play was analyzed in sex, weight and age matched couples of one NP and one LP mice that were confronted for the first time. Testing started at 17:00 h with lights set at 10 lx. Subjects were individually habituated to the experimental room without any food or water in a cage equal to their home cage. Next, each individual of the couple was placed in turns for 10 min in the experimental arena (21 × 30 ×23 cm, with black walls and covered with a thin layer of bedding), and a black stripe was painted in the tail of one of them for identification purpose. After this, both mice were placed in the arena and the pair was allowed to freely interact for 30 min [33]. The test session was recorded and subsequently scored for behavioral events. Parameters of juvenile mouse social behaviors were the same as those reported by Cox and Rissman [34] with the addition of "body sniffing" as a new investigation activity and jumping against the wall as a new non-social behavior. The complete list of parameters (slightly modified from Cox and Rissman) is as follows:

Social interactions (time spent):

1. Side-by-side sitting: sitting and/or sleeping in close contact with the other mouse.
2. Social grooming: allogrooming the other mouse.
3. Social other: spending time in close contact with the other mouse while self grooming

Nonsocial behaviors:

1. Exploring: investigating the walls or floor of the test cage (duration).
2. Self grooming: grooming any part of its own body while sitting alone (duration).
3. Sitting: sitting alone while the other mouse was engaged in other behaviors (duration).
4. Rearing with support of the walls (frequency).
5. Rearing without support (frequency).
6. Jumping against the walls (frequency).

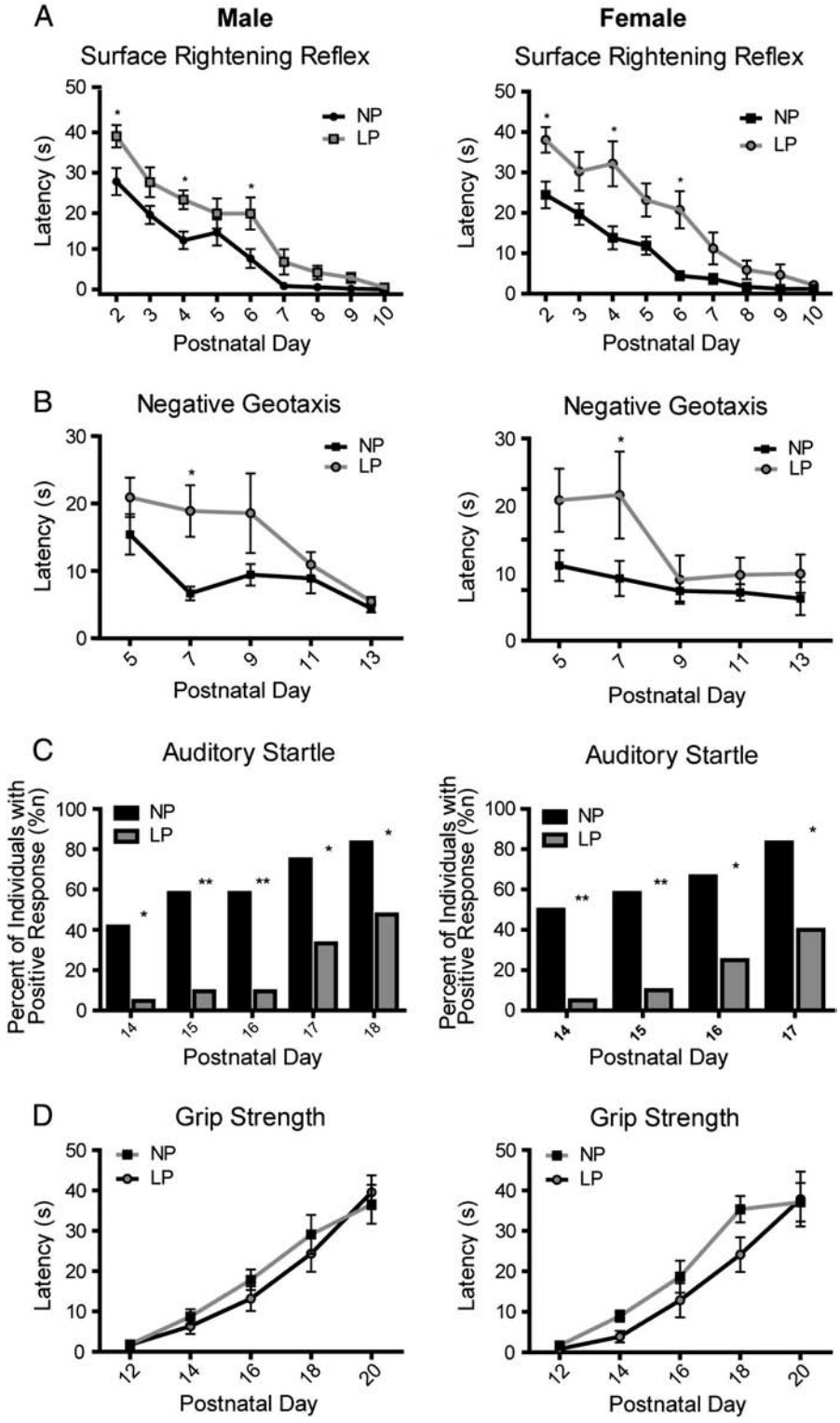

Fig. 3. Neurological reflexes. Male (left column) and female (right column) pups were analyzed separately. Latency to perform the surface righting reflex (A), the negative geotaxis (B), the percent of animals that displayed a startle response when presented with a sound (C) and to fall in the grip strength test (D), was measured at different postnatal days, as indicated in the x-axis. Time values for each litter (n) are an average of the time measured for each pup of the litter in the indicated PD for all reflexes with the exception of the startle response, where n was equal to the number of analyzed pups. Mean ± SEM. Two-way RM ANOVA, Bonferroni post hoc test. $n = 7$–12 for each treatment in (A), (B) and (D), and $n = 12$–20 for each treatment in (C). $^{*}p < 0.05$, $^{**}p < 0.01$.

Investigation (frequencies):

1. Anogenital sniff: sniffing the other mouse's anogenital region.
2. Nose sniff: sniffing the other mouse's nose.
3. Body sniff: sniffing the other mouse's body in any part other than the nose or the anogenital region.
4. Follow: walking behind and following the other mouse around the cage.

Play solicitation (frequencies):

1. Crawl: crawling over or under the other mouse.
2. Push: pushing between the other mouse, typically with the cage wall behind the mouse exhibiting the pushing.
3. Approach: approaching the other mouse head-on.

2.5.2. Social interaction

Mice were placed in a 15 × 41 × 25 cm rectangular cage made of black polystyrene with two transparent acrylic cylinders (d: 7 cm, h: 15 cm) set on opposite corners of one of the 41 cm sides. The cylinders had 1 cm diameter holes evenly distributed throughout their surface which allowed mice to examine its interior. They were let to explore the area and the cylinders for 5 min. Subsequently, a 3 weeks old novel mouse was placed inside one of the cylinders and a small film container in the other. Test mice were left to explore for another 10 min. Both stages were video recorded using the ANY-Maze™ Video Tracking Software (Stoelting) and time spent exploring the inside of each cylinder was quantified by an observer blind to the mouse treatment sitting outside the room with the computer.

2.5.3. Elevated plus maze

The apparatus consisted of a dark floor maze with a central platform (5 × 5 cm) from which four arms radiated. Two arms had polystyrene dark walls of 15 cm height (closed arms), and the other two had a small 0.5 cm ledge (open arms). The maze was elevated 50 cm above the floor and the length of each arm was 30 cm. Testing was performed placing the mouse in the central platform facing one of the open arms and allowing it to explore the apparatus for 5 min. A video camera was placed above the maze, and the ANY-Maze™ was used for both session recording and animal movement tracking. An observer sitting outside the room registered the number of rearings, head dippings, and protected head dippings (head dipping while the animal was still in the central platform) and the total time the animal spent self-grooming, using the computer. Lights were set at 100 lx for the duration of the test.

2.5.4. Open field

The open field consisted in a 45 cm side square arena with dark walls. A central square area of 23 cm sides was virtually delimited using the ANY-Maze™ software. Mice were placed in the peripheral area and left to explore the environment for 20 min. The whole session was video recorded and mice were tracked using the ANY-Maze™. An observer sitting outside the room registered the number of rearings and time of grooming using the computer. Lights were set at 100 lx.

2.5.5. Cage escape

Mice were placed in a cage identical to their home cage with 3 cm of clean bedding and left for 10 min. During that time the number of wall climbs (considered when the mouse forelimbs left the floor surface) and attempts of escape (when the mouse was entirely over the edge of the wall) were counted. After each attempt of escape mice were returned to the testing cage if necessary. Lights were adjusted at 100 lx.

2.5.6. Tail suspension test

Mice were suspended by the tail using cotton adhesive tape to fix them to the apparatus, which consisted of a thin wire supported by two sticks. Total immobility time was registered for the whole session (6 min). Lights were kept at 100 lx.

2.6. Statistical analysis

Statistical tests used throughout the paper are described in the figure legends and text. When data complied with normal distribution and equal variances between treatments, Student's *t*-test was applied. Welch's correction was used when data didn't meet homoskedasticity criteria. Mann–Whitney non-parametric test was employed when data failed to pass normality testing. For parameters analyzed in the same individual during a series of days (weight, surface righting reflex, hang wire and negative geotaxis), a two-way ANOVA with repeated measures was used with Bonferroni Post test to compare between treatments. Dam's percent of pregnancy (number of pregnant females × 100 / total of females set to mate) for each treatment was calculated in different experiments, being N the number of experiments. For developmental landmarks and neurological reflexes (with the exception of the startle reflex) N was equal to the number of litters. In these cases, the variable measured for every parameter or time point was averaged between littermates. For the startle reflex, a different set of mice was used. For this assay, two male and two female pups were chosen from every litter and marked with a permanent marker. Startle reflex was only performed in these animals and *n* was equal to the number of pups assessed and significance was tested using the Fisher's exact test for binomial variables. In tests performed after weaning, *n* was the number of tested individuals. Data were tested with Grubbs' method in order to detect the presence of outliers [35]. This is a statistical test used to detect outliers in a univariate dataset assumed to come from a normally distributed population. Values that were considered outliers ($\alpha = 0.05$) were removed from the dataset before statistical analysis.

3. Results

3.1. Body weight of dams during gestation and lactation

Body weights of mouse dams were measured throughout gestation and lactation (Fig. 1A and B). A two-way ANOVA with repeated-measures revealed a significant main effect for the diet ($F_{1,20} = 5.048$, $p = 0.0357$), time ($F_{20,420} = 373.44$, $p < 0.0001$) and their interaction ($F_{20,420} = 8.48$, $p < 0.0001$) during gestation. To further examine the body weight of mother mice on different time points, a Bonferroni post-test was followed. The body weight of dams was lower in the LP group than that in the NP group on E16 ($p < 0.05$), E17 ($p < 0.001$), E18 ($p < 0.001$) and E19 ($p < 0.001$). On the other hand, there was a significant main effect for time ($F_{21,441} = 13.74$, $p < 0.0001$) on dams weight during lactation but not for diet ($F_{1,21} = 1.408$, $p = 0.2486$). The interaction between diet and time was also significant ($F_{21,441} = 1.789$, $p = 0.0176$) (Fig. 1B).

3.2. Dam fecundity and maternal behavior

Maternal LP diet resulted in a significant decrease in the number of pups born compared to those born from NP dams ($t_{23} = 3.270$, $p = 0.034$), which ranged from 4 to 13 and 6 to 16 pups per dam, respectively (Fig. 1C). Moreover, the pregnancy percentage for female fed with a LP diet was less than the one for NP group ($U_{10} = 2.305$, $p = 0.0131$) (Fig. 1D).

To determine whether LP and NP dams provide different amount of care to their pups, we evaluated their maternal behavior during the first postnatal week. A significant group effect was found for maternal care with NP dams spending more time licking and grooming their pups ($t_{21} = 2.816$, $p = 0.0103$) (Fig. 1E). There were no effects of dietary regimen on nest condition, dam location, arched-back posture or the percentage of time engaged in nursing (Fig. 1F).

Notably, we observed a high degree of cannibalism in the LP mothers group rising to 30% of the litter in some cases. As it was not observed in mothers from NP group and, since CF1 strain had not been reported to exhibit such behavior, this conduct could be attributed to LP diet.

3.3. Pups weight and physical growth

The body weight of both male and female pups was measured during lactation (Fig. 2A). A two-way ANOVA with repeated-measures indicated that there were significant main effects for diet group (male: $F_{1,20} = 11.72$, $p = 0.0027$; female: $F_{1,18} = 18.05$, $p = 0.0005$), time (male: $F_{10,200} = 491.1$, $p < 0.0001$; female: $F_{10,180} = 409.7$, $p < 0.0001$) and interaction for diet group with time (male: $F_{10,200} = 9.563$, $p < 0.0001$; female: $F_{10,200} = 10.10$, $p < 0.0001$). No differences were observed for sex neither in the NP ($F_{1,18} = 0.0216$, $p = 0.8847$) nor in the LP group ($F_{1,20} = 0.3395$, $p = 0.5666$). Bonferroni post hoc analysis indicated that the body weights of LP feeding pups between PD13 and PD21, for male, and between PD9 and PD21 for female, were significantly lower than that of NP group (Fig. 2A).

We next analyzed the effect of maternal LP diet during pregnancy and lactation on the physical development of pups. LP diet delayed significantly the day of dorsal (male: $t_{19} = 2.833$, $p = 0.0053$; female: $t_{17} = 4.130$, $p = 0.0004$) and ventral (male: $U_{19} = 19.00$, $p = 0.0066$; female: $t_{17} = 4.017$, $p = 0.0004$) fur emergence, eye opening and ear canal opening (male: $U_{51} = 85.50$, $p < 0.0001$; female: $U_{46} = 25.50$, $p = 0.0007$) both in male and female pups (Fig. 2B–E).

Anogenital distance, a sexually dimorphic trait in rodents, was also determined. The anogenital distance of both the NP (male: 2.389 ± 0.044 mm; female: 1.736 ± 0.024 mm) and the LP pups (male: 2.387 ± 0.022 mm; female: 1.714 ± 0.023 mm) was measured on PD1. Unpaired *t* test indicated that no significant differences were observed between pups of both groups (male $t_{65} = 0.0585$, $p = 0.4767$; female $t_{60} = 0.6427$, $p = 0.2613$).

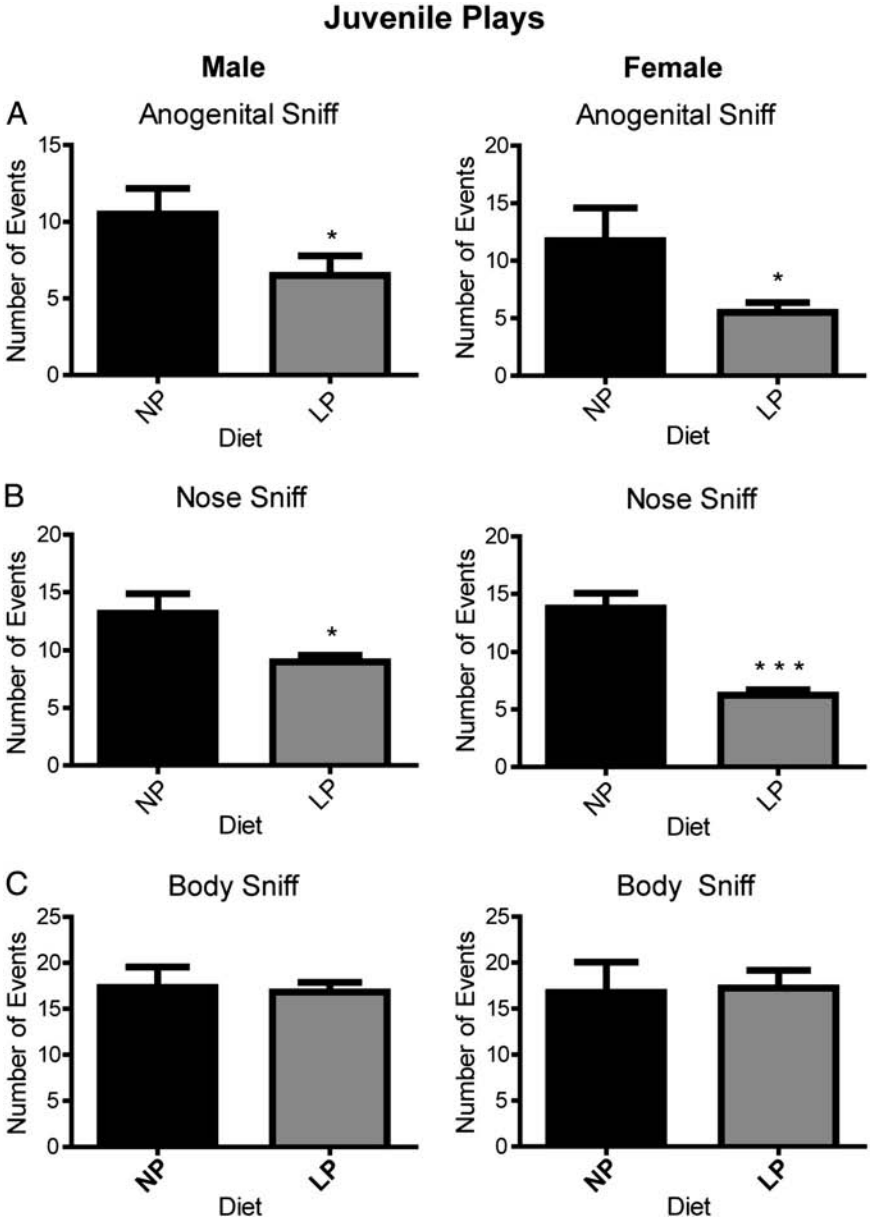

Fig. 4. Juvenile play. Male (left column) and female (right column) mice were analyzed separately. Number of times a mouse from the treatment indicated in the x axes, sniffed the anogenital region (A), the nose (B), the body (C), followed (D), crawled over (E), pushed (F), approached head on (G) the mouse from the other treatment. Mean + SEM Mann–Whitney or Student's *t*-test, for each treatment. $n = 4$ for females and $n = 6$ for males. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

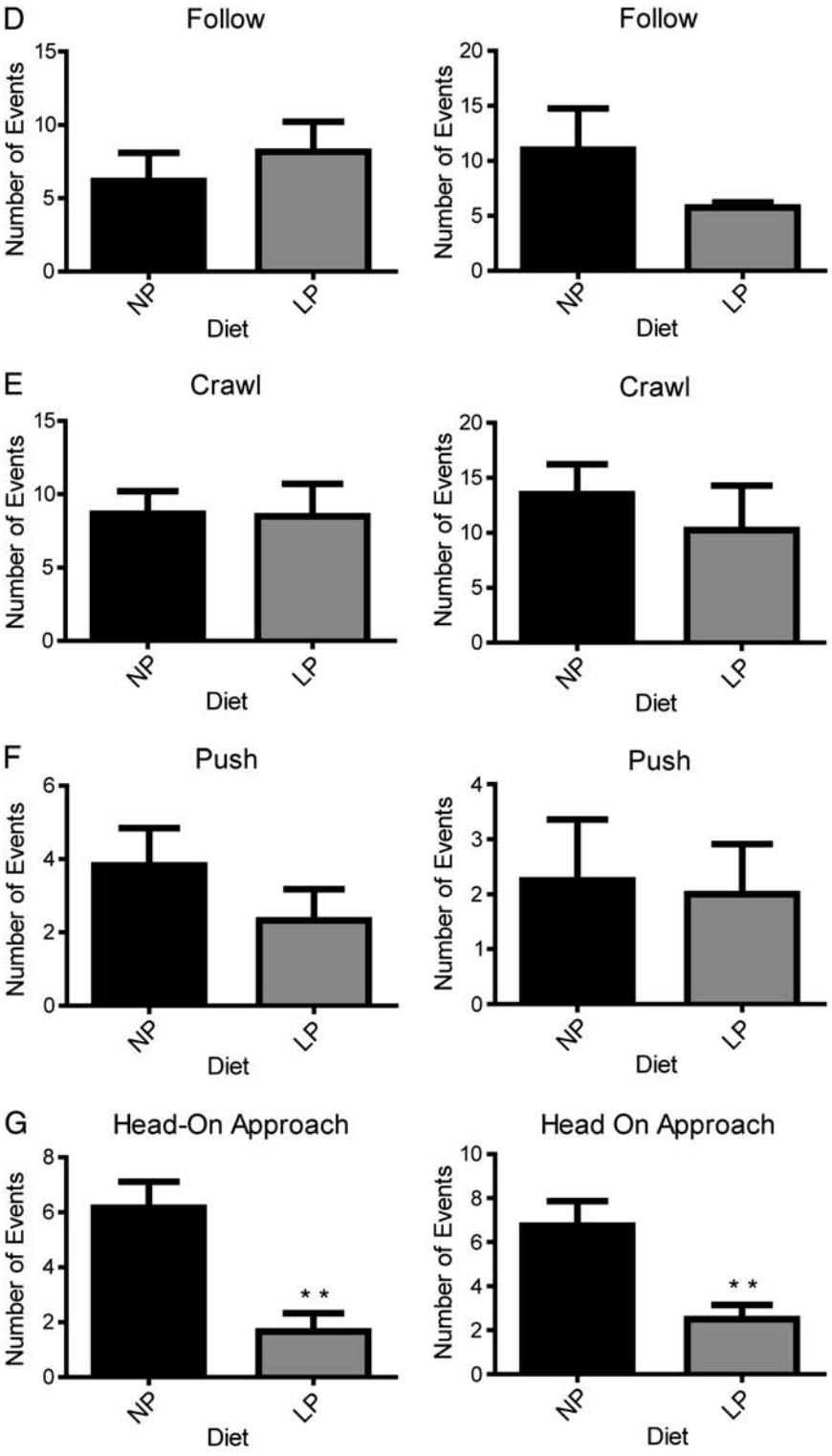

Fig. 4 (*continued*).

The body weight of both male and female mice was also measured from weaning until six weeks of age (Fig. 2F). A two-way ANOVA with repeated-measures revealed that there were significant main effects for diet group (male: $F_{1,24} = 45.41$, $p < 0.0001$; female: $F_{1,23} = 11.61$, $p = 0.0024$), time (male: $F_{5,120} = 723.5$, $p < 0.0001$; female: $F_{5,115} = 422.3$, $p < 0.0001$) and interaction for diet group with time (male: $F_{5,120} = 9.298$, $p < 0.0001$; female: $F_{5,115} = 5.787$, $p < 0.0001$). Bonferroni post hoc analysis indicated that the body weights of male LP mice continues to be significantly different to that of NP mice. On the other hand, body weight of female LP mice seems to approach to NP group from the fifth week of life.

3.4. Developmental reflexes

We next evaluated the impact of maternal LP diet on neurological reflexes of male and female pups. First, righting reflex, a sensorimotor development test, was measured between PD2 and PD10. Maternal LP diet resulted in a significant increase in the latency time required for male and female pups to right themselves when placed in a supine position compared to the NP group (Fig. 3A). A two way ANOVA with repeated-measures across this period revealed significant main effects for time (male: $F_{8,136} = 47.08$, $p < 0.0001$; female: $F_{8,136} = 28.64$, $p < 0.0001$) and diet group (male: $F_{1,17} = 13.14$, $p = 0.0021$; female: $F_{1,17} = 18.09$, $p = 0.0005$). Interaction was significant only for females ($F_{8,136} = 2.197$, $p = 0.0313$). Bonferroni pos hoc test indicated that latency time of right response attained in LP group was higher than the NP group on PD2, PD4 and PD6 for male and female pups. These differences disappeared at PD8 in both sex groups.

We also evaluated the negative geotaxis response of male and female pups, another sensorimotor test that measures the pup ability to orient themselves with the head upward on an inclined plane, from PD5 to PD13. We observed that nutritional condition affects the latency for pups to achieve a head-up position (Fig. 3B). The two way ANOVA showed a significant effect of diet (male: $F_{1,16} = 8.697$, $p = 0.0094$; female: $F_{1,14} = 14.39$, $p = 0.0020$) and time (male: $F_{4,64} = 5.369$, $p = 0.0009$; female: $F_{4,56} = 3.132$, $p = 0.0215$) but not in their interaction (male: $F_{4,64} = 1.238$, $p = 0.3037$; female: $F_{4,56} = 1.054$, $p = 0.3879$) on the latency time to achieve a head-up position. Post hoc analysis pointed out that on PD7 the time spent by the mice to straighten its head was increased in the LP group compared to the control one in both male and female pups.

In view of the differences observed in the ear canal opening between both mice groups, we assessed the effect of maternal LP diet on the acoustic startle reflex. The percentage of LP mice that displayed a startle response was significantly less between PD4 and PD18 in male (PD14: $p < 0.0159$, PD15: $p < 0.0046$, PD16: $p < 0.0046$, PD17: $p < 0.0252$, PD18: $p < 0.0471$) and between PD14 and PD17 in female (PD14: $p < 0.0057$, PD15: $p < 0.0057$, PD16: $p < 0.0254$, PD17: $p < 0.0197$) compared to mice from LP group (Fig. 3C). These differences disappeared on PD19 for male ($p < 0.2695$), and PD18 for female ($p < 0.5033$) mice.

We next employed the wire hang test to evaluate the neuromuscular strength of male and female pups from both nutritional condition groups (Fig. 3D). There were significant main effects for time (male: $F_{4,72} = 50.92$, $p < 0.0001$; female: $F_{4,64} = 48.55$, $p < 0.0001$), but no differences were observed for diet groups (male: $F_{1,18} = 0.3329$, $p = 0.5711$; female: $F_{1,16} = 1.410$, $p = 0.2524$).

As a whole these results suggest that physical growth and neurobehavioral development are detrimentally impacted by maternal protein malnutrition during gestation and lactation.

3.5. Juvenile play

Social play behaviors can result in useful strategies later in adult life and prepare the individual for future environmental contingencies. We asked whether juvenile play behaviors could be affected by maternal protein malnutrition. Investigative activities and play solicitation were measured in heterogeneous pairs formed by mice from different diet groups of the same age and gender. The frequency with which the NP member of the pair, either male or female, performed any investigative activity was significantly higher than the LP member did (male: $t_{10} = 1.862$, $p = 0.0461$; female: $t_6 = 2.005$, $p = 0.0459$) (Fig. 4A–D). The LP member of the pairs displayed significantly less anogenital (male: $t_{10} = 1.900$, $p = 0.0433$; female: $t_6 = 2.105$, $p = 0.0399$) and nose-to-nose sniffing (male: $t_{10} = 2.319$, $p = 0.0214$; female: $t_6 = 5.359$, $p = 0.0009$) while sniffing anywhere on the body was similar between both diet groups (male: $t_{10} = 0.2040$, $p = 0.4212$; female: $t_6 = 0.1307$, $p = 0.4501$). With regard to frequency of play solicitation, the number of times that the LP member approach head-on to the NP member of the pair was less than the head-on approach in the reverse direction either for male or female pups (male: $t_{10} = 3.889$, $p = 0.0015$; female: $t_6 = 3.313$, $p = 0.0081$) (Fig. 4E–G). On the other hand, no differences were observed in the time engaged in any other social or non social activities measured in both members of the pair.

These results indicate that investigative and play solicitation behaviors are weakened by early malnutrition.

3.6. Motivation and neuromuscular coordination are diminished in protein malnourished mice

We performed the cage escape test in order to evaluate the effect of protein malnutrition on motivation and neuromuscular coordination in adult young mice. Both male ($U_{44} = 169.0$, $p < 0.0219$) and female ($U_{38} = 76.00$, $p < 0.0004$) LP mice made significantly fewer climbing events compared to NP mice (Fig. 5A). Similarly, a reduced number of attempts to escape was observed in male ($U_{44} = 190.0$, $p < 0.0435$) but not female ($U_{36} = 158.0$, $p < 0.2611$) LP mice when compared to NP animals (Fig. 5B).

3.7. Early malnutrition promotes anxiety and depression-related behaviors

We first evaluated anxiety-related behavior in the open field test. Latency to enter the central zone was significantly higher in LP compared to NP mice (male: $U_{44} = 113.0$, $p = 0.0006$; female: $U_{36} = 87.00$, $p = 0.0038$) (Fig. 6A). LP mice spent less time in the center (male: $U_{44} = 128.0$, $p = 0.0036$; female: $U_{36} = 86.50$, $p = 0.0072$), walked less in the central zone (male: $U_{44} = 125.0$, $p = 0.0014$; female: $t_{36} = 90.0$, $p = 0.0049$) and showed reduced vertical exploration (male: $U_{44} = 115.5$, $p = 0.0007$; female: $U_{36} = 89.0$, $p = 0.0045$) (Fig. 6B–D). However, mice of both groups walked similar total distance indicating that they did not differ significantly in locomotive activity (male: $t_{44} = 1.463$, $p = 0.0753$; female: $U_{36} = 174.0$, $p = 0.4533$) (Fig. 6E).

We next assessed the performance of mice in the elevated plus maze test. Distance traveled in the open arms was significantly lower in LP compared to NP mice (male: $U_{26} = 55.00$, $p = 0.0424$; female: $t_{16} = 2.626$, $p = 0.0092$) (Fig. 7A). Moreover, LP mice exhibit a lower average speed in open arms (male: $U_{26} = 42.00$, $p = 0.0053$; female: $t_{24} = 2.640$, $p = 0.0072$) and perform a minor number of protected head dipping events (male: $t_{26} = 2.551$, $p = 0.0085$; female: $t_{24} = 3.789$, $p = 0.0004$) than NP mice (Fig. 7B and C). Head dipping activity in open arms showed no significant differences between both mice groups. However, the total distance traveled (male: $t_{25} = 4.054$, $p = 0.0002$; female: $U_{24} = 37.00$, $p = 0.0084$) were also significantly lower in LP mice compared to NP mice, suggesting that LP mice displayed a decreased exploratory activity (Fig. 7D). Interestingly, male mice seem to have an increased sensitivity to a diet with reduced protein content. LP male mice spend less time in the tips of the arms ($U_{26} = 60.00$, $p = 0.0177$) and display a lower number of rearings ($U_{26} = 35.00$, $p = 0.0020$) and entries to the open arms ($U_{26} = 39.50$, $p = 0.0036$) compared to NP male mice (Fig. 7E–G).

To further address the mice anxiety-related behavior we performed the social interaction test. Mice from both groups spend the same time in total sniffing activity (male: $t_{15} = 0.7980$, $p = 0.2187$; female: $t_9 = 0.4932$, $p = 0.3169$) and showed the same exploratory preference for the novel mouse (male: $t_{15} = 0.7992$, $p = 0.2183$; female: $t_9 = 0.0268$, $p = 0.4896$) (Fig. 8).

Behavioral despair, a depression-related behavior, was evaluated in the tail suspension test. We observed a significant effect of nutritional condition in female mice, with LP group spending more time immobile ($U_8 = 2.000$, $p = 0.0159$) comparing with female mice from NP group (Fig. 9).

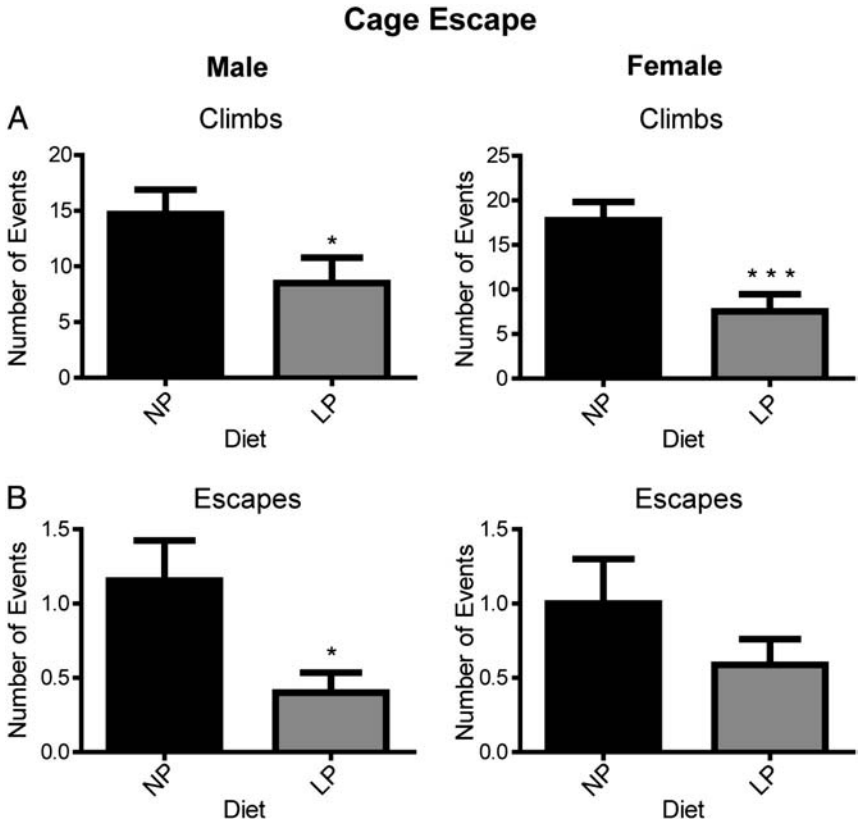

Fig. 5. Cage escape. Male (left column) and female (right column) mice were analyzed separately. (A) Number of climbs (number of the times the mouse's forelimbs left the floor surface). (B) Number of escapes (number of times the mouse's entire body was over the cage edge). Mean + SEM Mann–Whitney $n = 26$–20 males or $n = 19$–21 females for each treatment. $^{*}p < 0.05$ and $^{***}p < 0.001$.

3.8. Protein-malnourished dams display an anxiety-related behavior

Two weeks after weaning, dams were subjected to elevated plus maze and open field tests in order to detect potential diet effects on their behavior. Performance in the elevated plus maze revealed that LP dams spent less time in the open arms ($t_{22} = 1.856$, $p = 0.0384$), exhibited a lower head dipping activity ($U_{22} = 38.00$, $p = 0.0399$) and display a significant increase in latency to enter the tip zone of the open arms ($U_{22} = 40.00$, $p = 0.0474$) compared to NP dams (Fig. 10A–D). Furthermore, total distance traveled was significantly higher in LP compared to NP dams ($t_{18} = 3.009$, $p = 0.0075$) but the former moving more distance ($t_{22} = 2.901$, $p = 0.0088$) and spending more time ($t_{22} = 2.164$, $p = 0.0208$) in the closed arms. Rearing ($t_{22} = 1.695$, $p = 0.1042$) and grooming ($U_{22} = 64.00$, $p = 0.8554$) activities showed no significant differences between dams from both groups. On the other hand, dams from NP and LP groups showed a similar performance in the open field test. These results suggest that LP dams exhibit some traits of an anxious behavior.

4. Discussion

Maternal malnutrition has been associated with behavioral and neurological deficiencies such as impaired development of cognitive abilities and linked to both acute and long-term health-damaging effects. Although there is extensive literature regarding the effects of protein malnutrition, the present paper explores several issues that have received less attention and recapitulates a condition of malnutrition extended throughout the world. For these reasons, in this paper we investigated the consequences of middle degree perinatal protein malnutrition on physical growth and neurological development as well as social interaction and anxiety related behaviors in mice of both sexes.

Protein-malnourished dams exhibit a lesser increase in body weight during the last stage of pregnancy. However, pups display similar weight size at birth. This would be due to the minor number of pups born from LP compared to NP dams. Our results are in agreement with those obtained in rats [36]. In this report, authors demonstrated that differences between protein dietary groups in number of potential offspring were due mostly to lower ovulation and to a lesser extent to a preimplantation lost.

A consequence of a protein-deficiency for the offspring of both genders is a sustained shortfall in body size. By PD5 LP mice began to gain less weight per day than their NP counterparts. Protein restriction during gestation and lactation renders pups reaching 70% of body weight of control animals at weaning and even at later stages in life remain around 18% lighter than control mice. Protein deficient diets have been implicated as important regulators of metabolic programming and later health outcomes in rats [16,17]. Beside differences in body size, both LP male and female pups showed delayed dorsal and ventral fur appearance as compared to gender matched NP pups. Moreover, ear canal and eye opening were sooner in NP than in LP pups, suggesting a general delay in epithelial development and eye maturation [37].

Neurological development was also affected by perinatal protein malnutrition. LP pups showed a delay of in surface righting reflex and negative geotaxis response. These sensorimotor responses reflect maturation of cerebellar function. The cerebellum is important for making postural adjustments in order to maintain balance. Through its input

from vestibular receptors and proprioceptors, it modulates commands to motor neurons to compensate for shifts in body position or changes in load upon muscles [38]. Pups from both groups showed similar performances in hang wire test suggesting that protein-deficient diet does not promote motor neuromuscular impairment or affect muscular strength [39]. Therefore, since neonatal reflexes may be considered as an index of brain maturation, the present findings suggest that perinatal exposure to undernutrition affects embryological mechanisms responsible for the correct development of the brain.

When considering these findings, it is important to keep in mind that our protein malnutrition model of dams does not allow us to separate direct effects of the diet on pup growth with possible indirect effects on maternal behavior. It is, therefore, possible that the effects on preweaning growth and development may have been affected by variations in maternal behavior [40]. In fact, our results showed that mothers fed a LP diet spent significantly less time licking and grooming their pups in the early neonatal period. In addition those mothers display an anxious-like behavior. It is known, that disparities in maternal behaviors are associated with the development of individual differences in neurodevelopment and responses to stress in the offspring. As adults, the offspring of high-licking and grooming mothers are less fearful and shows more modest HPA responses to stress than the offspring of low-licking and grooming mothers that display altered ACTH and corticosterone responses to stress [41,42]. In animals, prenatal stress reduces maternal care and bonding and the offspring of mothers who exhibit reduced maternal care and bonding shows altered behavior [43,44]. However, the link between maternal nutrition, maternal care and offspring disease risk remains to be determined [7].

Social play is a prevalent behavior during a short period of time in rodents declining with the beginning of the sexual maturity and may serve to equip the animals with some basic skills and strategies essential for a variety of behaviors that are expressed in adulthood [45]. The development of social behaviors can be affected by several environmental conditions. The present study showed that early protein malnutrition produced changes in the development of the social play in mice, specifically a reduction in investigative and play solicitation behaviors. While similar decrease in social juvenile behavior have been described in malnourished rats [20,45], in our knowledge is the first report concerning perinatal protein malnourished mice. It is suggested by our results that changes in the expression of social play in mice is possibly due to the result of developmental retardation and neurological changes produced by the protein deficient diet. If play and other social behaviors early in life are crucial for the expression of adult behavior [46], then the well-nourished mice would be more adapted to cope with the challenges imposed by their environment in adulthood. Our results are consistent with this hypothesis.

In the present study, differences indicating altered levels of anxiety in the perinatal protein malnourished animals were observed after nutritional recovery. Behavioral observations in the elevated plus maze revealed differences in approach/avoidance behavior: LP offspring traveled minor distance and spent shorter time in the open arms and made lesser protected head dippings than the NP offspring. The results

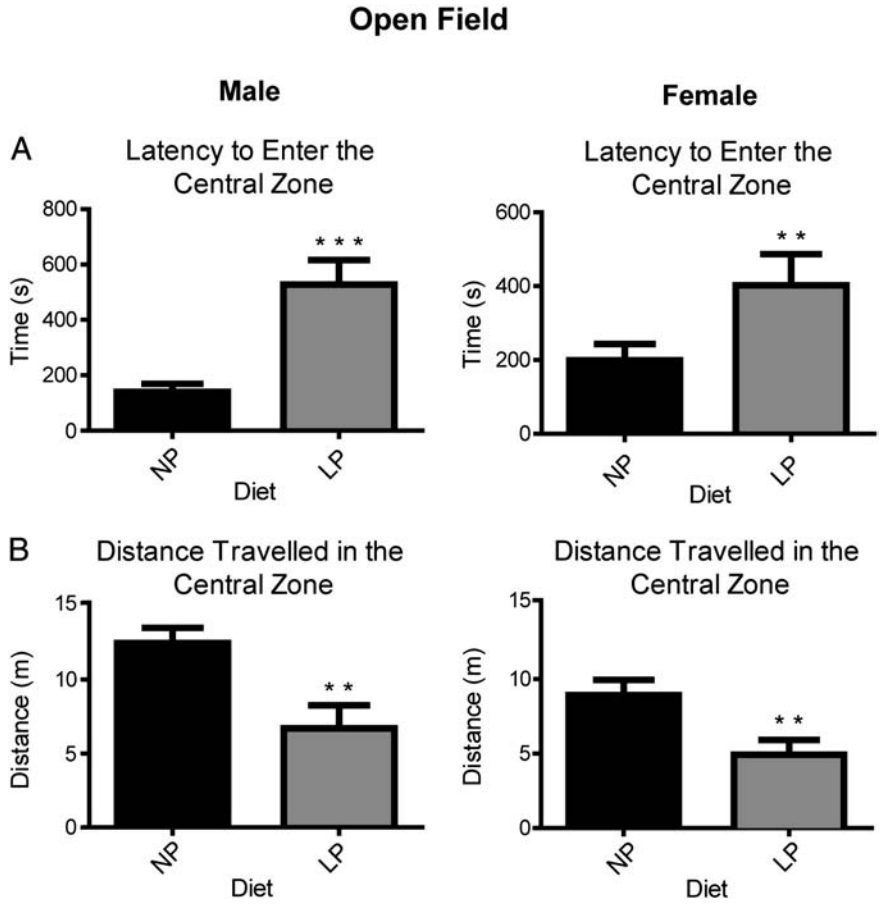

Fig. 6. Open field. Male (left column) and female (right column) mice were analyzed separately. Latency to enter the central zone (sec) (A). Time in the central zone (sec) (B). Distance traveled in the central zone (m) (C). Number of rearings in both areas (D). Total distance traveled in both areas (E) were measured using the ANY-Maze software. Mean + SEM Mann–Whitney or Student's *t*-test. $n = 26$–20 males or $n = 19$–21 females for each treatment. $^{**}p < 0.01$ and $^{***}p < 0.001$.

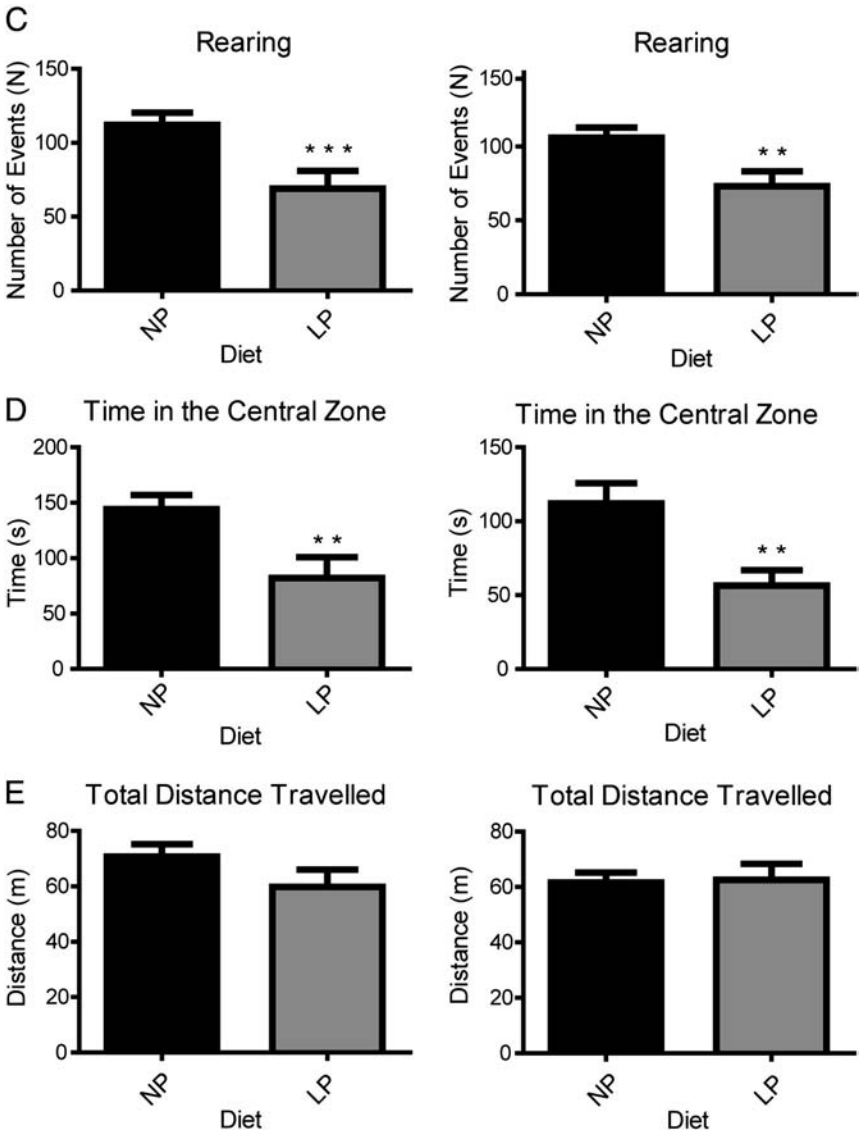

Fig. 6 (*continued*).

demonstrate the negative effect of reduced protein during pregnancy and lactation in male and female mice during risk assessment behaviors in this test. A similar anxious response was observed in the open field test. Perinatal protein restricted offspring traveled a minor distance and spent less time in the central zone and showed increased latency to leave peripheral zone characteristic response of avoidance risk behavior. In addition, female LP mice showed increased behavioral despair in the tail suspension test, a behavior sensitive to antidepressant drugs [47]. Considering the high comorbidity between major depression and generalized anxiety disorder [48], the behavioral phenotype of female LP mice in tail suspension test seems not to be unexpected.

Molecular basis for these responses remain to be uncovered. Structural and neurochemical changes in certain areas of the nervous system, like the GABAergic hippocampal system and glutamatergic activity, were observed in rats subjected to perinatal protein malnutrition suggesting that this condition may affect the excitatory and inhibitory interaction [49]. Recently, it was shown that protein restriction during gestation significantly decreases delivery to the fetus of essential fatty acids and lipids required for normal brain development in rat [50]. This deficiency in brain fatty acid content could affect important brain regions involved in approach/risk assessment behavior in the offspring.

Effects of early protein malnutrition on emotionality are supported by a number of studies. However, there does not seem to be consensus about the direction of these effects. While some authors indicate increased anxiety in early malnourished animals after nutritional recovery and in chronically malnourished animals, several studies report anxiolytic like effects of early malnutrition in rodents [16,17,51]. In this regard, it is important to consider potential species differences as well as the differences in techniques for inducing early protein malnutrition [52].

Interestingly, although the same distance was traveled by mice from both groups in the open field test, protein malnourished animals showed less locomotive activity and reduced average speed than their normal feeding counterparts in both arms of elevated plus maze device. This lack of activity is probably the result of adaptation to the situation and the need of the animals to save energy rather than an indication of locomotive impairment. Moreover, LP mice display less number of rearing in open field and made fewer climbs and escape attempts in the cage escape test. These results indicate deficit in exploratory

behavior and reduced motivation in pre and postnatal protein restricted offspring. These are complex behaviors that require not only motor coordination, but also integration of sensory input. Normal sensory function is critically important in social and exploratory behavior [53]. The change in exploratory behavior is particularly interesting because novelty seeking behavior is thought to be related to brain systems modulated by dopamine and is perturbed in many clinical manifestations [54]. Studies with severely malnourished children show a similar trend as found in the present study: marked behavioral impairments such as apathy, lack of activity, and exploration of the environment [6].

Several effects of maternal undernutrition have been described to be sex-dependent. However, in the present study negative effects on numerous development parameters and behaviors of the offspring following perinatal protein malnutrition were similar between both genders. Perhaps, the most remarkable difference was observed in tail suspension test where only female malnourished mice display behavioral despair. While we do not know the molecular bases for this difference, it would be consistent with epidemiological studies that show a higher incidence of major depressive disorder in female.

5. Conclusion

Epidemiological studies in humans have shown the negative effects on numerous developmental parameters such as affect, learning, memory, neuromuscular coordination, and behavioral development in offspring following perinatal malnutrition. However, the underlying molecular mechanisms affected are unknown. Coincidently, the present results show that maternal nutrition during pregnancy and lactation has significant effects on the physical and neurobiological development of mice offspring. In addition, findings about increased anxious and depressive-like behaviors and lack of exploration and motivation are indicative of the detrimental effects in offspring subsequent to insufficient protein during critical windows of brain development.

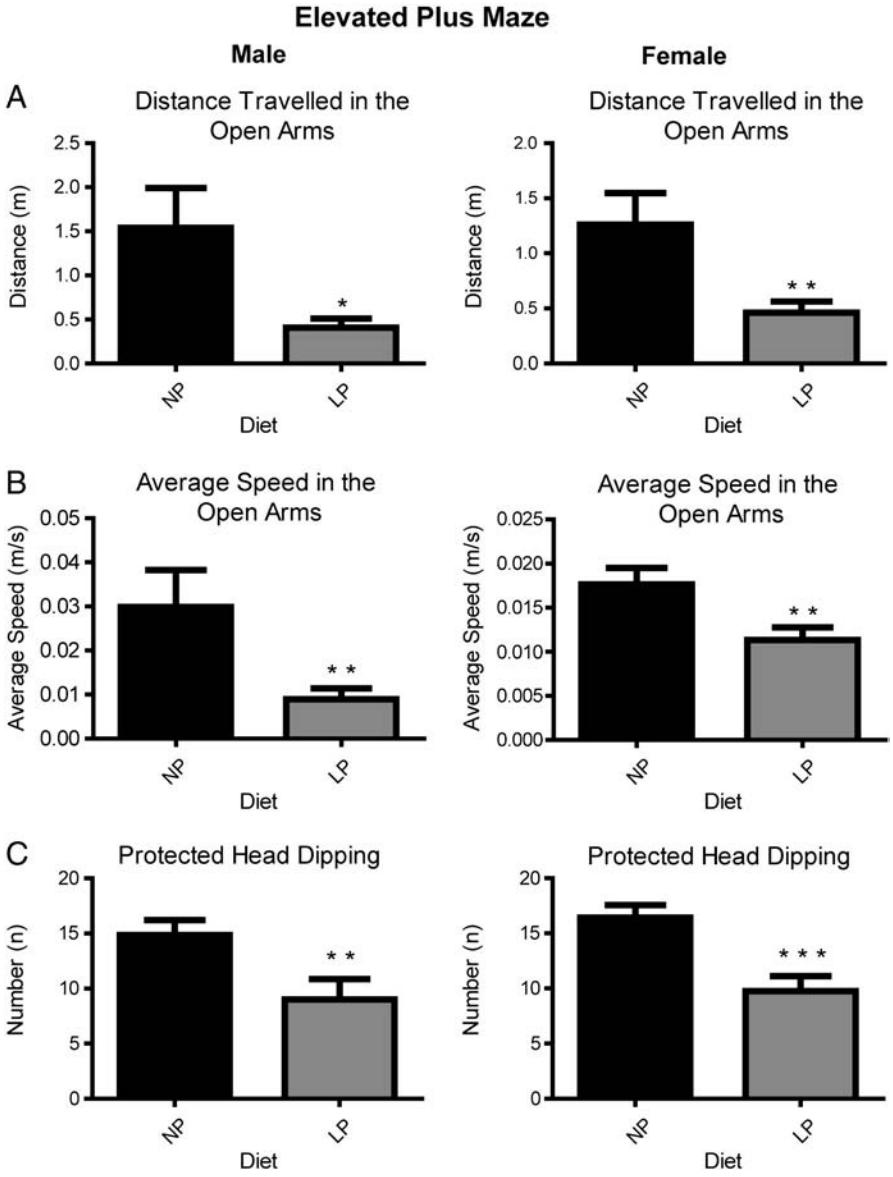

Fig. 7. Elevated plus maze. Male (left column) and female (right column) mice were analyzed separately. Distance traveled in the open arms (m) (A). Average speed in the open arms (m/s) (B). Number of protected head dippings (C). Total distance traveled (m) (D). Time in the tips zone (sec) (E). Number of rearings (F) and Number of entries to the open arms (n) were measured using the ANY-Maze software. Mean + SEM Mann–Whitney or Student's t-test. $n = 14$ males or $n = 12$–14 females for each treatment. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

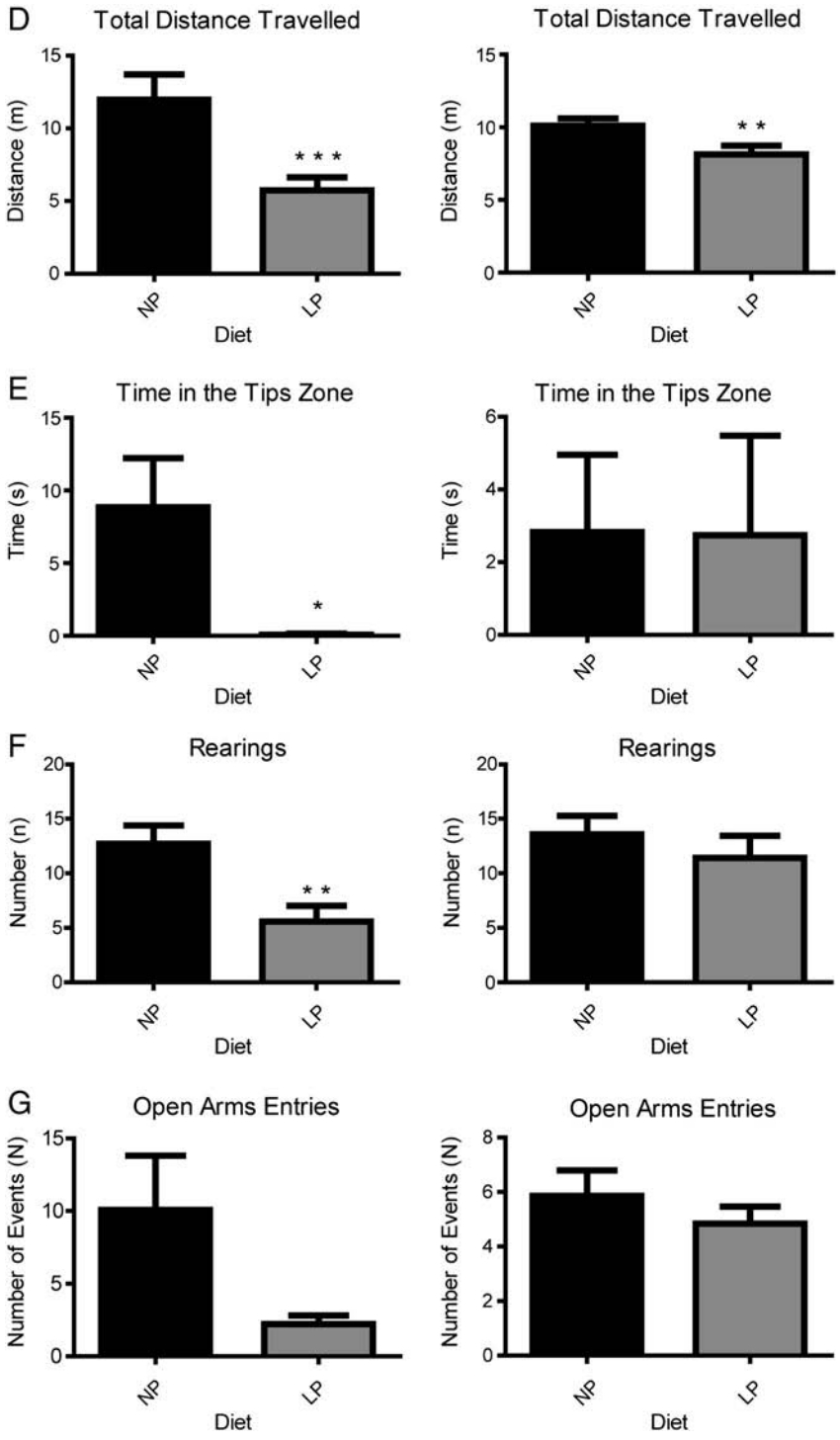

Fig. 7 (*continued*).

Hence, appropriate animal studies will help to understand the effect of maternal protein undernutrition on human perinatal development.

Acknowledgments

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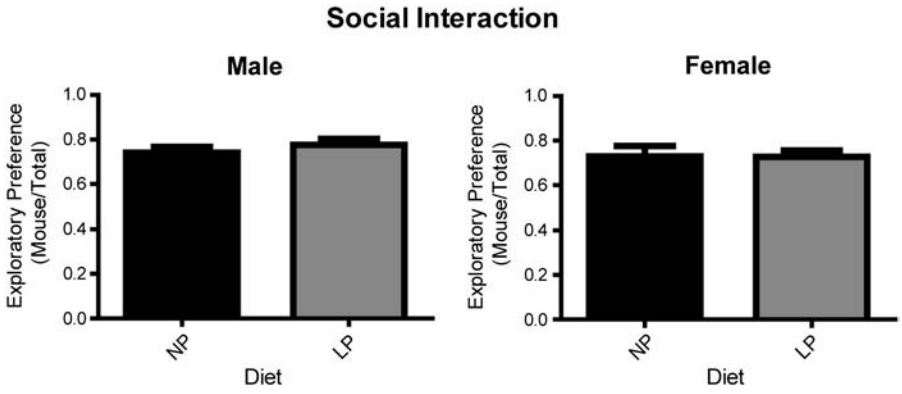

Fig. 8. Social interaction. Male (left column) and female (right column) mice were analyzed separately. Social preference is expressed as the time the mouse spent investigating the cylinder with the novel mouse, over the time it spent investigating both cylinders. Mean + SEM Student's *t*-test. $n = 6$–12 males or $n = 5$–7 females of each treatment.

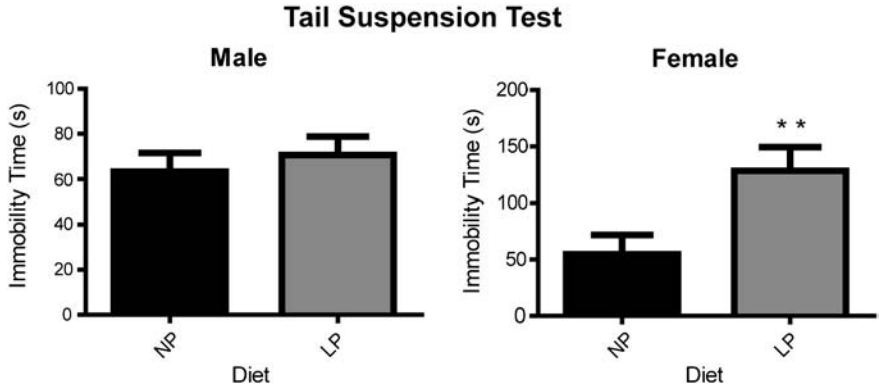

Fig. 9. Tail suspension test. Male (left column) and female (right column) mice were analyzed separately. Total immobility time (sec) for the 6 minute session. Mean + SEM Mann–Whitney. $n = 12$–6 males of $n = 6$–5 females for each treatment. $^{**}p < 0.01$.

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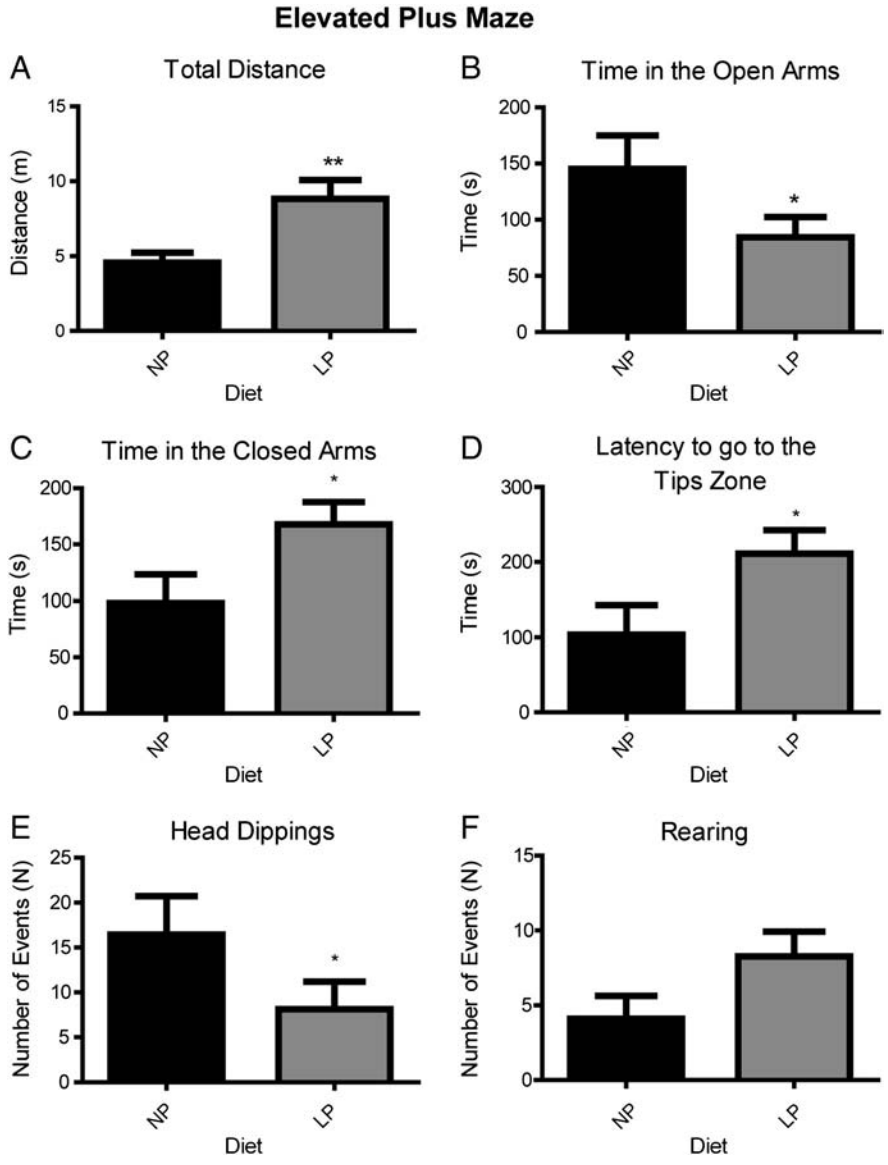

Fig. 10. Elevated plus maze of the F_0. Total distance traveled (m) (A). Time in the open arms (sec) (B). Time in the closed arms (sec) (C). Latency to go to the tips zone (sec) (D). Number of head dippings performed (E). Number of rearings (F), were measured using the ANY-Maze software. Mean + SEM Mann–Whitney or Student's *t*-test. $n = 8$–14 dams for each treatment. $^{*}p < 0.05$ and $^{**}p < 0.01$.

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