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Low or High Ethanol Intake during Gestation and Lactation: Effects on Rat Posnatal Development

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Abstract

Ethanol abuse is a disorder that affects the world, potentially reaching people of both sexes, different ages, and socioeconomic conditions. Ethanol abusive use leads to serious deleterious effects on various organic systems, and the use of this substance by pregnant women can cause developmental and metabolic problems in the fetus, in addition to the risk of fetal alcohol syndrome (FAS). The prevalence of complete FAS is around 1 - 2 per 1000 births, and it occurs when the pregnant woman consumes a large amount of ethanol. However, it is still unknown whether there is a safe dose of ethanol that can be consumed during pregnancy and whether there are consequences of using low doses on development. In addition to problems during pregnancy, the lactation phase can also be harmed by ethanol consumption. Besides nutritional and immunoprotective functions, lactation also impacts the proper development of the central nervous system of newborns. However, there are few reports on the consequences of ethanol consumption by the mother during this period. The existing reports are scarce, especially regarding evaluation of the consequences in periods after birth. Objectives: Investigate the effects of the consumption of low and high concentrations of ethanol by mothers during pregnancy and lactation on rat offspring. We analyzed nutritional parameters of the mother and pups, maternal behavior, and physical and neurobehavioral development of the offspring. The rats were allocated to one of four groups: Ethanol (2% ≈ 2.3 g/kg/day or 12% ≈ 10.1 g/kg/day), Control (pups without treatment that were subjected to tests), and Unmanipulated Control (pups that were not subjected

to tests or treatment). Results: The mothers in the 12% EtOH group showed a reduction in liquid consumption, feed intake, and calories consumed, mainly during lactation. The pups in the 2% EtOH and 12% EtOH groups showed delays in physical development parameters, such as the appearance of lanugo and ear opening. Conclusion: Ethanol causes harm to the offspring development, with a greater impact on females. However, further investigations are needed to determine whether these changes extend into adulthood. In any case, our results provide evidence for the recommendation to avoid ethanol consumption, even in low concentrations, during pregnancy and lactation.

Keywords

Ethanol, Gestation, Lactation, Physical Development, Neurobehavioral Parameters, Maternal Behavior

1. Introduction

Ethanol is the most consumed drug of abuse in the world. The use of this substance is not only legal but also encouraged in most cultures. According to a survey published by the World Health Organization [1], 43% of the world's population has consumed alcoholic beverages. The average per capita consumption in individuals above 15 years old is around 6.2 L/year [1] [2]; similar figures are reported by other authors [3]-[5]; with men being the highest consumers [6].

The mechanism of action of ethanol involves an increase in plasma membrane fluidity, impairing the conduction of nerve impulses and interfering with the function of neurotransmitters and receptors [7]-[10]. Ethanol has also direct actions on receptors, being the GABA-A receptor, one of the main targets. Ethanol increases the effects of the stimulation of this receptor [11]-[16]. Additionally, glutamatergic receptors, especially the kainate and NMDA subtypes, are also affected. By stimulating GABAergic transmission and inhibiting glutamatergic transmission, ethanol has a depressant effect on neurons [11] [17] [18]. Ethanol also increases serotoninergic transmission. Serotonin is implicated in the regulation of mood, satiety, sleep, among many other behaviors [19]-[23]. Ethanol's action on serotonin and dopamine is associated with the excitatory effects observed with low doses of this substance [20] [24]-[26].

Ethanol crosses the placental barrier and reaches the fetus at the same concentration present in the maternal blood, interfering with fetal development. Fetal alcohol syndrome (FAS) is the most well-described condition related to the use of ethanol during pregnancy. The condition is characterized by central nervous system abnormalities, facial alterations, and growth problems, among other signs [27]-[35].

Although not all children whose mothers consumed ethanol during pregnancy are diagnosed with FAS, they can present low birth weight, malformations, and cognitive problems [36]-[39].

Some studies in rats report that the consumption of high concentrations of ethanol by lactating mothers impairs the physical and neurological development of their offspring [40] [41]. Because ethanol is also rapidly transferred to breast milk, the infant may be ingesting it during breastfeeding. Some studies suggest that the concentration of ethanol in the milk is the same as in the mother's plasma [42]-[44], while others report that only 2% of the mother's concentration would pass to the milk, and this may vary among individuals [45]-[47].

Regardless of concentration, and despite the limited data, ethanol consumed during breastfeeding can cause alterations in offspring development such as impaired sleep quality, delayed neuromotor development, and learning difficulty [48]-[52].

Furthermore, experiments conducted with rats [50] showed that the alteration in GABAergic transmission promoted by alcohol caused negative impacts on maternal behavior. Thus, the mothers potentially dedicate less time to caring for their offspring during the lactation period [53]-[56].

It is important to highlight that the care provided by the mother is essential for the health and survival of rat offspring, as they are born immature. In some mammal species, it may take years for them to become self-sufficient [56]. In this respect, the impairment in maternal behavior caused by ethanol can also lead to problems in the development and maturation of the central nervous system of rats [52] [54] [57].

Studies with laboratory rats are important for investigating the effects of drug administration during pregnancy. Some of the advantages of this use are the short gestational period (21 days) and the number of offspring, which facilitates the observation of possible alterations and follow-up of the development in the postnatal period [33].

In studies focusing on FAS, there is a large variation in the amount and duration of ethanol administration during pregnancy, ranging from 6.6 g/kg/day to 300 g/kg/day, and from acute administration in a specific period of gestation to 10-day or more repeated treatments [2]-[19].

The British National Institute for Health advises women not to exceed a dose of 12 g/day of ethanol [20], while in other countries total abstinence is recommended [21] [22]. These diverse orientations can cause doubts in pregnant women, especially considering that the consumption of ethanol increases annually, with 55% of pregnant women using this drug [23]-[25]. In addition, despite several data in the literature, it is still not known whether there is a safe dose of ethanol that can be used during gestation and lactation. Therefore, our objective was to evaluate the effects of 2% or 12% ethanol as the exclusive source of liquid during gestation and lactation on physical and neurobehavioral development of rat offspring from birth to weaning.

2. Materials and Methods

Three-month-old Wistar rats (32 females and 32 males) were obtained from the

animal facility of Universidade Metodista de São Paulo. The environment was kept at controlled temperature (24˚C ± 1˚C) and humidity (45%) with a 12/12-hour light/dark cycle (lights on at 7 am and off at 7 pm). All experiments were approved by the Ethics Committee for Animal Use (CEUA-Metodista - Protocol No. 168/2016).

Pregnancy control

Starting five days before the females were placed with the males, their weight, liquid consumption, and food consumption were daily monitored. This was done to ensure balance among the experimental groups.

Each female was placed in a housing cage (20 × 30 × 12 cm) with a male. The vaginal lavage test was performed on the day after the pair formation to verify pregnancy. The female was considered pregnant when the presence of sperm was detected in the lavage. Immediately after pregnancy confirmation, the female was separated from the male and allocated to one of the 4 groups: control (CTRL) n = 8, unhandled control (UNC) n = 8, 2% ethanol (2% EtOH) n = 8, and 12% ethanol (12% EtOH) n = 9. The ethanol concentrations were chosen based on previous studies conducted in our laboratory.

Dams and their offspring in the control (CTRL) group received water during gestation and lactation and underwent the same experimental timeline as the ethanol (2% and 12% EtOH) groups. For the unhandled control (UNC) group, dams received water after pregnancy confirmation, but no interventions were performed during gestation or lactation. This group served as baseline control for the strain, with handling restricted to routine cage changes.

The groups allocation was randomized, and behavioral observation and developmental scoring were conducted blindly.

Ethanol administration, offspring birth, and litter standardization

Immediately after pregnancy confirmation, ethanol solutions (2% and 12%, respectively) were introduced as the exclusive source of liquid to the ethanol groups. Water was offered to the control groups. The day of birth was designated as postnatal (PN) day 1 (PN1) for all groups. Twenty-four hours after the offspring were born, the litter was randomly standardized to 4 females and 4 males. The number of male and female pups was respectively 35 and 27 in the CTRL group, 34 and 29 in the 2% EtOH group, and 40 and 32 in the 12% EtOH group.

Fluid consumption was recorded daily by weighing the drinking bottles before and 24 hours after being provided for the animals. After being measured, the liquid was discarded, the bottle was washed with running water, and a new ethanol solution was prepared with filtered water. In the control groups, the same procedure was performed, but with the addition of filtered water to the bottle.

On the 15th day of lactation (39th or 41th), each group was offered an additional bottle with filtered water. The control groups were given two bottles of water, while the ethanol group was given one bottle of ethanol (2% or 12%) and one bottle of water.

The total calories intake (feed intake plus ethanol consumption) by the dams

were calculated as follows:

Feed intake: calculation was performed by multiplying feed intake by 3.78 kcal, corresponding to the caloric content of 1 gram of standard chow.

Ethanol consumption: calculation was performed by multiplying the consumption by 0.02 (2%, corresponding to 0.2 g in 1 mL) or 0.12 (12%, corresponding to 1.2 g in 1 mL) × 0.792 (ethanol density) × 7 (caloric value of ethanol per gram: kcal/g).

The pups from the unhandled control (UNC) were not subjected to the procedures described below.

Body weight evaluation

The body weights of the pups were monitored from postnatal (PN) day 2 (PN2) to PN40, with daily weighing in the first 22 days and then every three days in the remaining period.

Analysis of physical development

From birth until the completion of development, determined by testicular descent and vaginal opening, pups were examined daily. Each pup was briefly removed from the dam to assess the latency to ear unfolding, ear canal opening, incisor eruption, eye opening, appearance of lanugo, fur development, vaginal opening, and testicular descent.

Analysis of neurobehavioral parameters

Palmar grasp reflex: consists of opening the animal's paw, placing the tip of a pencil on the paw, and timing how long it takes for the paw to close. This reflex was evaluated from PN2 to PN10.

Postural reflex: consists of placing the pup in a supine (dorsal decubitus) position and recording the time it takes to return to the normal prone (ventral decubitus) position, with the paws supporting the body. The parameter was evaluated from PN2 to PN10.

Negative geotaxis reflex: consists of placing the pup supported by all four paws on a ramp with a 30-degree inclination, with its head facing downwards, and timing how long it takes for the pup to position itself with its head facing upwards. This parameter was evaluated from PN5 to PN10.

Climbing reflex: The animal is placed with its four paws on a ramp with a 30˚ incline, with the side of its head facing the highest point, and it is observed whether the animal climbs, descends, or remains in place where it was initially placed. The time taken to perform the action is measured, with a maximum time of 300 seconds, from PN5 to PN10.

Grasping reflex: It consists of supporting the front paws of the puppy on a bar higher than the animal's size and verifying whether it grasps or not. This relflex was analyzed from PN5 to PN10.

Maternal behavior

On days 4, 7, and 10 after birth, the pups were removed from the mother for 30 minutes. After this time, the pups were placed back in the same cage in random positions. Then, the mother was placed in the center of the cage and filmed for 30

minutes to verify the time it took for the mother to retrieve the first pup, the time it took to retrieve all pups, the total number of pups retrieved during the observation period, and the time spent nursing the pups.

Statistical Analysis

Data normality and sphericity were evaluated by the Shapiro-Wilk and Mauchly tests, respectively. Data with non-normal distribution were analyzed using the Generalized Estimation Equations (GEE) model. Data with normal distribution were analyzed by ANOVA. When group effects, time effects, or interaction between these factors were detected, data were subjected to pairwise comparison with Sidak or Turkey post hoc tests. All analyses were performed using Statistical Package for the Social Sciences (SPSS) for Windows, version 26.0. The adopted significance level (α) was 5%.

Individual pups, rather than litter means, were used as the primary statistical unit to capture intra-litter phenotypic variability. Because maternal toxicant exposure yields heterogeneous transfer via placenta and milk, evaluating offspring individually is critical to map biological vulnerability [58]. While analyzing litter as single units can obscure treatment effects, utilizing individual data points coupled with GEE modeling accounts for litter-associated dependencies without sacrificing statistical power.

3. Results

MOTHERS

Three days prior to parturition, the dams were not handled. In **Figure 1**, the days represent the following periods: pre-pregnancy (1^{th} to 5^{th}), pregnancy (6^{th} to 23^{th}), lactation (24^{th} to 38^{th}), and late lactation (39^{th} to 44^{th}), whereas in the remaining figures, the periods correspond to pre-pregnancy (1^{th} to 5^{th}), pregnancy (6^{th} to 26^{th}), lactation (27^{th} to 40^{th}), and late lactation (41^{th} to 47^{th}).

Figure 1 illustrates the daily mass gain of mothers from the control (CTRL), unhandled control (UNC), and ethanol at concentrations of 2% EtOH (approximately 2.3 g/kg/day) or 12% EtOH (approximately 10.1 g/kg/day) groups. In the pre-pregnancy period, there was a time effect [$F(2, 69) = 6.416$; $p = 0.002$], but not a group effect [$F(3, 29) = 0.295$; $p = 0.829$] or an interaction between time and group [$F(7, 69) = 1.459$; $p = 0.195$]. During pregnancy, there was a time effect [$F(3, 89) = 530$; $p = 0.01$] and an interaction between time and group [$F(9, 89) = 2.884$; $p < 0.05$], but no group effect [$F(3, 29) = 0.373$; $p = 0.773$].

During lactation (24^{th} to 38^{th} day), there was a time effect [$F(7, 158) = 35$; $p < 0.001$], a group effect [$F(2, 22) = 3.708$; $p = 0.041$], and an interaction between time and group [$F(14, 158) = 4.859$; $p < 0.001$]. Pairwise comparison with Sidak correction indicated that the 12% ethanol group had a statistically lower average gain on the 28^{th} day and from the 34^{th} to the 38^{th} day compared to the control group, and lower than the 2% ethanol group from the 32^{nd} to the 38^{th} day. Regarding the late lactation period (39^{th} to 44^{th} day), when each cage received an additional water bottle, there was no time effect [$F(2, 60) = 0.496$; $p = 0.669$], group

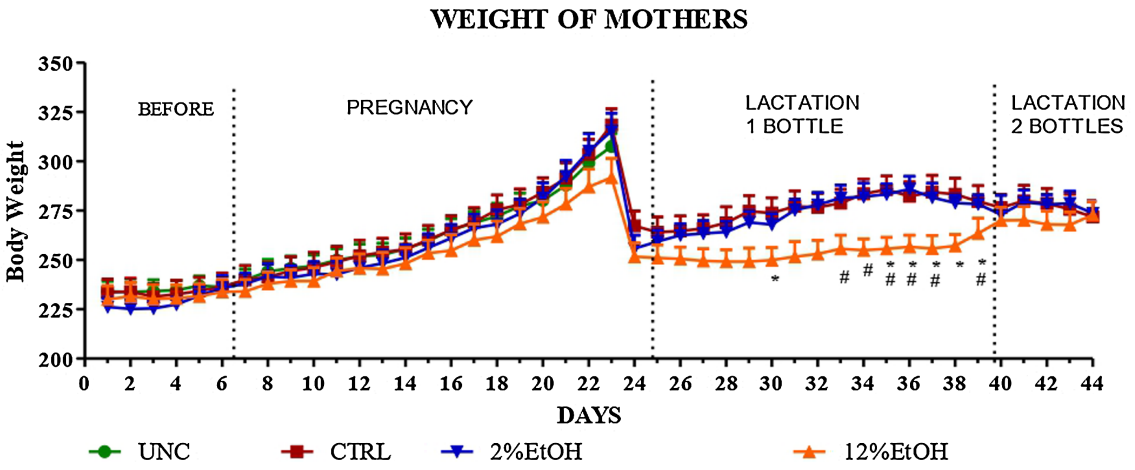

Figure 1. Daily variation in the body mass of mothers who consumed water or different concentrations of ethanol. Mothers in the control group (CTRL) consumed water throughout the observation period. Mothers in the group labeled unhandled control (UNC) drank water and were not subjected to the experimental procedures described. For mothers in the experimental groups, water was offered during the 5 days preceding pregnancy, ethanol was the sole source of liquid from day 6 to day 41, and optional ethanol along with the choice of water was provided from day 42 to 47. The group designated as low-dose ethanol (2% EtOH ≈ 2.3 g/kg/day) received 2% ethanol, while the high-dose ethanol group (12% EtOH ≈ 10.1 g/kg/day) received 12% ethanol. Each point represents the mean ± standard error of the mean of mass (g) (n = 8: CTRL, 2% EtOH and n = 9: 12% EtOH). There is no statistically significant difference between the groups before and during gestation. (*significance at $p < 0.05$ compared to the CTRL group was indicated. ANOVA followed by Sidak post hoc test was used. #significance at $p < 0.05$ compared to the 2% EtOH group was indicated. ANOVA followed by Sidak post hoc test was use.)

effect [F (2, 22) = 0.559; p = 0.580], or interaction between time and group [F (5, 60) = 1.017; p = 0.419].

Figure 2 illustrates the daily feed intake of mothers from the control (CTRL) and unhandled control (UNC) groups or ethanol at concentrations of 2% EtOH or 12% EtOH. In the pre-pregnancy period, there was a time effect [F (4, 116) = 6.870; $p < 0.001$], but not a group effect [F (3, 29) = 0.164; p = 0.920] or an interaction between time and group [F (12, 116) = 0.832; p = 0.617]. During pregnancy, there was a time effect [F (10, 291) = 21.283; $p < 0.001$], a group effect [F (3, 29) = 17.897; $p < 0.001$], and an interaction between time and group [F (30, 291) = 1.497; p = 0.05]. Pairwise comparison with Sidak correction indicated that the 12% ethanol group had a statistically lower average intake on days 7, 8, 10, 11, 14, 15, and 17th to 25th compared to the control group, on days 7th to 11th, 14th, 15th, and 17th to 25th compared to the UNC group, and on days 8th, 10th, and 19th to 24th compared to the 2% ethanol group.

During lactation (27th to 41st day), there was a time effect [F (6, 178) = 150.528; $p < 0.001$], a group effect [F (3, 29) = 24.255; $p < 0.001$], and an interaction between time and group [F (18, 178) = 2.715; $p < 0.001$]. Pairwise comparison with Sidak correction indicated that the 12% ethanol group had a statistically lower average intake on days 29th to 41st compared to the UNC group, and on days 32nd to 41st compared to the 2% ethanol group. Regarding the late lactation period (42nd to 47th day), when each cage received an additional water bottle, there was no time effect [F (3, 94) = 19.108; $p < 0.001$], group effect [F (3, 29) = 0.894; p =

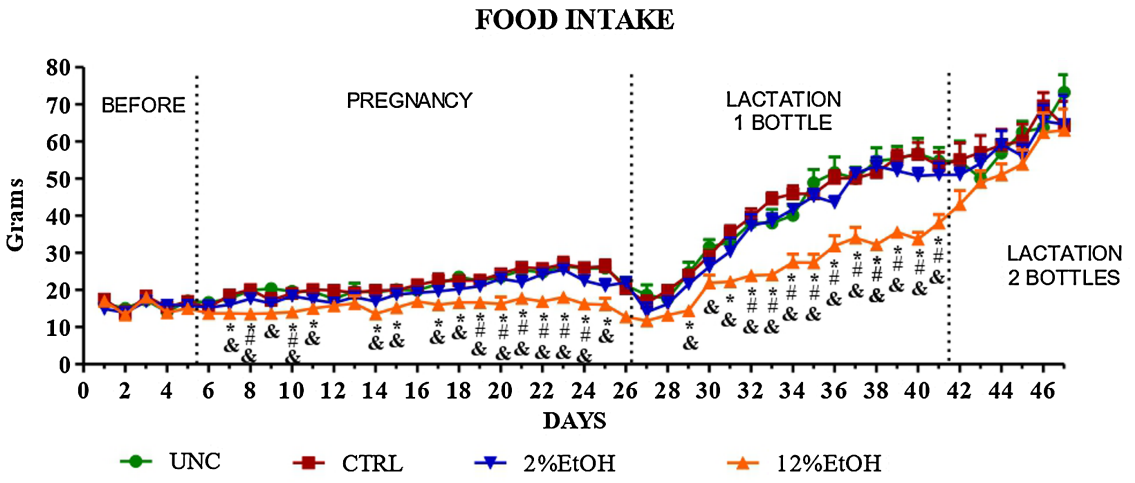

Figure 2. Daily variation in maternal food intake among those who consumed water or different concentrations of ethanol. Mothers in the control group (CTRL) consumed water throughout the entire observation period. Mothers in the unhandled control group (UNC) drank water and were not subjected to the experimental procedures described. For mothers in experimental groups, water was provided during the 5 days preceding pregnancy, ethanol was the sole source of liquid intake from day 6 to day 41, and optional ethanol along with the choice of water was offered from day 42 to 47. The group named low-dose ethanol (2% EtOH ≈ 2.3 g/kg/day) received 2% ethanol, while the high-dose ethanol group (12% EtOH ≈ 10.1 g/kg/day) received 12% ethanol. Each data point represents the mean ± standard error of the mean in mass (g) (n = 8: CTRL, 2% EtOH and n = 9: 12% EtOH). There is no statistically significant difference between the groups before gestation. (*significance at $p < 0.05$ compared to the CTRL group was indicated. ANOVA followed by Sidak post hoc test was used. #significance at $p < 0.05$ compared to the 2% EtOH group was indicated. ANOVA followed by Sidak post hoc test was use & significance at $p < 0.05$ compared to the UNC group was indicated. ANOVA followed by Sidak post hoc test was used.)

0.456], or interaction between time and group [F (9, 94) = 1.037; p = 0.418].

Figure 3 illustrates the daily liquid consumption of mothers from CTRL and UNC groups or ethanol at concentrations of 2% EtOH or 12% EtOH. In the pre-pregnancy period, there was a time effect [F (4, 116) = 6.552; $p < 0.001$], but not a group effect [F (3, 29) = 0.156; p = 0.925] or an interaction between time and group [F (12, 116) = 1.454; p = 0.152]. During pregnancy, there was a time effect [F (7, 217) = 40.811; $p < 0.001$] and a group effect [F (3, 29) = 7.007; p = 0.001], but not an interaction between time and group [F (22, 217) = 1.531; $p < 0.064$].

During lactation (27th to 41st day), there was a time effect [F (4, 137) = 39.278; $p < 0.001$], a group effect [F (3, 29) = 7.975; $p < 0.001$], and an interaction between time and group [F (14, 137) = 2.339; p = 0.006]. Pairwise comparison with Sidak correction indicated that the 12% ethanol group had a statistically lower average liquid consumption compared to the average of the control, unhandled control, and 2% ethanol groups from the 31st to the 41st day. Regarding the late lactation period (42nd to 47th day), when each cage received an additional water bottle, there was no time effect [F (2, 43) = 0.631; p = 0.595] or interaction between time and group [F (2, 43) = 1.132; p = 0.346], but there was a group effect [F (1, 15) = 25.730; $p < 0.001$]. Pairwise comparison with Sidak correction indicated that the 12% ethanol group had a statistically lower average liquid consumption compared to the average of the control, non-manipulated, and 2% ethanol groups from the 42nd to

the 47th day.

Figure 4 illustrates the dose of 2% or 12% ethanol consumed during gestation and lactation. Although not statistically significant, we observed a slight increase in the dose of ethanol 2% and 12% during lactation, while the 12% group showed a reduction in ethanol consumption after the water bottle was made available.

Figure 5 illustrates the parameters related to maternal behavior of mothers from the control (CTRL) and ethanol groups at concentrations of 2% EtOH or

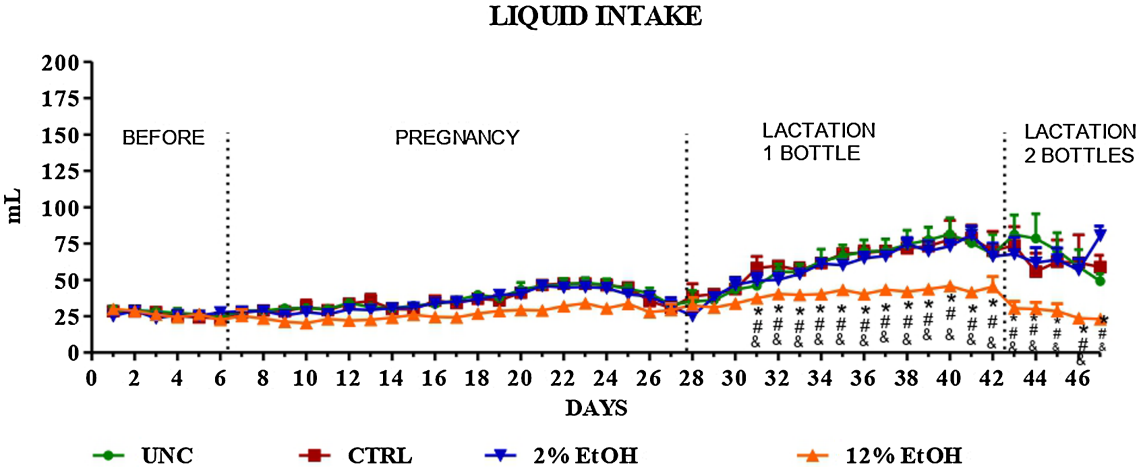

Figure 3. Daily variation in liquid intake by mothers who consumed water or different concentrations of ethanol. Mothers in the control group (CTRL) consumed water throughout the entire observation period. Mothers in the unhandled control group (UNC) drank water and were not subjected to the experimental procedures described. For mothers in the experimental groups, water was provided during the 5 days preceding pregnancy, ethanol was the sole source of liquid intake from day 6 to day 41, and optional ethanol along with the choice of water was offered from day 42 to 47. The group named low-dose ethanol (2% EtOH ≈ 2.3 g/kg/day) received 2% ethanol, while the high-dose ethanol group (12% EtOH ≈ 10.1 g/kg/day) received 12% ethanol. Each data point represents the mean ± standard error of the mean in mass (g) (n = 8: CTRL, 2% EtOH, and n = 9: 12% EtOH). There is no statistically significant difference between the groups before and during gestation. (*significance at $p < 0.05$ compared to the CTRL group was indicated. ANOVA followed by Sidak post hoc test was used. #significance at $p < 0.05$ compared to the 2% EtOH group was indicated. ANOVA followed by Sidak post hoc test was use & significance at $p < 0.05$ compared to the UNC group was indicated. ANOVA followed by Sidak post hoc test was used.)

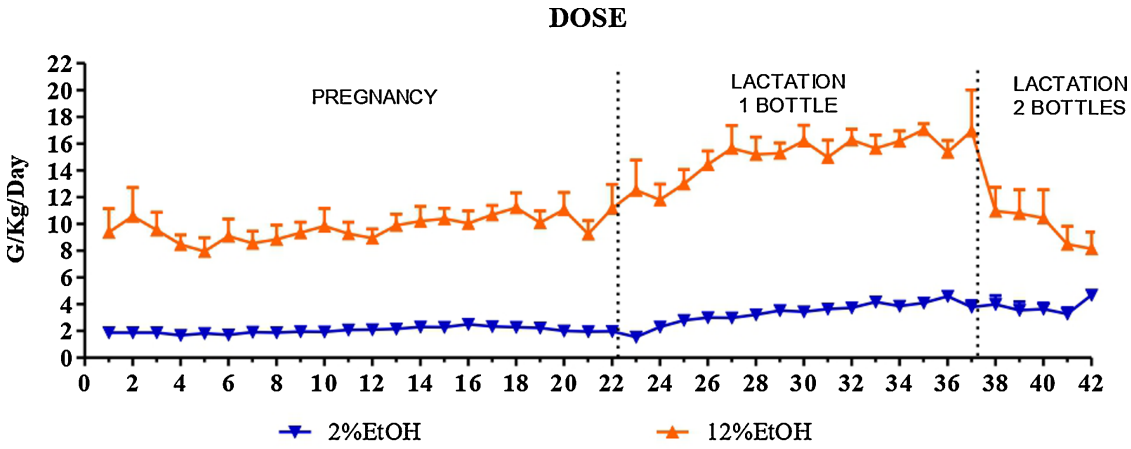

Figure 4. Daily variation in the ethanol dose ingested by mothers during gestation and lactation (n = 8: 2% EtOH and n = 9: 12% EtOH). There is no statistically significant difference within the same group compared to day 1.

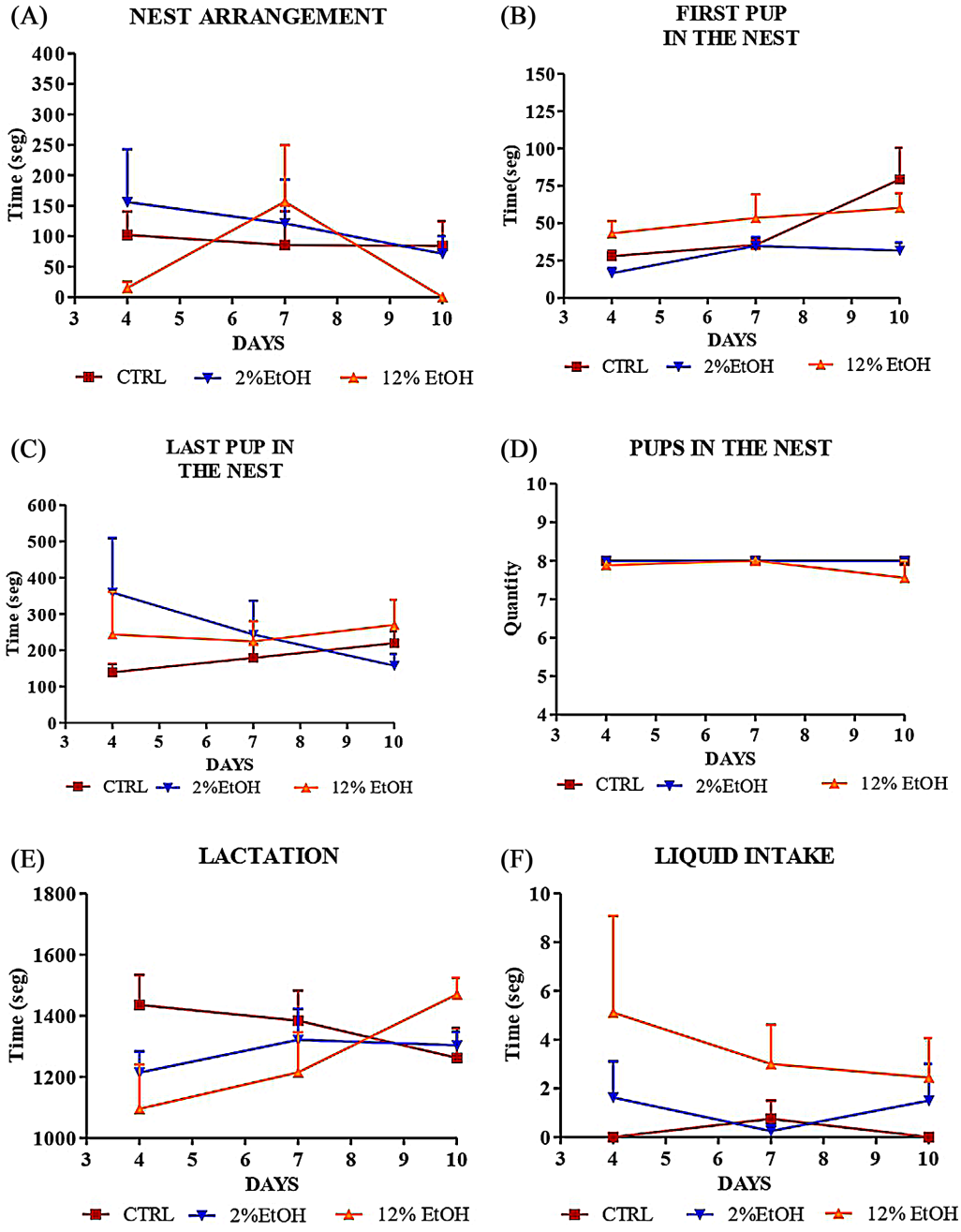

Figure 5. Maternal behavior of mothers who consumed water or different concentrations of ethanol. Mothers in the control group (CTRL) consumed water throughout the entire observation period. For mothers in experimental groups, ethanol was provided as the sole source of liquid intake. The group named low-dose ethanol (2% EtOH ≈ 2.3 g/kg/day) received 2% ethanol, while the high-dose ethanol group (12% EtOH ≈ 10.1 g/kg/day) received 12% ethanol. Each data point represents the mean ± standard error of the mean (n = 8: CTRL, 2% EtOH, and n = 9: 12% EtOH). There is no statistically significant difference between the groups in the parameters analyzed.

12% EtOH. For nest building (A), there was no time effect [F (1, 32) = 1.468; p = 0.243], group effect [F (2, 22) = 0.494; p = 0.617], or interaction between time and group [F (2, 32) = 1.317; p = 0.286]. For the first pup in the nest (B), there was a time effect [F (1, 32) = 7.650; p = 0.040], but not a group effect [F (2, 22) = 2.857;

p = 0.079] or interaction between time and group [F (2, 32) = 2.257; p = 0.100]. For the last pup in the nest (C), there was no time effect [F (1, 33) = 0.152; p = 0.801], group effect [F (2, 22) = 0.784; p = 0.469], or interaction between time and group [F (3, 33) = 0.843; p = 0.481]. For pups in the nest (D), there was no time effect [F (1, 22) = 0.846; p = 0.368], group effect [F (2, 22) = 0.880; p = 0.429], or interaction between time and group [F (2, 22) = 0.880; p = 0.429]. For nursing time (E), there was no time effect [F (2, 44) = 1.087; p = 0.346], group effect [F (2, 22) = 0.497; p = 0.615], but there was an interaction between time and group [F (4, 44) = 3.177; p = 0.022]. For liquid intake (F), there was no time effect [F (2, 44) = 0.394; p = 0.677], group effect [F (2, 22) = 1.618; p = 0.221], or interaction between time and group [F (4, 44) = 0.445; p = 0.776].

4. Offspring

Physical Development

Figure 6 illustrates the weight gain of females (A and B) and males (C and D) during lactation and after weaning. The GEE model for females indicated a significant effect of time ($\chi^2_{(28)}$ = 1753; p < 0.001), group ($\chi^2_{(2)}$ = 17.883; p < 0.001), and interaction between time and group ($\chi^2_{(56)}$ = 1072.789; p < 0.001). Pairwise comparisons with Sidak correction revealed that the 12% ethanol group had lower average weight gain compared to the control and 2% ethanol groups from the 11th to the 21st day of lactation. For males, the GEE model indicated a significant effect of time ($\chi^2_{(28)}$ = 1160; p < 0.001), group ($\chi^2_{(2)}$ = 29.852; p < 0.001), and interaction between time and group ($\chi^2_{(56)}$ = 1056.790; p < 0.001). Pairwise comparisons with Sidak correction showed that the 12% ethanol group had lower average weight gain compared to the control group from the 8th to the 21st day of lactation, and on the 11th and 12th days of lactation compared to the 2% ethanol group.

Figure 7 illustrates the daily growth of females (A and B) and males (C and D) during lactation and after weaning. The GEE model for females indicated a significant effect of time ($\chi^2_{(28)}$ = 131,023.167; p < 0.001), group ($\chi^2_{(2)}$ = 14.757; p = 0.001), and interaction between time and group ($\chi^2_{(56)}$ = 667.814; p < 0.001). Pairwise comparisons with Sidak correction revealed that the 12% ethanol group had lower average daily growth than the control group on the 15th and 17th day of lactation, and from the 14th, 15th, and 17th to the 21st day of lactation compared to the 2% ethanol group. At weaning, there was a reduction on the 22nd day compared to the control and 2% ethanol groups. For males, the GEE model indicated a significant effect of time ($\chi^2_{(28)}$ = 210,591.767; p < 0.001), group ($\chi^2_{(2)}$ = 30.004; p < 0.001), and interaction between time and group ($\chi^2_{(56)}$ = 939.989; p < 0.001). Pairwise comparisons with Sidak correction showed that the 12% ethanol group had lower average daily growth than the control and 2% ethanol groups from the 12th to the 21st day of lactation. At weaning, there was a reduction on the 22nd and 23rd day compared to the control group, and on the 22nd day compared to the 2% ethanol group.

Figure 8 and Figure 9 illustrate the latency (in days) to: ear unfolding (A), ear

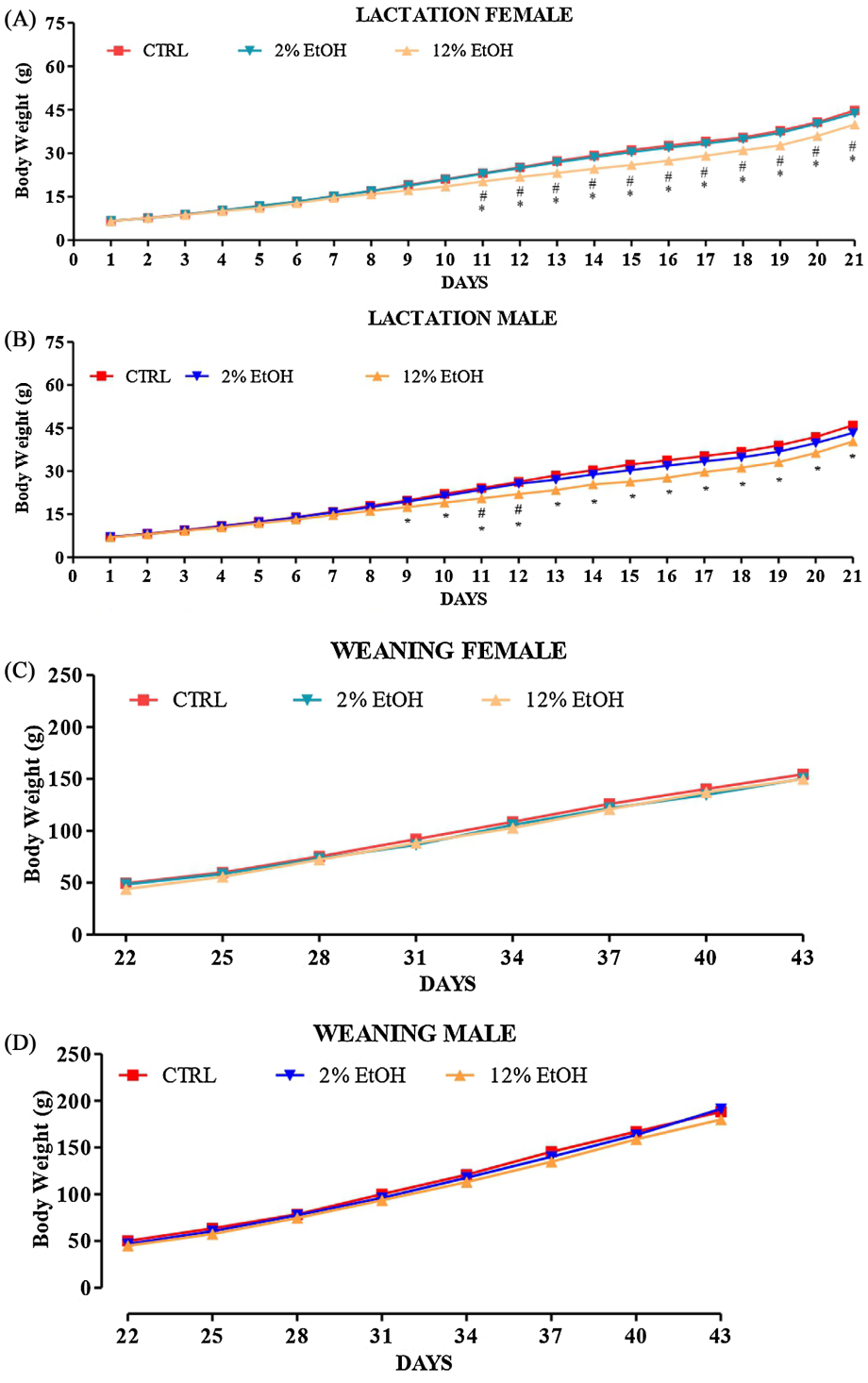

Figure 6. Weight gain of the offspring during lactation and weaning. In the offspring of the control group (CTRL), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group (2% EtOH) and the 12% ethanol group (12% EtOH), mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10.1 g/kg/day), respectively, as the sole source of liquid intake. During lactation (days 1 to 21), the offspring were weighed daily. After weaning (days 22 to 43), weight measurement occurred every three days. Each data point represents the mean ± standard error of the mean. For females, CTRL n = 27, 2% EtOH = 29, and 12% EtOH n = 32; for males, CTRL n = 35, 2% EtOH n = 34, and 12% EtOH n = 40. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test.)

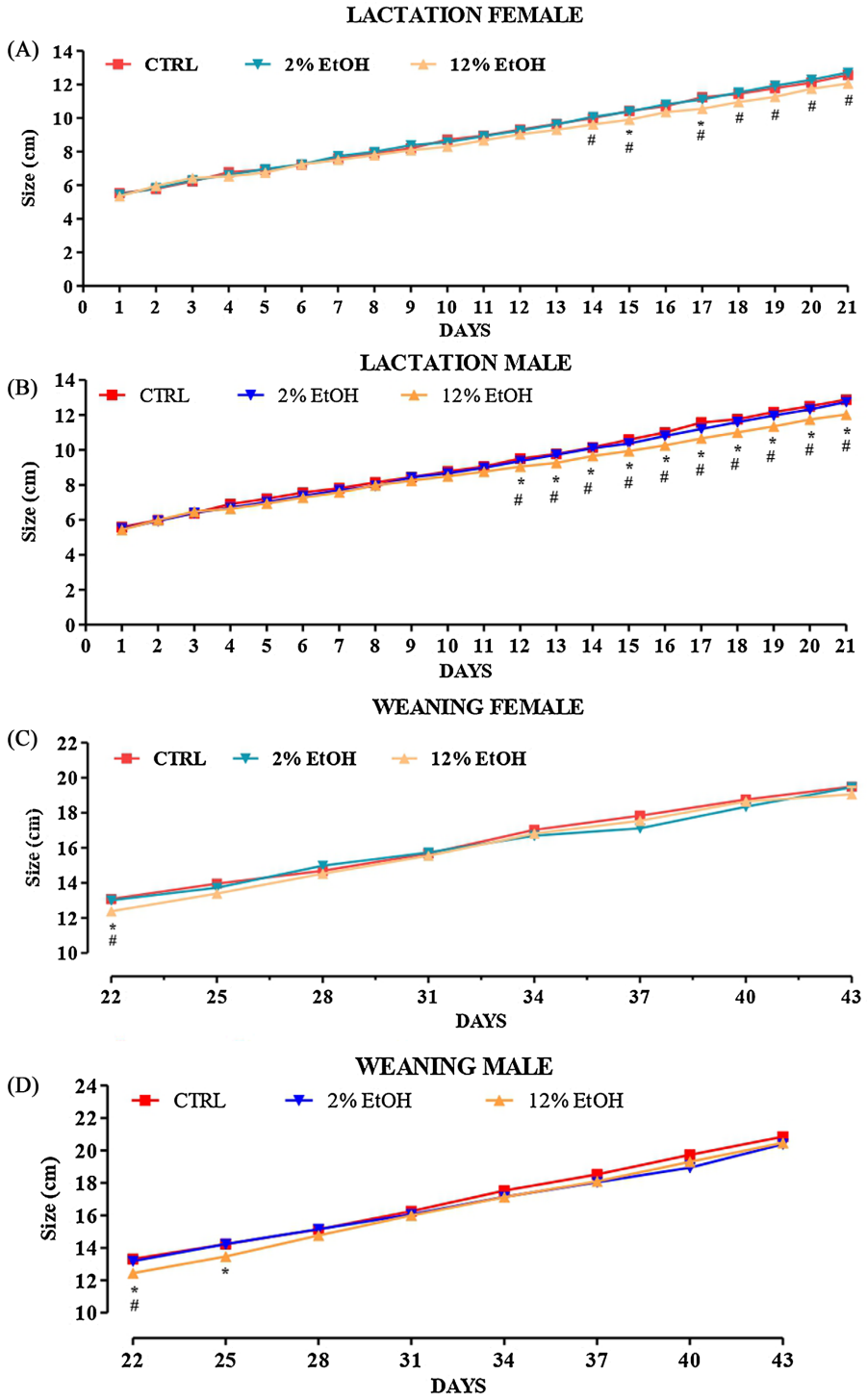

Figure 7. Size of the offspring during lactation and weaning. In the offspring of the control group (CTRL), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group (2% EtOH) and the 12% ethanol group (12% EtOH), mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10.1 g/kg/day), respectively, as the sole source of liquid intake. During lactation (days 1 to 21), the offspring were weighed daily. After weaning (days 22 to 43), weight measurements occurred every three days. Each data point represents the mean ± standard error of the mean. For females, CTRL n = 27, 2% EtOH = 29, and 12% EtOH n = 32; for males, CTRL n = 35, 2% EtOH n = 34, and 12% EtOH n = 40. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test.)

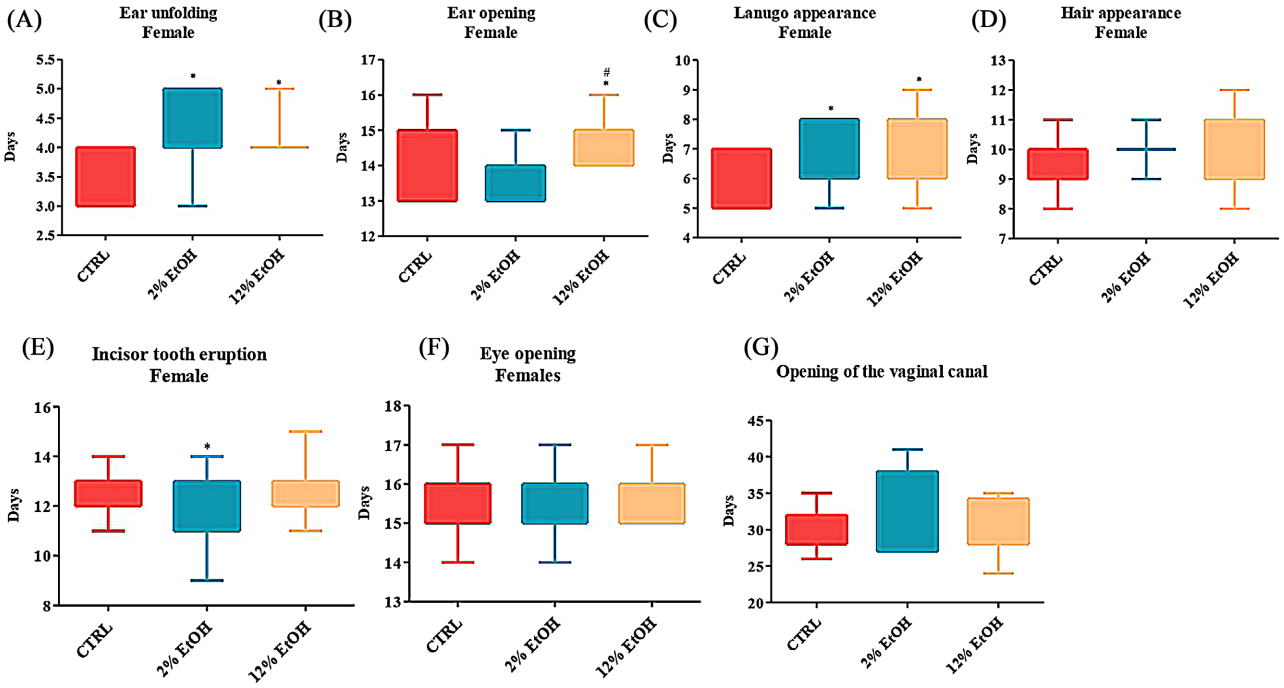

Figure 8. Parameters of physical development in female offspring. Ear unfolding (A), Ear canal opening (B), Lanugo appearance (C), Hair appearance (D), Incisor teeth eruption (E), Eye opening (F), and Vaginal canal opening (G). In the offspring of the control group (CTRL), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group (2% EtOH) and the 12% ethanol group (12% EtOH), mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10.1 g/kg/day), respectively, as the sole source of liquid intake. Each data point represents the median ± standard error of the mean. CTRL n = 27, 2% EtOH = 29, and 12% EtOH n = 32. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test.)

opening (B), appearance of lanugo (C), appearance of hairs (D), eruption of incisor teeth (E), eye opening (F) and vaginal opening or testicular descent (G) in female and male pups, respectively. Females, the generalized linear model indicated a significant effect of group on ear unfolding ($\chi^2_{(2)} = 12.940$; $p = 0.002$), ear opening ($\chi^2_{(2)} = 23.649$; $p < 0.001$), lanugo appearance ($\chi^2_{(2)} = 19.654$; $p < 0.001$), and tooth eruption ($\chi^2_{(2)} = 7.782$; $p = 0.002$). Pairwise comparisons with Sidak correction indicated the following: (A) The 2% and 12% ethanol groups had statistically lower mean compared to the 12% ethanol group; (B) The 12% group had statistically higher mean compared to the 2% ethanol group and control group; (C) The 2% and 12% ethanol groups had statistically higher mean compared to the control group; (D) The 12% ethanol group had statistically lower mean compared to the 12% ethanol group. For the other parameters, there was no significant effect of the groups: hair appearance ($\chi^2_{(2)} = 3.545$; $p = 0.170$), eye opening ($\chi^2_{(2)} = 2.238$; $p = 0.327$), vaginal opening ($\chi^2_{(2)} = 4.934$; $p = 0.085$).

For males, the generalized linear model indicated a significant effect of group on ear unfolding ($\chi^2_{(2)} = 7.556$; $p = 0.023$), ear opening ($\chi^2_{(2)} = 33.191$; $p < 0.001$), lanugo appearance ($\chi^2_{(2)} = 9.660$; $p = 0.008$), and eye opening ($\chi^2_{(2)} = 7.337$; $p = 0.026$). Pairwise comparisons with Sidak correction indicated the following: (A, B,

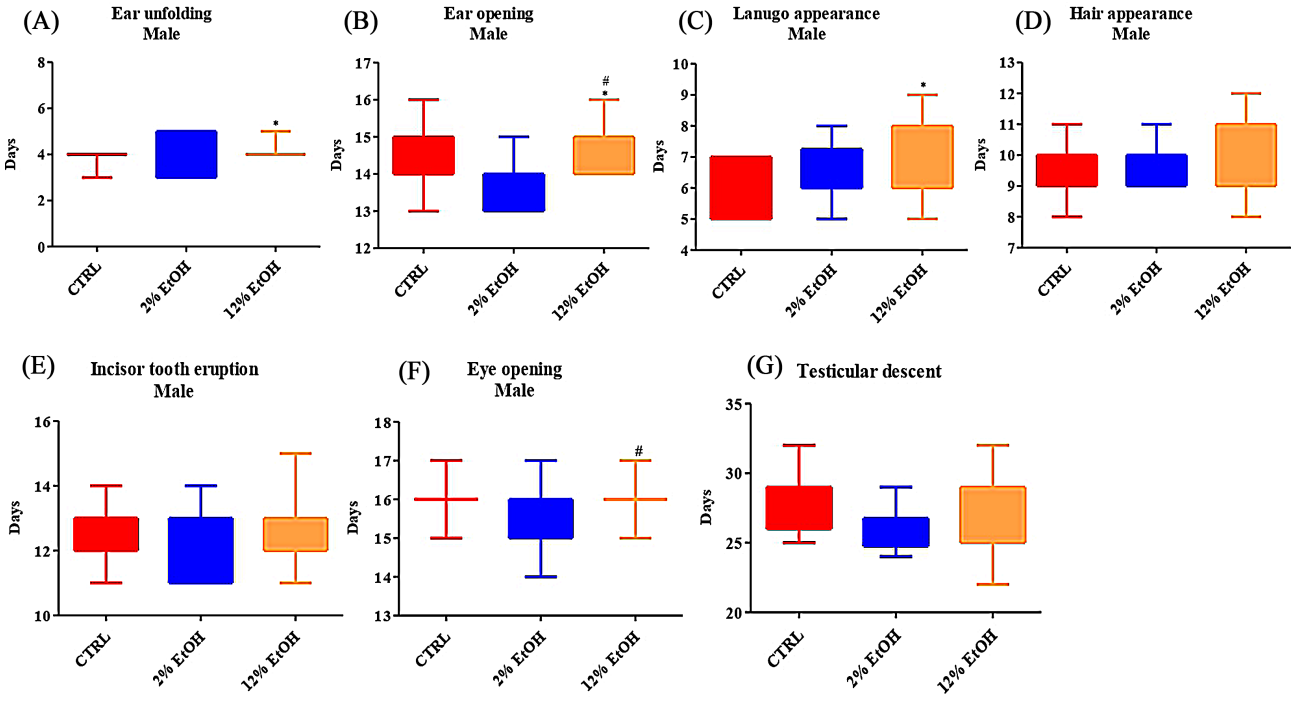

Figure 9. Parameters of physical development in male offspring. Ear unfolding (A), Ear canal opening (B), Lanugo appearance (C), Hair appearance (D), Incisor teeth eruption (E), Eye opening (F), and Testes descent (G). In the offspring of the control group (CTRL), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group (2% EtOH) and the 12% ethanol group (12% EtOH), mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10.1 g/kg/day), respectively, as the sole source of liquid intake. Each data point represents the median ± standard error of the mean. CTRL n = 35, 2% EtOH n = 34, and 12% EtOH n = 40. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test.)

C) The 12% ethanol group had statistically higher mean compared to the control group; (B, F) The 12% ethanol group had statistically higher mean compared to the 2% ethanol group. For the other parameters, there was no significant effect of the groups: hair appearance ($\chi^2_{(2)}$ = 0.633; p = 0.729), tooth eruption ($\chi^2_{(2)}$ = 4.724; p = 0.094), testicular descent ($\chi^2_{(2)}$ = 4.816; p = 0.090).

Neurobehavioral development

Figure 10 and Figure 11 show the results of the evaluation of palmar grip (A), postural reflex (B), grasping reflex (C), negative geotaxis (D), and climbing reflex (E) in female and male pups, respectively.

For females, the GEE model indicated the following results. Palmar grasp: There was a significant effect of time ($\chi^2_{(8)}$ = 77.752; p < 0.001), but no effect of group ($\chi^2_{(2)}$ = 0.387; p = 0.824) or interaction between time and group ($\chi^2_{(16)}$ = 17.951; p = 0.327). Postural reflex: There was a significant effect of time ($\chi^2_{(8)}$ = 1332.663; p < 0.001) and interaction between time and group ($\chi^2_{(16)}$ = 44.122; p < 0.001), but no effect of group ($\chi^2_{(2)}$ = 0.394; p = 0.821). Grasping reflex: There was a significant effect of time ($\chi^5_{(5)}$ = 450.713; p < 0.001) and interaction between time and group ($\chi^2_{(10)}$ = 38.526; p < 0.001), but no effect of group ($\chi^2_{(2)}$ = 2.328; p = 0.312). Negative geotaxis: There was a significant effect of time ($\chi^5_{(5)}$

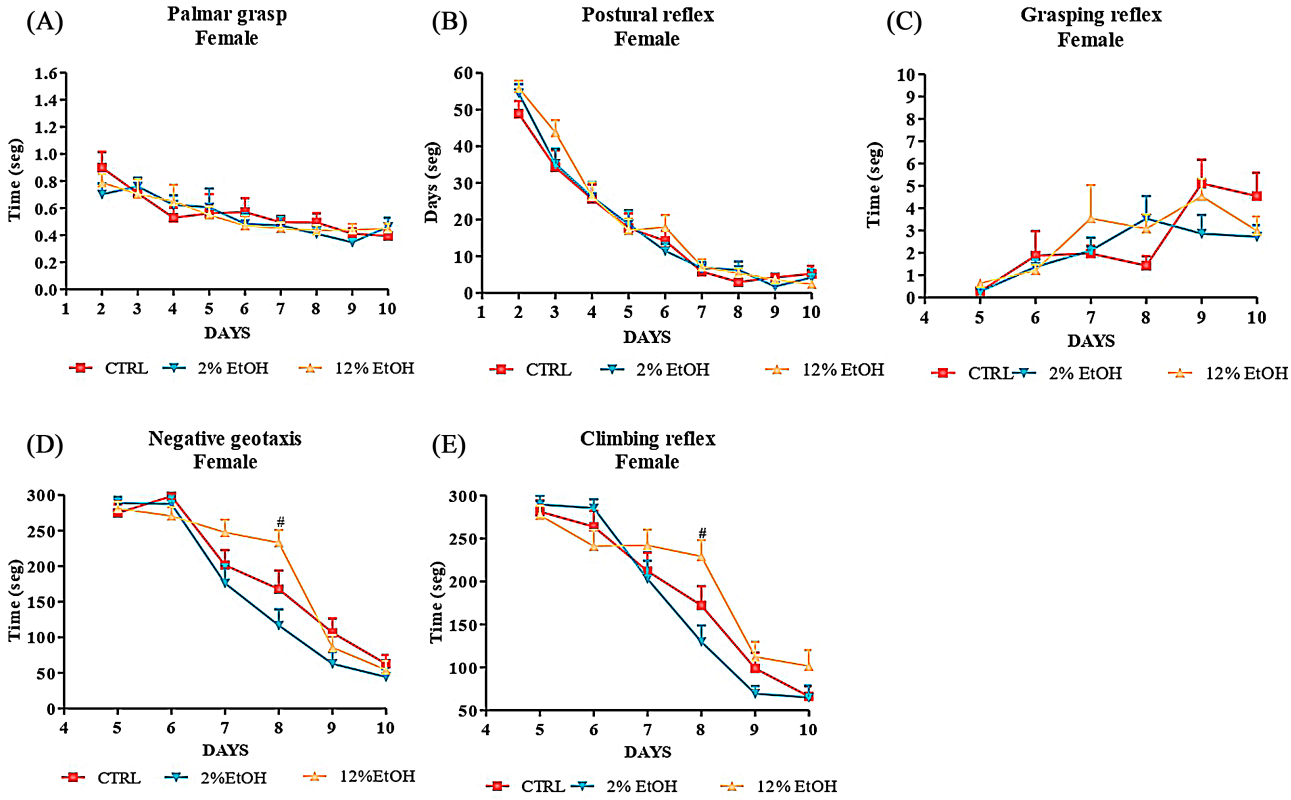

Figure 10. Neurobehavioral development of female offspring. Palmar grasp reflex (A), Postural reflex (B), Grasping reflex (C), Negative geotaxis (D), Climbing reflex (E). In the offspring of the control group (CT), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group (EBD) and the 12% ethanol group (EAD), mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10.1 g/kg/day), respectively, as the sole source of liquid intake. Each data point represents the median ± standard error of the mean. CTRL n = 27, 2% EtOH = 29, and 12% EtOH n = 32. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test.)

= 244.367; $p < 0.001$) and interaction between time and group ($\chi^2_{(10)}$ = 27.752; p = 0.002), but no effect of group ($\chi^2_{(2)}$ = 5.492; p = 0.064). Pairwise comparisons with Sidak correction indicated that the 12% ethanol group had a higher mean time on the 8th day compared to the 2% ethanol group. Climbing reflex: There was a significant effect of time ($\chi^5_{(5)}$ = 276.139; $p < 0.001$), group ($\chi^2_{(2)}$ = 8.111; p = 0.017), and interaction between time and group ($\chi^2_{(10)}$ = 30.221; p = 0.001). Pairwise comparisons with Sidak correction indicated that the 12% ethanol group had a higher mean time on the 8th day compared to the 2% ethanol group.

For males, the GEE model indicated the following results. Palmar grasp: There was a significant effect of time ($\chi^2_{(8)}$ = 95.952; $p < 0.001$) and interaction between time and group ($\chi^2_{(16)}$ = 34.562; p = 0.005), but no effect of group ($\chi^2_{(2)}$ = 0.387; p = 0.824). Postural reflex: There was a significant effect of time ($\chi^2_{(8)}$ = 2720.580; $p < 0.001$) and interaction between time and group ($\chi^2_{(16)}$ = 41.755; $p < 0.001$), but no effect of group ($\chi^2_{(2)}$ = 1.348; p = 0.510). Grasping reflex: There was a significant effect of time ($\chi^5_{(5)}$ = 190.346; $p < 0.001$), group ($\chi^2_{(2)}$ = 14.716; p = 0.001), and interaction between time and group ($\chi^2_{(10)}$ = 49.427; $p < 0.001$).

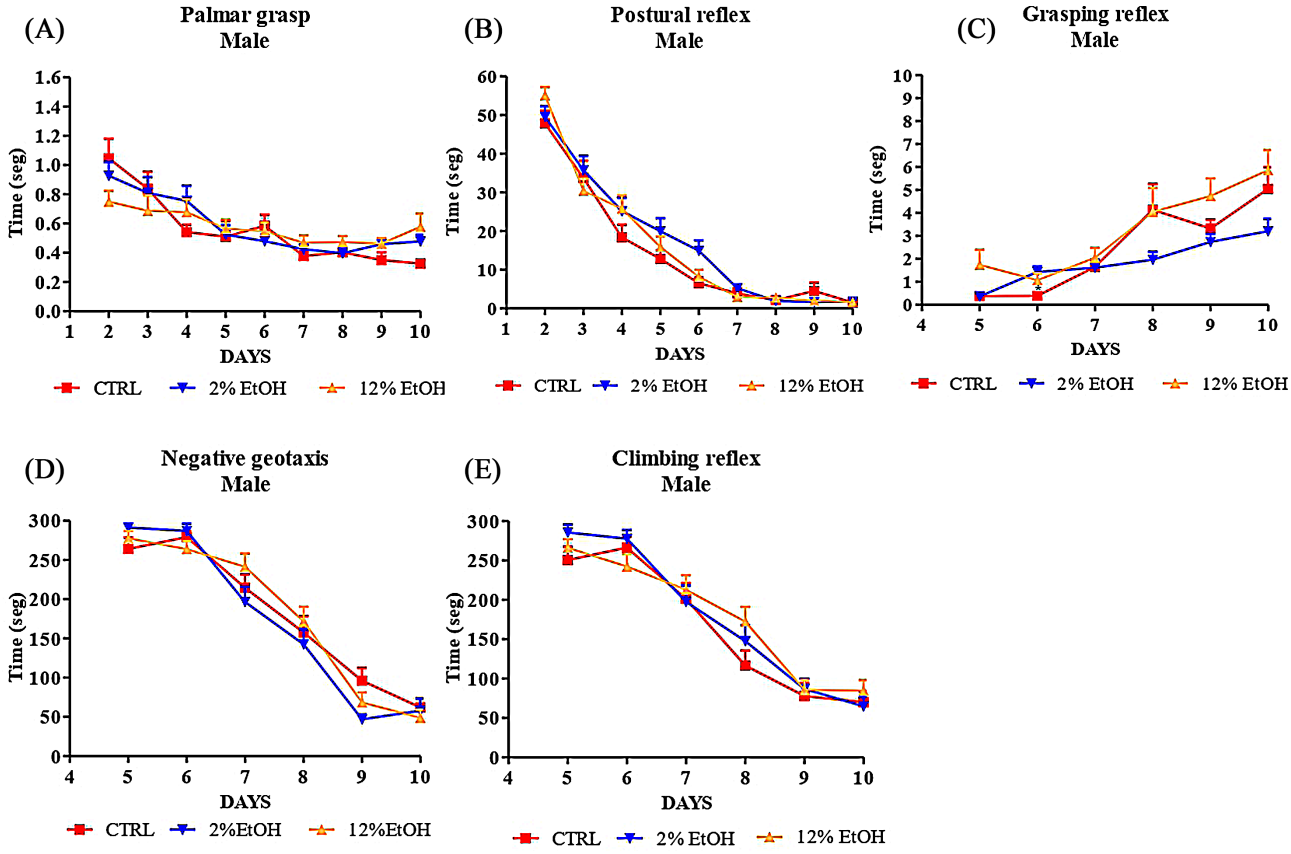

Figure 11. Neurobehavioral development of male offspring. Palmar grasp reflex (A), Postural reflex (B), Grasping reflex (C), Negative geotaxis (D), Climbing reflex (E). In the offspring of the control group (CTRL), mothers consumed water throughout the entire observation period. For the offspring of the 2% ethanol group and the 12% ethanol group, mothers consumed 2% ethanol (≈2.3 g/kg/day) and 12% ethanol (≈10 g/kg/day), respectively, as the sole source of liquid intake. Each data point represents the median ± standard error of the mean. CTRL n = 35, 2% EtOH n = 34, and 12% EtOH n = 40. (*Indicates $p < 0.05$ compared to the CTRL group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test. #Indicates $p < 0.05$ compared to the 2% EtOH group - Generalized Estimating Equations (GEE) followed by Sidak post hoc test).

Negative geotaxis: There was a significant effect of time ($\chi^5_{(5)}$ = 346.400; $p < 0.001$) and interaction between time and group ($\chi^2_{(10)}$ = 24.172; $p = 0.007$), but no effect of group ($\chi^2_{(2)}$ = 2.504; $p = 0.286$). Climbing reflex: There was a significant effect of time ($\chi^5_{(5)}$ = 282.669; $p < 0.001$), but no effect of group ($\chi^2_{(2)}$ = 1.494; $p = 0.474$) and interaction between time and group ($\chi^2_{(10)}$ = 10.969; $p = 0.360$). There were no statistically significant differences between the groups.

5. Discussion

The consumption of ethanol during gestation and lactation promoted changes in the analyzed parameters of the mothers, mainly during lactation. Regarding the offspring, the observed changes were more evident in the parameters related to physical development, such as weight gain and size. These are interesting findings that may indicate problems not only directly related to ethanol but also to nutritional factors.

Mothers

The mothers from the groups that ingested 12% ethanol generally had a lower

consumption of feed (**Figure 2**) throughout gestation and lactation, while the reduction in liquid consumption (**Figure 3**) and weight gain (**Figure 1**) was only observed during lactation.

Several authors have reported a reduction in feed consumption associated with the ingestion of different concentrations (5% - 20%) of ethanol during gestation and lactation [15] [2] [26]-[28].

The decreased feed intake by the 12% ethanol group could be related to the caloric content associated with the consumed ethanol, potentially resulting in a substitution of calories obtained from feed with those obtained through ethanol consumption [29] [30], because the ethanol is a caloric molecule, providing 7.1 kcal/g [29] [31] whereas the standard feed (Nuvilab®) used in our experiments provides 2.9 kcal/g.

We did not observe a reduction in caloric intake (data not shown) in the group that received 12% EtOH during gestation. However, a reduction was observed during lactation, until an additional bottle of water was provided, after which no differences were observed between the groups.

Lactation demands significant physiological effort from the mother, requiring nutrients and increased fluid intake; therefore, rats increase their consumption [32]. In the 12% EtOH group (**Figure 3**), it is possible that the caloric demand to maintain lactation exceeded intake, which was reflected by the reduced caloric intake in this group.

Ethanol, by inhibiting vasopressin, has a diuretic effect [33] [32], increasing fluid loss; moreover, it is also known to reduce milk production and the duration of breastfeeding. The reduction observed in the 12% EtOH group (**Figure 3**) may represent a mechanism to prevent this loss and, consequently, avoid impairing lactation [1] [2] [59]. More studies are needed to clarify this result. Nevertheless, the reduction in liquid and feed consumption during lactation may be related to the weight loss (**Figure 1**) exhibited by the mothers in the 12% ethanol group.

Maternal behavior

Several studies demonstrate that ethanol consumption, particularly during lactation, negatively impacts maternal behavior and compromises offspring development [34]-[36]. Maternal deprivation, depending on the postnatal period, can lead to alterations in neuropeptides within the nervous system and increased dopamine levels due to higher tyrosine hydroxylase expression [37]. The interaction of ethanol with primarily GABAergic receptors leads to central nervous system depression [36] [38] which leads to reduced locomotion, negatively impacting the mother's behavior (for example, carrying pups to the nest).

Although the literature indicates that ethanol impairs maternal behavior, we did not observe statistically significant differences between groups (**Figure 5**). Nevertheless, mothers of the group 12% ethanol showed a tendency towards general impairment in maternal behavior. The lack of significant difference could be due to the sample size, or differences in the experimental protocol compared to previous studies or even the reduction in ethanol intake observed in the 12%

group.

Offspring

Regarding physical development parameters, we observed that the offspring of the 2% EtOH and 12% EtOH groups presented delays compared to the CTRL group.

Ethanol also leads to an imbalance in the hypothalamic-pituitary-adrenal axis, increasing plasma levels of cortisol and adrenaline, blood glucose, and the alert system in the mothers, similar to a fight-or-flight situation, *i.e.*, stress [39] [40]. The increase in cortisol in mothers is passed on to the offspring through the circulation during gestation and through milk during lactation [41]-[43]. This increase dysregulates the hypothalamic-pituitary axis of the pups, and this probably also contributes to the delay in the developmental parameters observed in our experiments.

Approximately during the first 10 days of lactation, we did not observe a difference in weight gain (**Figure 6**) and size (**Figure 7**) between the groups. Comparing these data with the development parameters, possibly the delay occurred because the energy demand was shifted, used to maintain weight gain and size, ensuring the survival of the offspring.

Despite not showing a statistically significant difference ($p = 0.051$), we observed a longer time for vaginal canal opening in the 2% EtOH group (**Figure 8**) compared to the CTRL group, which indicates a delay in sexual maturation. This may be due to the inhibitory effect of ethanol on the hypothalamic-pituitary-gonadal axis, which causes a decrease in the release of gonadotropin-releasing hormone [44] [45] and consequently of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Furthermore, this condition could be aggravated by cortisol [46] [47]. However, it remains to be understood why the higher concentration of ethanol (12% EtOH) did not promote the same effect.

The study of neurobehavioral development evaluates the reflexes present in the offspring during lactation, being relevant for the study of nervous system maturation. As an involuntary response, and in conjunction with physical development, it helps in the survival of the species, as they are involved with spatial representation and motor skills [48]-[51]. The presence of reflexes is related to the maturation of the nervous system, mainly the vestibular system, the structures of the brainstem, the cerebellum, in addition to myelination processes [50] [48].

The nervous system, especially during development, is sensitive to the deleterious effects of ethanol. Indeed, there is evidence of anatomical and histological alterations related to neuronal migration, receptor expression, among others [52]-[55]. However, we observed few differences in neurobehavioral development parameters. Significant differences were observed only in females (**Figure 10**) in the 12% EtOH group (spent more time performing negative geotaxis and climbing reflex) on the 8th day of observation in relation to 2% EtOH. As it was a punctual difference, it may not reflect the use of ethanol.

Analyzing the data from the offspring, females displayed a trend toward greater

effects associated with ethanol exposure. However, this observation should be interpreted with caution, as the study was not designed to directly compare sexes and no formal statistical analysis was performed to test sex differences. The observed pattern may be related to sex-specific variations in hepatic biotransformation, stress sensitivity, or hormonal regulation [56] [57] [59]. Additionally, comparison with the existing literature remains challenging, as most studies and evaluated parameters are predominantly based on male offspring.

A limitation of the experimental protocol lies in the difficulty of isolating the mechanisms involved in the effects of ethanol. In the dams, ethanol interferes with the hypothalamic-pituitary axis, affecting cortisol, adrenaline, and thyroid hormone levels [39] [40], which triggers an increase in stress levels. Potentially, the offspring suffer from the impacts of maternal stress and hormonal imbalances, combined with the direct action of ethanol, which crosses the placental barrier. These factors can lead to neuroanatomical alterations, impaired neuronal migration, reduced BDNF levels, and disruptions in hormonal and neurotransmitter systems—such as GABA, glutamate, dopamine, and serotonin—all of which are well-documented in the literature [39] [40] [60] [61].

Additionally, most studies evaluating the effects of ethanol rely on experimental designs focused on single endpoints—such as assessing memory, neuroanatomical changes, or receptor expression—and a large portion of these protocols are conducted exclusively on males. Consequently, comparing our findings with the existing literature proved challenging; however, it allowed us to broaden the scope of investigation regarding ethanol use during pregnancy and lactation, paving the way for future research.

6. Conclusion

In general, we conclude that maternal consumption of ethanol can affect pregnancy and lactation differently. There was a decrease in weight gain and food intake during pregnancy, while caloric gain and liquid consumption were affected during lactation. Furthermore, there was a delay in the physical development of the offspring, especially in females. These data reinforce the importance of avoiding ethanol consumption, regardless of concentration, during pregnancy and lactation, as it can have an impact on both maternal health and offspring development.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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