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Maternal Choline Supplementation Is Associated with Lasting Improvements in Behavioral Effects of Prenatal Alcohol Exposure in the C57BL/6J Mouse

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Abstract

Background. Prenatal alcohol exposure (PAE) causes changes in growth, metabolic outcomes, and behavior that can last into adulthood. Treatment with choline has proven efficacious in the short term in animal models and improves some outcomes in people with Fetal Alcohol Spectrum Disorder. Here we tested the hypothesis that maternal supplementation with choline would mitigate behavioral effects of PAE through adulthood. **Methods**. Pregnant C57BL/6J mice were given alcohol (ALC; 3 g/kg body weight) and choline (Cho; 100 mg/kg) once per day from embryonic day (E) 8.5 through E17.5. Control mice received isocaloric maltodextrin via gavage. Animals that did not receive choline were handled but not injected. Male and female offspring were weighed and tested at 2 months of age (2 mo), 6 mo, 12 mo, and 18 mo on the open field, accelerating rotarod, and fear conditioning tests. **Results**. Body weight increased with age; however, ALC mice had greater body weight than age-matched controls (CON), and Cho decreased body weight in both CON and ALC mice. Both CON and ALC mice performed better on motor behaviors (open field and rotarod) at younger ages and better on fear conditioning in mid-adulthood (6 and 12 mo). CON mice typically outperformed same-age, same-sex ALC mice, and ALC-Cho mice performed better than ALC mice on fear conditioning but not on motor outcomes. **Conclusions**. Our data suggest that maternal choline supplementation can modulate body weight increase through later adulthood in both CON and ALC offspring and also confers lasting behavioral benefits in the context of PAE. The similar age-related trends in behavior for both groups suggest that PAE did not accelerate the behavioral decline.

Keywords: ethanol; gestation; cognition; pregnancy; supplementation

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1. Introduction

Alcohol use during pregnancy is a significant public health problem. In the U.S., almost 14% of pregnant women self-report alcohol consumption, and around one-third of these women (5.2%) report binge drinking [1]; these rates have not changed since the 1980s, and they correlate well with recent estimations of Fetal Alcohol Spectrum Disorder (FASD) incidence in the general population. In a survey of four U.S. communities, 4.1% of first-grade children met the criteria for an FASD diagnosis, yet only ~1% of them previously received a diagnosis [2]. An unbiased sampling of newborn bloodspots from across Texas

found that 8.4% (range 0% to 18%) of infants tested positive for phosphatidylethanol, a metabolite of alcohol, with higher rates in affluent counties [3]. Similar findings are reported for West Virginia, with a positive test for 8.1% (range 2% to 17%) of infants [4]. Although four independent studies found that ~10% of the U.S. population experienced prenatal alcohol exposure (PAE), relatively little is known about the long-term consequences of PAE.

Clinical research on PAE largely focuses on children and adolescents, although some recent studies investigated older ages. Results from a survey of ~500 people with a diagnosed FASD suggest higher than normal incidences of many physical ailments, including mental health disorders [5]. The cohort had a mean age of 27.5 years of age (range <16–60+ years of age), and early-onset dementia was apparent at 209 times the rate for the general population. Similarly, they reported a high incidence of executive dysfunction, including problems with short- and long-term memory (81.5% and 67.5%, respectively). Kable and colleagues reported that adults 29–64 years of age with PAE may have a wider range of negative health effects and with higher frequency than people without PAE [6]. Deficits in fluid cognition were also reported in the same cohorts [7]. And a recent report from hospital records [8] identified 57 comorbidities in people (age <1–75+ years of age) with FASD. In that report, ~20% of people with FASD also had intellectual and developmental disorders, compared with 0.8–2.3% in the control populations.

Animal models of PAE report a wealth of negative effects but seldom investigate beyond young adulthood. We previously investigated the potential effects of PAE and age on behavior and metabolic outcomes from adolescence to older adulthood [9]. In that study mice were tested in adolescence (6 weeks of age (wk)) and mid- and late-adulthood (10 months of age (mo) and 17 mo) for males and females and again at old age (24 mo) for females only [10]. In a test of motor performance, we found that PAE worsened rotarod performance in females, and this was most apparent between 10 mo and 24 mo. With respect to learning and memory, PAE decreased freezing behavior in mice at 10 mo when assessed in a fear conditioning test. Moreover, at 18 mo, alcohol-exposed mice were more likely to have elevated fasting blood glucose than controls, and alcohol-exposed female mice had higher adiposity than controls.

Prenatal choline supplementation has been shown to improve behavioral deficits that result from PAE in rodent models [11,12]. A recent meta-analysis showed that in humans prenatally exposed to alcohol, choline supplementation pre- or postnatally supported neurocognitive development [13]. We recently reported that prenatal choline supplementation mitigated the effects of PAE on body weight, fat mass, and glucose tolerance in older mice (13 and 19 mo) and that these improvements in metabolic outcomes were also seen in aging control mice [14]. In the current study we aim to (1) describe the effects of age and PAE on behavioral measures in this same cohort of animals, (2) test whether prenatal choline supplementation mitigates those effects, and (3) test whether weight outcomes contribute to behavioral deficits.

2. Materials and Methods

2.1. Animals

Female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at five weeks of age (5 wk). They were housed in a temperature- and humidity-controlled facility with a 12 h light–dark cycle (lights on 7 a.m.–7 p.m.) and given ad libitum access to water and a defined diet (AIN-93G; Teklad Envigo, Madison, WI, USA) [15]. Between 8 and 10 wk, females were mated overnight, and the first day a plug was seen was defined as embryonic day (E) 0.5. Pregnant females were randomly assigned to one of four experimental groups: control (CON), CON supplemented with choline (CON-Cho), alcohol-exposed (ALC), and ALC supplemented with choline (ALC-Cho). ALC animals received a

daily gavage of 3.0 g/kg alcohol (200 proof; Koptek, King of Prussia, PA, USA) from E8.5 through E17.5. CON animals received isocaloric maltodextrin (Teklad Envigo). Choline-treated animals were given a single daily subcutaneous injection of choline in sterile water (100 mg/kg body weight; Balchem, New Hampton, NY, USA). Animals that did not receive choline were handled similarly but not injected. Blood alcohol concentrations in this model were 211 ± 14 mg/dL 30 min post-gavage [16].

Animals typically gave birth on E18.5, and pups were left with their dam until weaning on postnatal day (P)21 and then housed in groups of 2–4 same-sex littermates where possible. Offspring were weighed periodically and regularly assessed for signs of illness. Body weights taken at the time of behavior testing were tested for statistical differences. Mice were euthanized at 20 months of age (20 mo) or earlier if health issues arose. Over seven of the eight groups, 16 animals were euthanized between 12 and 18 mo due to weight loss greater than 10% (see Table 1). These protocols were approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus.

Table 1. Number of mice (litters) per group.

Group	Male 2 mo	Male 18 mo	Female 2 mo	Female 18 mo
CON	14 (9)	13 (9)	15 (8)	13 (8)
CON-Cho	19 (8)	17 (8)	16 (7)	16 (7)
ALC	12 (7)	10 (7)	14 (7)	12 (6)
ALC-Cho	15 (8)	11 (6)	15 (9)	12 (7)

Number of mice (and litters) at 2 mo and 18 mo. Numbers at 6 mo and 12 mo are the same as for 2 mo. ALC, alcohol-exposed; CON, control; Cho, choline-treated; mo, months of age.

2.2. Behavior

Male and female offspring (n = 10–19 per group; 1–4 per sex per litter; Table 1) underwent behavioral testing by a blinded investigator at 2 mo, 6 mo, 12 mo, and 18 mo as done previously [9,16–18] and described briefly below in the following order.

Activity: Mice were placed in a rectangular arena (40 cm × 29.5 cm; height 40 cm) and videotaped for 5 min. Distance traveled was recorded using Ethovision software (Noldus Information Technology, Leesburg VA). Due to technical issues, data for this behavior are missing from two CON litters at 2 mo.

Accelerating Rotarod: Mice were placed on a rotarod (BioSeb, Pinellas Park, FL, USA), a cylinder that accelerates from 3 revolutions per minute (rpm) to 30 rpm over 5 min. Mice received one training trial, and after a 45 s rest they performed a test trial where latency to fall from the rod was recorded.

Fear conditioning: On day 1 (conditioning), mice were placed in the conditioning chamber (Video Fear Sound Attenuating Cubicles, Med Associates, Fairfax, VT, USA) and left to explore for 2 min; then, a tone (2.8 kHz, 85 db) played for 30s co-terminating with a 2 s mild foot shock (0.75 mA). The tone–shock pairing occurred four times with an 80 s interval between. Percent time freezing during exploration and during each tone presentation was recorded. On day 2 (context), mice returned to the chamber for 5 min, and percent time freezing was recorded. On day 3 (cue), mice were placed in a chamber with different features (floor and walls were changed and an odor was added). After 2 min of exploration the tone was played for 3 min. Percent time freezing was recorded during the test. The cue background was the freezing in the 2 min prior to cue onset, the cue was the average percent time freezing during the 3 min cue presentation, and cue reactivity was calculated as the difference in percent time freezing during background and during the first minute of cue presentation.

2.3. Statistical Analysis

For analysis of body weight, each animal was treated as an individual, whereas behavior data were averaged across animals of the same sex within each litter. Group means and standard deviations are shown for weight, and means and standard errors are shown for behavior data. Data are presented as histograms with individual datapoints (body weight) or litter averages (behavior data) and also as line graphs to facilitate the visualization of age- and group-related differences. Dam and litter outcomes were analyzed using factorial analysis of variance (ANOVA). Body weight and behavioral outcomes were analyzed using linear mixed-effects models (LMMs), as not all animals contributed data at every age [19,20]. Post hoc analyses used Bonferroni tests with an alpha value of 0.05; trends are reported for post hoc tests if $0.05 < p \leq 0.08$. Tests were performed using SPSS (v.31; IBM SPSS Statistics, Armonk, NY, USA). Model results (degrees of freedom, F and p values) are reported in the text for significant outcomes and trends, and the 95% confidence intervals (CIs) for these are in Supplemental Material S1 (SM1).

Behavior data were analyzed sequentially to determine the following: (1) the effect of aging in CON mice, independent of choline treatment using CON data only; (2) the effect of aging in ALC mice, using ALC data only (no choline-treated animals); (3) the effect of ALC in aging mice, comparing CON and ALC (no choline-treated animals); and (4) the effect of choline treatment, using data from all four groups.

Finally, we wanted to model how all these isolated effects work together as a system. To test whether the effect of PAE on activity was mediated by body weight and whether this mediation was conditional on choline treatment, we implemented a moderated mediation model within a linear mixed-effects framework, with repeated observations nested within subjects [21]. The effect of exposure (CON vs. ALC) on weight was estimated by path a, and the effect of weight on activity was modeled as a function of exposure and choline treatment, estimated by path b. A conceptual model is depicted in Figure 1. In this model, the conditional indirect effect of exposure on activity depends on whether mice were exposed to prenatal alcohol exposure or not [22]. Indirect effects (path a $*$ path b) were computed separately for each combination of exposure and treatment. Fixed effects of age and sex were included as covariates. Assuming within-mice variation in development with age, random slopes of age for each subject were further included in the model. Additionally, since litter size incidentally differed and mice from the same litter are not considered independent, we also included fixed effects of litter size and dam ID as a random effect. We decomposed the mediator (weight) into between-subject (mean weight) and within-subject (deviation from individual means) components, allowing us to examine both between- and within-level indirect effects. No within-subject effects of weight were found; as such, this was not included in the final model. Categorical predictors (exposure, treatment, sex) were coded with the following reference levels: exposure (CON), treatment (no treatment), and sex (male). To facilitate the interpretability of the effects, continuous variables (weight, activity) were standardized (z-scored); thus, all effects are expressed in standard deviation units. Indirect effects were estimated using nonparametric bootstrap resampling with 5000 samples, generated with 95% confidence intervals; results excluding 0 were considered significant [22]. Mediation analyses were conducted in MATLAB (MATLAB R2025a, MathWorks, Natick, MA, USA) using the fitlme function.

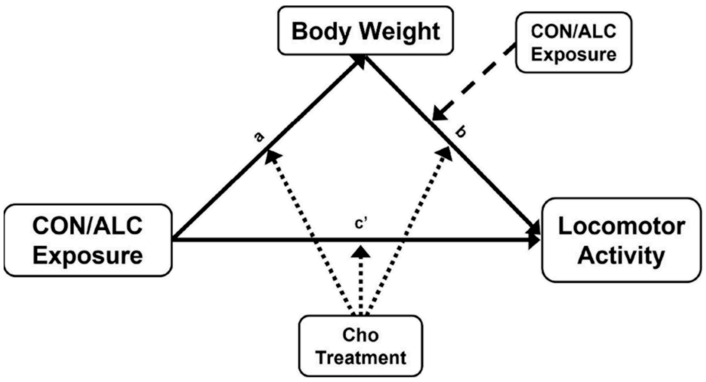

Figure 1. Conceptual model depicting the mediating role of body weight and moderating roles of exposure and treatment on activity while controlling for age and sex. a × b = indirect effect; c′ = direct effect. Exposure: CON, control; ALC, alcohol-exposed; Cho, choline-treated.

3. Results

3.1. Dam and Litter Outcomes

3.1.1. Pregnancy

Neither pregnancy weight gain nor weight gain across the gavage period was affected by exposure or treatment (smallest $p = 0.120$; Table 2); overall, dams gained ~13 g during pregnancy, and ~11 g of that was during the gavage period (E8.5–E17.5). The number of pups per litter was not affected by exposure or treatment (overall average was 5.5 pups; smallest $p = 0.113$), and neither was the percentage of male pups (overall average 53% male; smallest $p = 0.259$; Table 2).

Table 2. Pregnancy outcomes.

Group	CON	CON-Cho	ALC	ALC-Cho
Pregnancy weight gain (g)	13.54 ± 1.56	14.19 ± 2.52	13.49 ± 1.43	12.08 ± 3.12
Gavage weight gain (g)	11.14 ± 1.90	11.88 ± 2.39	11.38 ± 1.81	9.59 ± 3.20
Number of pups	5.9 ± 1.70	6.5 ± 2.80	5.5 ± 2.20	4.4 ± 2.70
% male pups	47.9 ± 15.70	55.3 ± 26.40	61.5 ± 23.7	49.3 ± 33.4

Data are mean (standard deviation). ALC, alcohol-exposed; CON, control; Cho, choline-treated; mo, months of age.

The number of animals (and litters) in each group was the same at 2, 6, and 12 mo, but is lower at 18 mo; therefore, only the numbers at 2 and 18 mo are shown in Table 1. The percent survival was not significantly different among the groups ($p = 0.121$).

3.1.2. Offspring Weight

Data for individual animals underwent LMM with exposure, treatment, age, and sex as fixed factors and litter as a random factor. There were main effects of treatment ($F_{1,26.479} = 95.684$; $p < 0.001$), sex ($F_{1,403.659} = 49.797$, $p < 0.001$), and age ($F_{3,394.618} = 304.612$; $p < 0.001$) on body weight at all ages (Figure 2), as well as interaction effects of treatment × age ($F_{3,394.618} = 3.921$; $p = 0.009$), sex × age ($F_{3,394.019} = 4.927$; $p = 0.002$), and exposure × treatment × sex ($F_{1,403.659} = 13.978$; $p < 0.001$). The treatment × age interaction indicated that weight significantly increased between each age (largest $p = 0.001$) and that choline-treated mice weighed less than untreated mice at all ages (all p's < 0.001). The sex × age interaction indicated that males trended to being heavier at 2 mo ($p = 0.064$), with significant sex differences observed at all subsequent ages (largest $p = 0.003$). The exposure × treatment × sex interaction showed that choline treatment decreased body weight in all four groups (both sexes, CON or ALC, all p's < 0.001), that untreated ALC males were heavier than

CON males ($p = 0.010$), and that significant sex differences were seen in CON-Cho and untreated ALC mice (both p's < 0.001), with a trend in ALC-Cho mice ($p = 0.076$) but not in untreated CON mice. Litter accounted for a substantial proportion of the variance in body weight not explained by the fixed effects (adjusted intraclass correlation = 0.16).

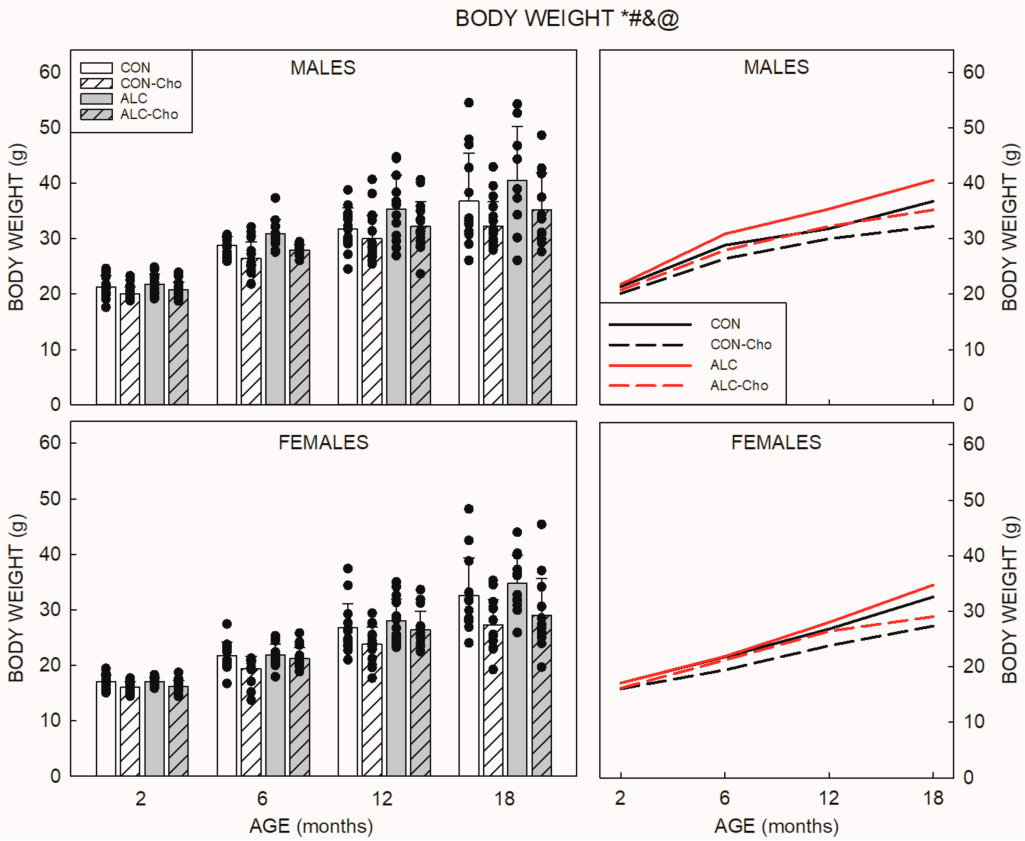

Figure 2. Body weights were taken at ages used for behavior. Alcohol-exposed (ALC) mice weighed more than control (CON) of the same sex and treatment. Choline-treated (Cho) animals weighed less than untreated mice of the same sex and exposure. Males weighed more than females at all ages. Left: Bars are mean, T-bars are standard deviation. Dots show individual datapoints. Right: Line graphs show mean to allow easy identification of age and group differences. n per group is shown in Table 1. Main effects: * significant ($p < 0.05$) effect of exposure; # significant effect of treatment; & significant effect of sex; @ significant effect of age. Interactions are described in the text. ALC, alcohol-exposed. Cho, choline-treated. CON, control. mo, months of age.

3.2. Behavior

Effect of aging in CON mice: We first ran LMMs with CON data from males and females to understand the effects of age and sex on the behavior of CON mice. Most behaviors showed significant age differences, but there were no sex differences or interactions of age with sex. Activity-related outcomes (Figure 3) tended to decrease with age ($F_{3,56} = 3.914$, $p = 0.013$); mice traveled further at 2 mo than at 12 mo ($p = 0.031$) and 18 mo ($p = 0.017$). Age differences were also seen in the rotarod test ($F_{3,60} = 4.888$, $p = 0.004$); mice remained on the rod longer at 2 mo than at 12 mo ($p = 0.049$) or 18 mo ($p = 0.028$), and there was also a trend for mice to remain on the rod longer at 6 mo than at 18 mo ($p = 0.061$). Together, these findings suggest that in CON mice motor activity peaks in young adulthood.

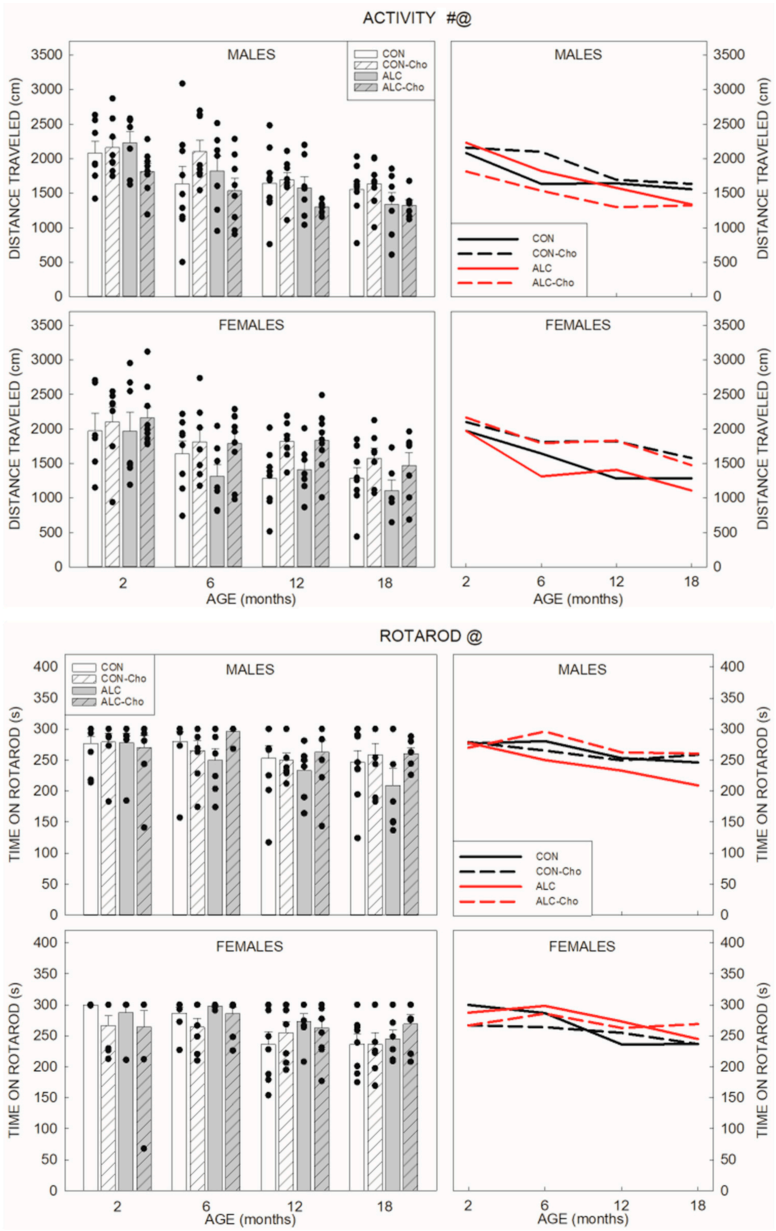

Figure 3. Locomotor behaviors. TOP. Activity was determined as the distance traveled in an open field over 5 min. Overall, CON mice traveled further than ALC, Cho mice traveled further than untreated animals, and younger animals traveled further than older. BOTTOM. Time spent on the rotarod over 5 min was mostly affected by age, where animals spent more time on the rod at 2 and 6 mo than 12 or 18 mo. Left: Bars are mean, T-bars are standard error. Dots show datapoints for each litter. Right: Line graphs show mean to allow easy identification of age and group differences. n per group is shown in Table 1. Main effects: # significant effect of treatment; @ significant effect of age. Interactions are described in the text. ALC, alcohol-exposed. Cho, choline-treated. CON, control. mo, months-of-age.

Analysis of the data for conditioning on day 1 (Supplementary Figure S1) showed effects of sex ($F_{1,300}$ = 9.434, p < 0.001), age ($F_{3,300}$ = 46.814, p < 0.001), test phase ($F_{4,300}$ = 76.756, p < 0.001), and interactions of sex × phase ($F_{4,300}$ = 3.499, p = 0.008) and age × phase ($F_{12,300}$ = 2.342, p = 0.007). As expected, freezing typically increased with repeated exposure to CSUS. Although 2 mo mice showed the lowest freezing for all CSUS presentations (largest p = 0.002), they showed more freezing for CSUS3 and CSUS4 compared to background, CSUS1, and CSUS2 (largest p = 0.042), suggesting that mice learned the association. Both 6 mo and 12 mo mice showed similar freezing, with higher

freezing to CSUS presentations than background and higher freezing to CSUS3 and CSUS4 than to CSUS1 (largest $p = 0.004$). Background freezing was highest at 18 mo compared to all other ages (largest $p = 0.016$), showed a trend to increase for CSUS2 ($p = 0.075$) and was significantly higher for CSUS3 and CSUS4 (p's < 0.001). The sex × phase interaction showed that females froze more for CSUS3 and CSUS4 than males (largest $p = 0.009$).

Freezing in the fear conditioning test increased with age for context ($F_{3,60} = 3.154$, $p = 0.031$); it was higher at 18 mo than 2 mo ($p = 0.021$) (Figure 4). Cue background showed that activity in the novel chamber prior to cue onset was affected by age ($F_{3,60} = 2.797$, $p = 0.048$), but there were no between-group differences in the post hoc test (Supplementary Figure S2). Response to the cue (during the 3 min presentation) was not different among the groups (Figure 4). Cue reactivity also showed an effect of age ($F_{3,60} = 8.101$, $p < 0.001$); it increased between 2 mo and 6 mo ($p = 0.028$) and was lower at 18 mo than 6 mo ($p < 0.001$) or 12 mo ($p = 0.012$; Supplementary Figure S3).

Effect of aging in ALC males and females: We next ran LMMs to understand the effects of age and sex on the behavior of ALC mice. Main effects of age and sex were observed, but there were no interaction effects. Males traveled further than females ($F_{1,47} = 4.887$; $p = 0.032$; Figure 3), and age differences ($F_{3,47} = 7.635$, $p < 0.001$) indicated that 2 mo mice traveled further than mice at all other ages (largest $p = 0.036$). Females outperformed males on the rotarod ($F_{1,47} = 8.379$, $p = 0.006$), and age differences ($F_{3,47} = 4.514$, $p = 0.007$) showed that mice performed better at 2 mo and 6 mo than at 18 mo ($p = 0.009$ and 0.038, respectively; Figure 3).

Conditioning on day 1 (Supplementary Figure S1) showed effects of sex ($F_{1,235} = 24.445$, $p < 0.001$), age ($F_{3,235} = 69.186$, $p < 0.001$), test phase ($F_{4,235} = 89.204$, $p < 0.001$), and an age × phase interaction ($F_{12,235} = 2.307$, $p = 0.008$). Females froze more than males, and freezing typically increased with repeated exposure to CSUS with all ages freezing more for CSUS3 and CSUS4 than CSUS1 or CSUS2 (largest $p = 0.025$). Mice were less active during background at 18 mo than all other ages (largest $p = 0.038$), and 2 mo mice froze less during each phase than older animals (largest $p = 0.022$).

In fear conditioning (Figure 4), females froze more than males in the context ($F_{1,47} = 30.122$, $p < 0.001$) and to the cue ($F_{1,47} = 10.797$, $p = 0.002$), and freezing increased with age ($F_{3,47} = 7.889$, $p < 0.001$ for context, $F_{3,47} = 7.168$, $p < 0.001$ for cue). In context mice froze more at 12 mo ($p = 0.007$) and 18 mo ($p < 0.001$) than at 2 mo and showed a trend to freeze more at 18 mo than 6 mo ($p = 0.054$). Freezing to the cue was lower at 2 mo than 6 mo ($p < 0.001$) or 12 mo ($p = 0.022$). There were no between-group differences in freezing in the novel environment (cue background; Supplementary Figure S2), but the response to the cue showed an effect of age ($F_{1,47} = 9.901$; $p < 0.001$); it was greater at 6 mo than 2 mo or 18 mo (both p's < 0.001; Supplementary Figure S3). Overall, the age-dependent activity outcomes were similar to the CON, where younger mice were better at motor behaviors but performance in the fear conditioning test was best between 6 mo and 12 mo.

Effect of ALC in aging males and females: To look specifically at effects of PAE, we next compared CON and ALC mice and focused on the main effects of or interactions with exposure. No effects of exposure were found for activity or freezing to the cue, but there was an interaction of exposure × sex for rotarod ($F_{1,107} = 4.032$, $p = 0.047$) and for context fear conditioning ($F_{1,107} = 4.209$, $p = 0.043$). For rotarod, ALC males spent the least time on the rotarod (Figure 3), significantly less than ALC females ($p = 0.007$), and showed a trend to spend less time than CON males ($p = 0.058$). For context (Figure 4), ALC males spent the least time freezing, significantly less than CON males ($p = 0.023$) and ALC females ($p < 0.001$). CON females showed a trend to freeze more than CON males ($p = 0.061$). Together, these findings suggest that males were most affected by PAE. There were no

effects of exposure on acquisition, freezing in the novel environment on day 3, or the response to the cue (Supplementary Figures S1–S3).

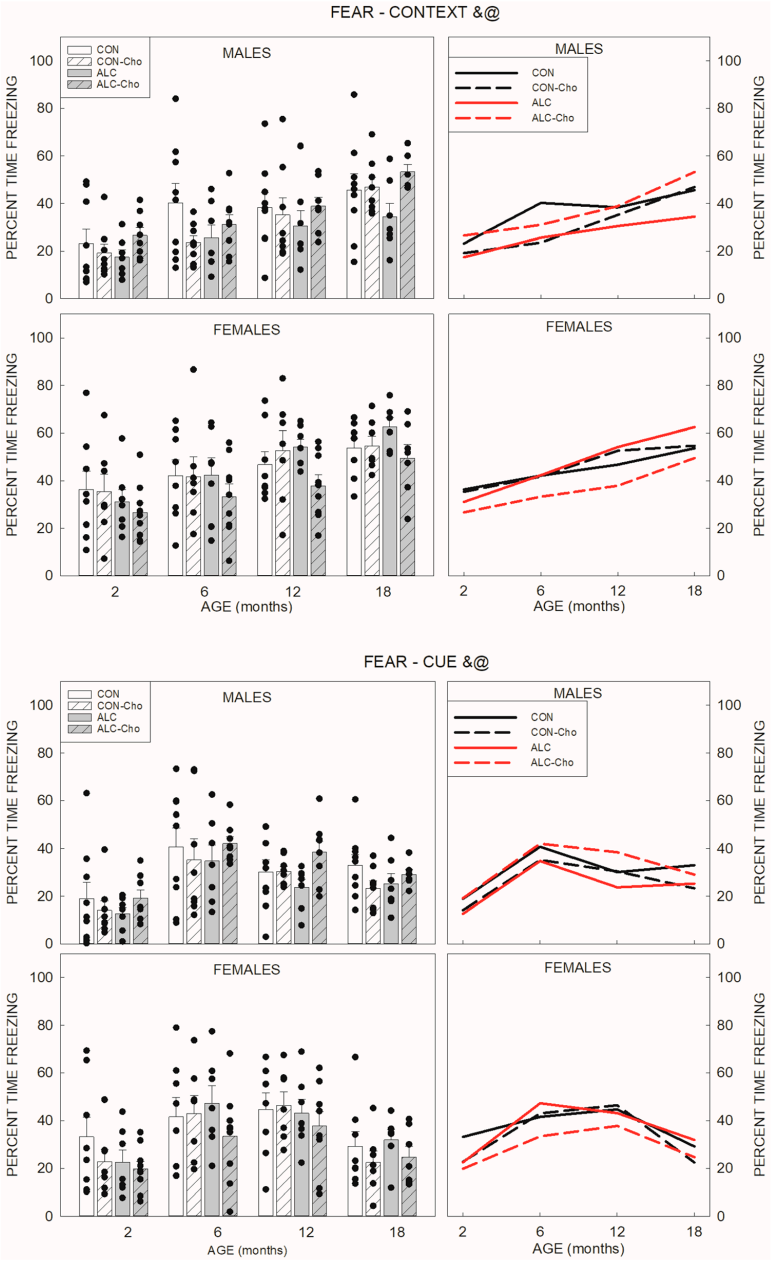

Figure 4. Fear conditioning. TOP: Freezing in the context was higher in females than males and increased with age. ALC males froze less than CON males and this is rescued by choline. In contrast, choline decreased freezing in ALC females. BOTTOM: Freezing to the cue was higher in females than males and increased from 2 to 6 mo, remained stable between 6 and 12 mo, and then decreased between 12 and 18 mo. Choline showed trends to increase freezing in ALC males but decrease it in ALC females. Left: Bars are mean; T-bars are standard error. Dots show datapoints for each litter. Right: Line graphs show mean to allow easy identification of age and group differences. n per group is shown in Table 1. Main effects: & significant effect of sex; @ significant effect of age. Interactions are described in the text. ALC, alcohol-exposed. Cho, choline-treated. CON, control. mo, months of age.

Effect of treatment with choline: Here we analyzed data from both sexes for all four experimental groups (CON and ALC +/− choline) and focused on the main effects and interactions with the choline treatment.

For activity (Figure 3) there was a main effect of treatment ($F_{1,211}$ = 5.651; p = 0.018) and interactions of treatment × sex ($F_{1,211}$ = 9.337; p = 0.003) and treatment × exposure × sex ($F_{1,211}$ = 4.393; p = 0.037). Overall, choline increased distance traveled in females; ALC-Cho mice traveled further than ALC (p = 0.002) and CON-Cho traveled further than CON (p = 0.021). But in males, choline decreased the distance traveled by ALC mice (p = 0.042), resulting in CON-Cho males traveling further than ALC-Cho males (p < 0.001). As above, no sex differences were seen in CON, but in ALC mice males traveled further than females (p = 0.020), and this was opposite following Cho; ALC-Cho females traveled further than ALC-Cho males (p = 0.006).

For rotarod (Figure 3), there was a trend for an interaction effect between treatment × sex ($F_{1,215}$ = 3.551, p = 0.061), where there was a sex difference in untreated mice (females outperformed males, p = 0.038) that was not seen in choline-treated animals.

Acquisition of fear conditioning (Supplementary Figure S1) showed an effect of treatment ($F_{1,1090}$ = 7.689, p = 0.006), a significant treatment × age interaction ($F_{3,1090}$ = 7.517, p < 0.001), and a trend for an interaction of treatment × exposure × age ($F_{3,1090}$ = 2.592, p = 0.051). Choline-treated animals showed less freezing than untreated mice. In CON mice, freezing was lowest at 2 mo (all p's < 0.001) and was higher at 18 mo than 6 mo (p = 0.013). A similar pattern was seen in CON-Cho mice: freezing was lowest at 2 mo (all p's < 0.001) and was higher at 12 mo than 6 mo (p = 0.028). In contrast, in ALC mice, freezing was significantly different among all ages and increased with age (largest p = 0.028). The pattern for ALC-Cho mice was more like CON and CON-Cho, where freezing was lowest at 2 mo (all p's <0.001) and showed a trend to be higher at 12 mo than 6 mo (p = 0.066).

For context (Figure 4), there was an interaction effect between treatment × exposure × sex ($F_{1,215}$ = 12.117; p < 0.001), which was largely driven by changes in the ALC mice, as Cho does not significantly alter freezing in CON mice. ALC males froze less than CON males (p = 0.014), and this was rescued by choline, with ALC-Cho males freezing more than ALC males (p = 0.013). For females, choline-treated ALC mice froze less than untreated ALC mice (p = 0.009) and CON-Cho females (p = 0.022). Sex differences were seen in the CON (p = 0.043), CON-Cho (p < 0.001), and ALC groups (p < 0.001), where females froze more than males.

Freezing to the cue also showed interactions of treatment × exposure × sex ($F_{1,215}$ = 4.307; p = 0.039). Trends were seen for effects of treatment in ALC mice; in males, choline increased freezing (p = 0.053), but in females choline decreased freezing (p = 0.075). There was a significant sex difference in ALC mice (p = 0.005) and a trend in CON-Cho mice (p = 0.075) where females froze more than males. There were no effects of choline on freezing in the novel environment or the response to the cue (Supplementary Figures S2 and S3).

3.3. Mediation Analysis

Results from the mixed-effects models are shown in Table 3. The first linear mixed-effects model tested whether exposure influenced body weight while controlling for age, sex, and litter size (Figure 5). There was no direct effect of exposure (alcohol) on weight (p = 0.36). We found a significant main effect of age, such that older mice weighed more than younger mice (β = 0.12, SE = 0.01, p < 0.001). We found a significant main effect of sex, such that females weighed less than males (β = −0.80, SE = 0.05, p < 0.001), and a significant main effect of treatment, with Cho-treated mice weighing less than untreated mice (β = −0.23, SE = 0.06, p < 0.001). The interaction between exposure × treatment was not significant (p = 0.32). Together, these results suggest that body weight was primarily

influenced by sex and treatment, with no evidence for exposure-related differences or interaction effects.

Table 3. Mixed-effects models.

Path a: Mediator Model (exposure→weight)					
Predictor	β	SE	t	*p*	95% CI
Intercept	−0.482	0.061	7.92	**<0.001**	[0.36, 0.60]
Female	−0.800	0.045	−17.72	**<0.001**	[−0.89, −0.71]
Age	0.121	0.005	23.68	**<0.001**	[0.11, 0.13]
Litter Size	0.014	0.011	1.24	0.217	[−0.01, 0.04]
Alcohol	0.062	0.068	0.92	0.360	[−0.07, 0.20]
Choline	−0.229	0.064	−3.54	**<0.001**	[−0.36, −0.10]
ALC × Cho	0.092	0.093	0.99	0.323	[−0.09, 0.27]
Path b: Outcome Model (weight→activity)					
Predictor	β	SE	t	*p*	95% CI
Intercept	0.064	0.192	0.33	0.741	[−0.31, 0.44]
Female	−0.216	0.154	−1.40	0.162	[−0.52, 0.09]
Age	−0.064	0.006	−11.17	**<0.001**	[−0.08, −0.05]
Litter Size	−0.067	0.037	−1.81	0.072	[−0.14, 0.01]
Alcohol	−0.181	0.231	−0.79	0.433	[−0.63, 0.27]
Choline	0.344	0.243	1.41	0.158	[−0.13, 0.82]
Weight	−0.197	0.231	−0.86	0.393	[−0.65, 0.26]
ALC × Weight	0.267	0.271	0.98	0.326	[−0.27, 0.80]
ALC × Cho	−0.301	0.330	−0.91	0.362	[−0.95, 0.35]
Cho × Weight	0.120	0.274	0.44	0.663	[−0.42, 0.66]
ALC × Cho × Weight	−1.019	0.397	−2.57	**0.010**	[−1.80, −0.24]

Bold numbers indicate significance at $p < 0.05$. ALC, alcohol-exposed; β, standardized beta coefficient; Cho, choline-treated; CI, confidence interval; SE, standard error.

A second linear mixed-effects model tested whether the relationship between body weight and activity was moderated by exposure, treatment, and their interaction, controlling for age, sex, and litter size (Table 3, Figure 5). We found that activity decreased significantly with age (β = −0.06, SE = 0.01, $p < 0.001$) but did not change as a function of sex (β = −0.22, SE = 0.15, $p = 0.16$) or weight (β = −0.20, SE = 0.23, $p = 0.39$). There was also no interaction effect between exposure and weight on activity ($p = 0.33$), nor between exposure and treatment ($p = 0.36$), or treatment and weight ($p = 0.66$). We did, however, find a significant higher-order interaction term between exposure, treatment, and weight (β = −1.02, SE = 0.40, $p = 0.01$), such that activity decreased with weight among ALC-Cho mice.

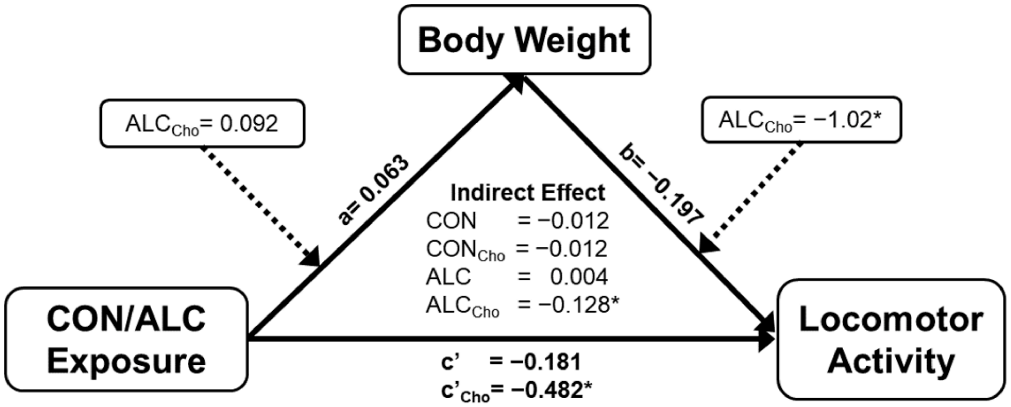

Figure 5. Resulting moderated mediation model showing conditional indirect associations between alcohol exposure, body weight, and locomotor activity across levels of exposure and treatment while controlling for age and sex. Indirect effects (a × b) suggest significantly reduced activity, mediated through body weight, among ALC-Cho mice (β = −0.128, 95% CI [−0.272, −0.023]). Direct effects (c′) suggest a significant effect of choline treatment in reducing activity among ALC-exposed mice (β = −0.48, SE = 0.24, p = 0.04). Values shown are standardized beta weights. c′ = direct effect. Exposure: CON, control; ALC, alcohol-exposed; Cho, choline-treated; ALCCho = interaction effect between alcohol exposure and choline treatment. * $p < 0.05$.

Direct and indirect effects of exposure on activity are shown in Table 4.

Table 4. Direct and indirect effects of PAE on activity.

Group	Direct Effect	SE	p	Indirect Effect	95% CI
Control	-	-	-	−0.012	[−0.071, 0.047]
Control Choline	-	-	-	−0.012	[−0.116, 0.078]
Alcohol	−0.181	0.231	0.433	0.004	[−0.025, 0.045]
Alcohol Choline	−0.482	0.239	0.044	−0.128 *	[−0.272, −0.023]
Index of Moderated Mediation					
Estimate	95% CI				
−0.132 *	[−0.275, −0.025]				

* $p < 0.05$. β, standardized beta coefficient; CI, confidence interval; SE, standard error.

We did not find any direct effect of exposure on activity among ALC mice (p = 0.43) but did find a significant effect among ALC-Cho mice (β = −0.48, SE = 0.24, p = 0.04; Figure 2, path c′), indicating reduced activity. The index of moderated mediation was significant (index = −0.13, 95% CI [−0.28, −0.03]), indicating that choline altered the indirect effect of prenatal alcohol exposure on activity through weight [23]. Specifically, the indirect effect was significant in ALC-Cho mice (β = −0.13, 95% CI [−0.27, −0.02]; Figure 5).

To test whether the indirect effect of exposure on activity was conditional on age, we included age as an additional moderator variable on path b. Nested models were compared using likelihood ratio tests estimated via maximum likelihood. Non-significant improvements in model fit indicated that the additional interaction terms with age did not significantly improve model fit, $\chi^2(3) = 6.46$, p = 0.091.

4. Discussion

Overall, we found that behaviors changed with age in both CON and ALC mice; motor behaviors peaked in younger mice (at 2 mo or 6 mo), whereas cognitive behaviors were better in mid-adulthood (6 mo and 12 mo mice) than in younger or older animals. The

age-related changes in behavior in the control mice were similar to those reported by others. For example, activity is typically higher in younger mice than older [24–26]. Freezing to the context was higher in aged males than younger, and freezing to the cue was higher in 5–6 mo males compared with older [24,26]. However, we found no interactions of age with exposure, suggesting that the effect of age on behavior was not altered by PAE. Although the maximal performance on most of the tests was worse in mice that experienced PAE than in control mice, the aging-related changes were typically similar. That is, both groups of mice performed better on the activity-related measures at younger ages and better in the cognitive test in middle adulthood. This suggests that PAE did not accelerate the behavioral decline in those animals as they aged. This is consistent with a prior study that followed PAE mice to age 20 mo [9]. In that cohort, mice were tested at 6 wk, 10 mo, and 17 mo for males and females and at 24 mo for females only. We found that PAE worsened rotarod performance in females, and this was most apparent between 10 mo and 24 mo. In the fear conditioning test PAE mice froze less than CON at 10 mo. Thus, the present study is congruent with those findings. Another study utilized the 3xTg mouse strain, which is engineered to express human presenilin, amyloid precursor protein, and tau, and thus is a model for Alzheimer's disease. PAE again caused cognitive deficits in the fear conditioning test, but there was no evidence that it interacted with either age or Alzheimer's disease risk to further worsen those cognitive measures [17]. Thus, three independent studies of aging mice found no evidence that prior PAE accelerated the acquisition of age-related behavioral deficits in those offspring. It will be important to establish if this outcome is generalizable to other animal models or to humans with PAE.

This study also found that PAE appeared to have greater behavioral effects on males than females. ALC males spent less time on the rotarod and less time freezing in the fear conditioning test compared with both CON males and ALC females. The greater effect on males may be a feature of the model; we previously reported that at embryonic day (E)17.5 PAE decreased placental weight for male but not female fetuses [27]. In children with FASD, although both males and females are adversely and similarly harmed by PAE, fewer males were born to pregnancies having heavy alcohol exposure, and the females had somewhat more severe dysmorphologies and poorer scores with respect to executive functioning and verbal and non-verbal IQ performance [28]. Although the mechanisms underlying such sex differences are unclear, epigenetic influences on the placenta and fetus, as well as the biological parents, are potential influences on those differences [14,29].

Prenatal choline supplementation largely rescued the negative effects of PAE on behavior, especially in males, perhaps because alcohol had more severe consequences to those male behaviors. The sex difference in the effect of choline on behavior may reflect differences in choline needs or utilization. Importantly, for both sexes, the benefits of prenatal choline extended from early through later adulthood. This suggests that the extra choline during the in utero period contributed to fundamental, permanent changes in the animals' development and physiology. The persistence of those benefits is mirrored in a continuing longitudinal study of choline supplementation in children diagnosed with FASD. Although the supplementation occurred when the children were 3-to-8 yr old, follow-up assessments as late as seven years thereafter still report improvements in multiple behavioral measures [30]. Although that intervention trial targeted the alcohol-exposed offspring, our intervention could have had direct effects on the fetoplacental unit and/or benefits to the alcohol-exposed mother, and further study is needed. Our assessment of choline metabolites in this PAE model found that supplemental choline had its greatest impact upon choline-related metabolites in the mother [31] and others concur [32]. However, analysis of the same animals reported herein revealed that this choline supplementation also mitigated the adiposity increases and greatly improved

glucose handling as the PAE mice aged [14]. Both glucose intolerance and the accompanying excess adiposity are known risk factors for cognitive impairment [33], and we cannot discount that those improvements might also contribute to their behavioral improvements.

Results from our moderated mediation analysis suggest that body weight's effect on activity is dependent on PAE exposure and treatment, with reduced activity observed among alcohol-exposed mice that received choline treatment. Initially, these findings may seem incongruent with our earlier linear mixed-effects model results, which found that choline treatment generally increased distance traveled, and specifically among female mice with PAE. However, this model did not account for body weight as a mediating factor, which our moderated mediation analysis did. One limitation to this design, however, is that we cannot establish the temporal order of weight and activity, given they were measured concurrently. The indirect effects reported here should be interpreted as consistent with, rather than evidence for, a causal pathway through weight. Additionally, despite a main effect of sex on weight, we had limited statistical power to explore higher-order interactions with exposure, treatment, and age. Due to the risk of model overparamaterization, future studies should consider a larger sample size to accommodate larger groups per parameter used in such complex statistical models.

Limitations of this study include a relatively small sample size (10–19 mice from 6–9 litters per group), that female estrus stage was not accounted for during behavior testing, and that the untreated mice were handled but not injected. Because mice underwent the same tests at multiple ages, it is possible that age-related improvements may be due to prior experience; however, we previously found that age-related changes in these behaviors were independent of prior exposure [9]. Finally, the ceiling effect in the rotarod data may mask between-group differences, especially at younger ages.

5. Conclusions

Our data suggest that maternal choline supplementation (and/or injection) can modulate body weight increase through later adulthood in both CON and ALC offspring and also confers lasting behavioral benefits in the context of PAE. The similar age-related trends in behavior for both groups suggests that PAE did not accelerate the behavioral decline.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/brainsci16111148/s1, Supplementary Figure S1: Fear conditioning at 4 ages; Supplementary Figure S2: Cue background; Supplementary Figure S3: Cue reactivity; Supplemental Material S1: Tables reporting outcomes of linear mixed models.

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Data Availability Statement: Data has been deposited in the UNC Dataverse and have been made public as of 20 October 2026: https://dataverse.unc.edu/dataset.xhtml?persistentId=doi:10.15139/S3/LARKKD.

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Abbreviations

The following abbreviations are used in this manuscript:

ALC	Alcohol-exposed
ANOVA	Analysis of variance
Cho	Choline-treated
CI	Confidence interval
CON	Control
E	Embryonic day
FASD	Fetal Alcohol Spectrum Disorder
LMM	Linear mixed-effects model
Mo	Months of age
PAE	Prenatal alcohol exposure
SD	Standard deviation of the mean
SE	Standard error of the mean
Wk	Weeks of age

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