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Low-Level Lead Exposure During Development Differentially Affects Neurobehavioral responses in Male and Female Mouse Offspring: A Longitudinal Study

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ABSTRACT

Early life low-level lead (Pb) exposure is still an alarming child health issue. To date, animal studies investigating the effects of low doses of Pb since early stages of life to adulthood are scarce. We investigated in a mouse model the behavioral effects of developmental exposure to low-level Pb yielding blood levels similar to those observed in child clinical literature. CD1 outbred mouse dams received Pb (25- or 100-ppm) via drinking water from two weeks pre-mating until the end of lactation. Offspring of both sexes underwent a longitudinal assessment of motor, socio-emotional, and cognitive endpoints from neonatal to adult stage. Pb levels were determined in several matrices (blood, brain and bone) up to six months after the end of exposure. We found that new born pups exposed to Pb have slightly altered motor patterns and reduced preference for the nest odor. Offspring of both sexes exposed to the lowest Pb dose showed diminished interest for social novelty stimuli as adults. Moreover, sex-dependent effects of Pb exposure were observed in the spatial learning and memory task, where males were selectively impaired. Finally, blood, brain and bone Pb levels were elevated in a dose dependent fashion up to six months after termination of exposure. We observed marked accumulation of Pb in bones, with higher Pb levels in 100-ppm exposed females than in males at 7 months of age. In conclusion, developmental Pb exposure caused mild alterations in early- and late-life behavioral domains, particularly involving olfactory and cognitive responses. These findings confirm the importance of animal models to understand how early chronic low-level lead exposure impacts on health in a life-course perspective.

KEYWORDS

Lead; behavior; olfactory discrimination; spatial memory; biomonitoring; mouse

1. INTRODUCTION

Lead (Pb) is a well-recognized toxic metal with various adverse neurological effects. Pb developmental neurotoxicity is still an issue of major concern for its short-, medium- and long-term effects on mental health. The most common routes of Pb exposure are oral ingestion, inhalation, or dermal absorption via sources such as contaminated dust, soil, drinking water, leaded gasoline, Pb-based paint or pipes (Prüss-Üstün et al., 2004; Vorvolakos et al., 2016). Once Pb is introduced into bloodstream, it is distributed to soft tissues, including the brain through blood-brain-barrier (BBB) and bones. Pb neurotoxicity mechanisms are multiple (from divalent cation mimicry at the synapse to selective mitochondrial dysfunction), involve different glutamatergic receptors and oxidative mechanisms [for a review see (Virgolini and Aschner, 2021)].

Developing fetus and child are more vulnerable to Pb because of several factors that include mobilization of maternal bone Pb during gestation and lactation, permeability to Pb of placenta and immature BBB, and higher risk to be exposed of infant and toddler due to hand-to-mouth behavior (Caserta et al., 2013; Dorea and Donangelo, 2006; Naranjo et al., 2020). Thus the developing brain is sensitive to Pb at levels much lower than those affecting adult brain function (Caito and Aschner, 2017; Grandjean and Landrigan, 2006). Indeed, although there is no safe threshold for Pb, 5 µg/dL has been identified as blood Pb reference value for children by Center for Disease Control and Prevention (CDCP, 2012). Robust data indicate that significant effects are evident at levels below 5 µg/dL; thus, in October 2021, the CDC proposed to reduce the reference value of blood Pb to 3.5 µg/dL (CDC, 2021). Not less interesting, non-linear dose-response relationships between blood Pb concentrations and intelligence scores (IQ) have been reported, with a greater impact of low levels of Pb on IQ scores in children than the impact at higher levels of exposure (Lanphear et al., 2005; Rothenberg and Rothenberg, 2005). In addition, detrimental effects of prenatal and postnatal Pb exposure on brain development can lead to functional impairments and disease not only in childhood (Canfield et al., 2003; Hu et al., 2006; Jedrychowski et al., 2009; Mamtani et al., 2008) but also at adulthood (Lasley, 2018; Mason et al., 2014; Spivey, 2007).

Moreover, Pb exposure has also been linked with pathogenesis of neuropsychiatric disorders such as Attention Deficit Hyperactivity Disorder (ADHD) (Dórea, 2019; Ma et al., 2019) and schizophrenia (Ma et al., 2019; Opler et al., 2008). A growing number of studies have also reported sex differences in the neurotoxicity of Pb in children (Geier et al., 2017), but results are often inconsistent across different studies. Similarly, several animal studies, mostly in rats, have explored the impact of Pb exposure during critical windows of brain development on behavior, glutamatergic receptors, brain gene expression, and epigenetic mechanisms, reporting sex-related effects of Pb exposure (Anderson et al., 2016; Anderson et al., 2013; Faulk et al., 2014; Kasten-Jolly et al., 2012; Leasure et al., 2008; Montrose et al., 2017; Tartaglione et al., 2020; Weston et al., 2014). However, most of these studies focused primarily on cognitive tasks and locomotor activity in post weaning animals, whereas life-span behavioral screening tests relevant from a translational perspective are scarce. Despite

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accumulating evidence on developmental toxicity of Pb at low-level exposures, the majority of the experimental studies in rodents have considered doses of Pb producing blood levels (BLLs) significantly higher than those reported in epidemiological studies. Indeed, only a limited number of animal studies have explored the impact of developmental Pb exposure on behavioral phenotype since the very early neonatal stage till adulthood for low Pb doses (lower or equal to 100 ppm) in female and male offspring.

The goal of the present study was to examine the effects on the behavior of early chronic low-level Pb exposure yielding BLLs similar or close to those reported in cord blood or children in large cohort studies worldwide [(Lanphear et al., 2005; Lucchini et al., 2019; Polanska et al., 2018; Sanchez-Guerra et al., 2019; Schoeters et al., 2017); see also the review by (Gundacker et al., 2021)]. To this aim, we exposed female mice during pre-conception, gestation, and lactation to two Pb concentrations among the lowest used in rodent studies [see (Rocha and Trujillo, 2019) for extensive review]. We assessed the behavioral profile in offspring of both sexes at different life stages, applying a comprehensive behavioral test battery to detect potential Pb-induced changes in age-specific abilities. We determined Pb levels in blood, brain, and bone samples at two and seven months of age, long after termination of Pb exposure in both sexes**.**

2. MATERIALS AND METHODS

2.1. Animals

Female outbred CD1 Swiss mice (Charles River, Italy) were randomly assigned to treatment groups (0, 25-ppm, or 100-ppm Pb acetate solutions) via drinking water 2 weeks before mating throughout gestation and lactation until offspring weaning [postnatal day (PND) 21]. On PND 2 (birth PND 0), litters were culled to 8 pups (4 males and 4 females, when possible). At weaning (PND 21), mice were housed with same-sex siblings. All animals were kept in standardized conditions (temperature 20 ± 2 °C; humidity 60-70 %) with access to ad libitum food and water under a reversed 12h-12h dark/light cycle (lights on from 7:00 p.m. till 7:00 a.m.). All experimental procedures were carried out under the European and Italian legislation (2010/63/EU, Dl 26/2014) and were approved by the local Ethical Committee and by the Italian Ministry of Health (n. 25255).

2.2. Pb Exposure

Lead acetate (Pb(Ac)2·3H2O) was purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Test solutions for animal treatment were prepared in soft tap water, slightly acidified (pH 4.6) with acetic acid, and containing no Pb acetate (control) or Pb acetate at a concentration of 25 mg/L (25-ppm) or 100 mg/L (100-ppm). Acidified water ensured complete dissolution of the Pb salt; Pb solutions intake levels were comparable to those of plain tap water. The Pb concentration in the drinking solutions was analytically checked; since stability had been previously verified for five days (Tartaglione et al., 2020), and new solutions were administered every 3-4 days.

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2.3. Behavioral Testing

Behavioral testing was performed in a timeline, as showed in Figure 1. One male and one female from each litter underwent behavioral testing using different couple of subjects at each developmental stage (PND 8-23, PND 50-55, PND 167-206). Different age-specific tests were selected (Branchi and Ricceri, 2002): spontaneous locomotor activity was recorded at the end of the first postnatal week (PND 8) when ultrasound vocalization rate reaches the highest levels; homing test, that is primarily based on olfactory cues was performed on PND 11, before eyelid opening; the open field test was performed on PND 23 (just after weaning) to evaluate short-term effects of Pb exposure on locomotor, explorative and emotional responses (Bignami, 1996); three-chamber social interaction test was carried out between PND 50 and 55, i.e. at late adolescence, an age characterized by maximal interest for social cues (Spear, 2000). Finally, long-term effects of Pb exposure were also evaluated in fully adult (six-seven month-old) mice on cognitive-related abilities, namely olfactory habituation/dishabituation and spatial learning water maze tests.

All behavioral tests were performed in a single day (including water maze). Behavioral scoring was performed by an observer not aware of animal treatment.

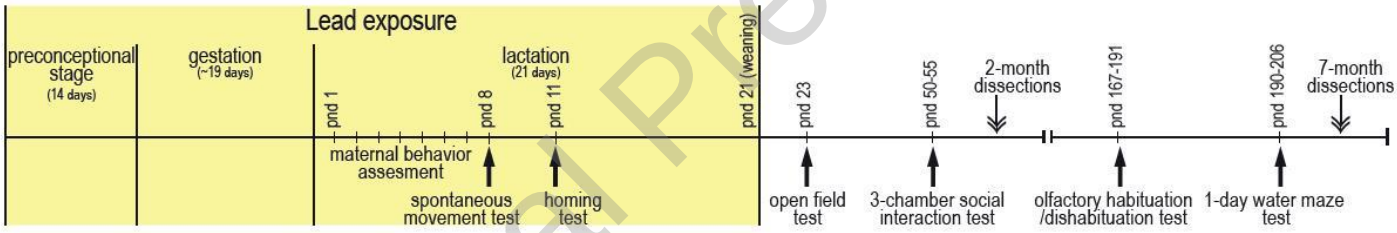

Figure 1. Experimental design and timeline. Pb exposure started at the preconceptional stage up to weaning of the offspring (indicated in highlighted area).

2.3.1. Maternal Behavior

From PND 1 to PND 7, maternal care was daily observed for three 60 min sessions, distributed across the dark phase of the diurnal cycle (at 9:00 a.m., 1:00 p.m. and 6:00 p.m.), by instantaneous sampling at an interval of four minutes (min) (45 samples per day for each dam). The experimenter scored maternal care in the home cage without moving the cage from the mouse rack. After having assessed whether the dam was inside or outside the nest, the dam behavior was recorded, considering: active nursing (kyphosis: high-crouched nursing posture by dam's body arched over the pups; licking and grooming: dam licking and grooming the pups mainly directed toward the anogenital region); passive nursing (supine nursing: dam lying on her side to provide maximum space for the pups; blanket nursing: dam lying flat on the pups); locomotion (dam mobile outside the nest); resting (dam lying motionless outside of nest); foraging (dam feeding); self-grooming (dam wiping, licking, combing or scratching any part of own body).

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2.3.2. Spontaneous Activity Test and Ultrasound Vocalizations (PND 8)

On PND 8, pups were removed from the litter and individually placed in a glass container (height: 7.5 cm; diameter: 14 cm). An Ultrasound Microphone (Avisoft Ultrasound Gate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10-180 kHz was placed about 20 cm above the pup to record ultrasound vocalizations (USVs), settings as in (Venerosi et al., 2009). Spontaneous movements and number of USVs were recorded for a 3 min session. At the end of the session, each pup was weighted and its axillary temperature measured by gentle insertion of the thermal probe (CHY 502 A, 2biological instruments; website www.2biol.com) in the skin pocket between upper foreleg and chest of the animal for about 20 seconds (s). For individual identification within the litter, after behavioral testing on PND 8, pups were permanently marked with a single dot in either left or right paw (Ketchum permanent Tattoo Inks green paste, Ketchum Manufacturing Inc., Brockville ON Canada). Tests were video recorded (Sony Handycam HDR CX240E) and subsequently analyzed using Observer XT software (Noldus, The Netherlands). The following behavioral items were scored: immobility (no visible movement of the body, all four paws on container's floor), head rising (a single rising of the head up and forward), wall climbing (forelimb placing movements on the container's wall), nose probing (various orientation movements of the nose), pivoting (locomotor activity involving only use of forelimbs) and walking (general translocation of the body by use of four paws).

2.3.3. Homing Test (PND 11)

In each litter, on PND 11, the same male and female pups tested on PND 8 were separated from the dam and kept for 30 min in an incubator (Elmed Ginevri 0GB 1000, Italy) at 28 ± 1 °C. Individual pups were then transferred to a polycarbonate cage (height × width × length: 15 × 21 × 42 cm) with one-sixth of the floor covered with sawdust from home cage (not cleaned in the previous five days; named as home bedding) and the rest with clean sawdust (named as clean bedding). Pups were gently placed in the middle of the area and video recorded for three min (Sony Handycam HDR CX240E). Video files were subsequently analyzed using Observer XT software (Noldus, The Netherlands). The following behavioral items were scored: locomotion (general translocation of the body by use of four paws); immobility (no visible movement of the pup body with all four paws placed on container's floor); head rising (a single rising of the head up and forward); wall climbing (forelimb placing movements on the container's wall); sniffing (a rhythmic inhalation and exhalation of air through the nose). Latency to, time spent in and number on entries in the home bedding area were also collected.

2.3.4. Open Field Test (PND 23)

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Animals of both sexes were placed in a single corner of the square open field (OPF) apparatus (40 × 40 × 40 cm) and video recorded for 15 min with dim 3 lux light (Calamandrei et al., 1997). By using ANY-maze software (Stoelting Co., Germany), the following parameters were scored: total distance, time spent in central area, distance traveled in central area, time spent in periphery, distance traveled in periphery, latency to first entry into central area.

2.3.5. Three Chamber Social Interaction Test

Young adult mice of both sexes were tested in a Plexiglas box (60 × 40 cm) divided into three compartments (with doorways along each dividing wall to access to the two side compartments) as described previously by (Venerosi et al., 2008). The subject mouse was initially placed in the apparatus for 20 min, 10 min in the central compartment with the doors closed [session 1 (S1)], followed by 10 min in the entire empty arena with the doors open (S2). The subject was then briefly confined to the central compartment while an inverted, empty wire cage (10 cm height, 10 cm bottom diameter, bars spaced 0.8 cm apart) was introduced into one of the side compartments. An unfamiliar, same-sex and strain mouse enclosed in an identical wire cage as a stimulus partner and was placed in the other side compartment. Doors were re-opened, and the subject was allowed to access to all three compartments for 10 min (S3). The subject was again confined to the central compartment, and another unfamiliar same-sex and strain mouse was placed in the remaining empty wire cage as a novel stimulus partner; doors were re-opened and the subject allowed to access to all three compartments and mouse behavior recorded for additional 10 min (S4). Side compartment location of the familiar and novel partners was randomized across subjects. Measures taken included time spent in each compartment and time spent sniffing each wire cage. Two video cameras were used to record the test: one above the Plexiglas box to track mouse movements with ANY-maze software. The second one in front of the Plexiglas box to get details of mouse behavioral responses (sniffing, cage rearing), and these video files were subsequently analyzed with Observer 10XT (Noldus, NL). Whole apparatus was cleaned with 70% ethanol after each mouse testing.
Animals used as partners were same-sex, same-age CD1 subjects; before the start of experiments, these mice were habituated to the apparatus and to the wire cage enclosure, 10 min per day for three consecutive days.

2.3.6. Olfactory Habituation/Dishabituation Test

Tests were performed in a polycarbonate cage (33 cm × 15 cm × 13 cm) with clean sawdust, and the test animal was exposed to a cotton swab introduced through the cage lid. Each test animal was habituated with a clean cotton swab in the test cage for 1-hour prior to the experiment. Estrous cycle of female mice was checked 1-day prior to the experiment by vaginal smear staining (Cora et al., 2015) and only sexually non-receptive females, in metestrous or diestrous, were included to the experiment. The test consisted of sequential exposure to different non-social and social odors. Each odor was presented in three consecutive trials (t1, t2, and t3) of two min each. Scented cotton swabs were prepared daily before the experiments, by soaking the swabs' top to neutral and non-social odor solutions; water, vanilla extract solution (1:100), almond

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extract solution (1:100) or by repeatedly wiping the cotton swabs across the bottom of unfamiliar mouse (same-strain and same- or opposite sex) cage beddings (De Felice et al., 2014) and stored in sealed plastic bags during the day. The sequence of the odors was the following; water 1, water 2, water 3, vanilla 1, vanilla 2, vanilla 3, almond 1, almond 2, almond 3, same-sex 1, same-sex 2, same-sex 3, opposite sex 1, opposite sex 2, opposite sex 3. Olfactory responsiveness was evaluated by interaction time with the cotton swabs (sniffing and chewing) recorded by a trained researcher with a silenced stopwatch.

2.3.7. Water Maze Test

One-day protocol was applied to avoid the effects of hormonal changes due to female estrous cycle (Frick et al., 2000). Estrous cycle of female mice was checked 1-day prior to the experiment by using vaginal smear staining (Cora et al., 2015) and only sexually non-receptive females, in metestrous or diestrous, were included to the experiment. This test was performed at least two weeks after the olfactory habituation/dishabituation test. The apparatus was a circular pool (diameter 110 cm, height 30 cm) with dark color inner-surface and located in middle of the test room. The pool was filled to depth of 40 cm water and maintained at 21 ± 2 °C. The pool virtually divided into four quadrants (North, South, West, East) and a platform (diameter 8 cm) placed in the North quadrant. Each mouse underwent four training sessions (S1 to S4), four trials in each session with the hidden escape platform (located 0.5 cm below water), 30 min intersession intervals, and a final probe trial without platform (Zuena et al., 2013). Training trials lasted 60 s, during which the mouse had to find the platform, climb onto it (and allowed to remain there for 10 s). If the animal did not find the platform, it was gently guided with a grid and allowed to stay for 10 s. Probe trial lasted 60 s. Experiments were video recorded, tracking and behavioral scoring were performed by ANY-maze software. The following parameters were scored for training sessions and probe trial: latency to reach the platform (platform location during probe), average velocity, path efficiency, number of entries, time spent and distance travelled in each quadrant.

2.4. Sample Collection and Lead Biomonitoring

2.4.1. Sample Collection

Animals were sacrificed by decapitation and tissue samples (blood and total brain) were collected at two and seven months of age (blood in metal free EDTA tubes, BD Royal Blue Blood Collection Tubes K2EDTA – 368381). Bone samples from tibia were obtained in 7-old-month animals only. Use of metal surgery tools and aluminum foil was minimized, to prevent metal contamination during sampling. After collection, blood samples were stored at +4 °C, brain and bone samples at -80 °C, till further mass spectrometry analyses.

2.4.2. Lead biomonitoring

Analyses were carried out in 2-month (blood n=29, brain=36) and 7-month (blood n=41, brain=39, bone=40) samples. As for blood, sample preparation and analysis were carried out using a method fully validated and

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accredited following the ISO/IEC 17025 guidance (Ruggieri et al., 2016). As for brain, drying procedure was applied by using an oven at 80 ± 3°C overnight, and a mineralization cycle performed in 15 mL Falcon polystyrene plastic tubes at 80 °C on a hot plate (Mod Block CPI International, Santa Rosa, CA, USA) for 3 h, via adding 3 mL of ultrapure HNO_3 (Normatom, Leuven, Belgium). The same protocol was applied for bone analysis. Analytical quality was assured by the repeated analysis of Certified Reference Materials (CRMs) Seronorm Trace Elements Blood (SERO AS, Billingstad, Norway) and bovine liver 185R (Institute for Reference Materials and Measurements, IRMM, Geel, Belgium) were used for brain and bone analysis; they were treated accordingly with the digestion and dilution procedures as samples. The Pb determination was carried out by using ICP mass spectrometry, namely iCAP Q model (Thermo Fisher Scientific Inc., Waltham, MA, USA), configured for ultra-trace elemental analysis in KEDS mode. The main instrument configuration and operation parameters are reported in Table S1.

The iCAP Q used in this study was equipped with a PFA ST MicroFlow nebulizer (ESI, Omaha, NE, USA), a peltier cooled quartz cyclonic spray chamber operating at 3 °C, a 2.0 mm ID quartz injector, a demountable quartz torch and interface Nichel cones. Lead was quantified with the standard addition calibration and Indium was used as the internal standard at the concentration of 1 ng mL−1 in the analytical solution. The limits of detection (LoD) was calculated using the 3σ criteria with a LoD of 0.045 microg/dL.

2.5. Statistical Analysis

Data were reported as mean value of ± SE (standard error). Behavioral data were analyzed by analysis of variance (ANOVA) with repeated measures (with litter as statistical units, and male and female subjects as replicates in case of offspring data) with treatment as between factor and sex/trial/partner/zone/quadrant/day (when present) as within subject-factors by using Statview 5.1 software. Post-hoc comparisons were performed using Tukey's HSD test when the p-value on the relevant interaction was significant or close to the significance. Potential correlation between maternal active nursing behavior and selected neonatal, juvenile, and adulthood behaviors were assessed by one-way ANOVA with treatment as between factor and maternal active nursing data as covariant using median of both sexes; followed by bivariate scattergram with regression analysis.

Pb biomonitoring data were analyzed by one-way ANOVA with treatment and sex as between factors by using Statview 5.1 software. Post-hoc comparisons were performed using Tukey's HSD test when the p-value on the relevant interaction was significant or close to the significance.

A 95% level ($p<0.05$) was considered statistically significant.

3. RESULTS

3.1. Reproductive Performance

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No effect of prenatal treatment was evident on litter size [F (2, 27) = 0.543 p=0.58] and sex ratio [F (1, 27) = 0.196 p=0.66].

3.2. Maternal Behavior

Maternal behavior assessment in the first week after parturition revealed increased active nursing in 100-ppm treated dams [Figure 2A; main effect of treatment, F (2, 24) = 3.280 p=0.05; control *vs* 100-ppm: p<0.05, 25-ppm *vs* 100-ppm: p<0.05] whereas no difference was evident in passive nursing. No treatment effect was evident in other behavioral items recorded.

3.3. Body Weight

Pb-exposed pups weighted more than controls only on PND 23 [Figure 2B; treatment × age interaction, F (6, 72) = 2.142 p=0.05; control PND 23 *vs* 25-ppm PND 23: p<0.01, control PND 23 *vs* 100-ppm PND 23: p<0.01], not at any older age considered (data not shown).

3.4. Offspring Behavioral Tests

3.4.1. Spontaneous Activity and USVs (PND 8)

Pb exposed pups, both 25-ppm and 100-ppm, showed increased nose probing frequency than control pups [Figure 2C; main effect of treatment, F (2, 19) = 4.101 p<0.05; control *vs* 25-ppm: p<0.05, control *vs* 100-ppm: p<0.05]; nose probing duration also showed the same profile [main effect of treatment, F (2, 19) = 3.788 p<0.05; control *vs* 100-ppm: p<0.05, data not shown]. As for pivoting, 100-ppm female pups displayed shorter responses than control female pups, whereas no difference was evident in males [Figure 2C; treatment × sex interaction, F (2, 19) = 3.481 p=0.05; control females *vs* 100-ppm females: p<0.05]. Likewise, for immobility, 100-ppm female pups spent more immobile time than 25-ppm females, yet this difference did not reach significance (data not shown). Treatment did not affect other motor responses. As for USVs, Pb-exposed pups emitted a number of calls comparable to control pups [main effect of treatment, F (2, 19) = 0.913 p=0.41; data not shown]. However, reduction in number of USVs emitted by females of the 100-ppm group in comparison with control and 25-ppm groups just missed statistical significance [(mean values ± SE) control: 118 ± 26, 25-ppm: 113 ± 15, 100-ppm: 69 ± 11].

3.4.2. Homing Test (PND 11)

Only control animals showed the expected preference towards the nest odor, spending more time in nest scented home bedding area than in the clean bedding area [Figure 2D; treatment × zone interaction, F (2, 21) = 1.952 p=0.16; control home bedding *vs* control clean bedding: p<0.05], whereas in both 25-ppm and 100-

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ppm animals, time over the home bedding did not differ from time over clean bedding. As shown in left panel of Figure 2D, females appeared more vulnerable than males to 100-ppm Pb exposure, as confirmed by analyses of confidence intervals (CI) for mean values of total time spent over home bedding [control males: 116.42 ± 73.95, control females: 92.61 ± 65.91, 25-ppm males: 58.84 ± 46.23, 25-ppm females: 83.96 ± 58.61, 100-ppm males: 87.54 ± 63.08. 100-ppm females: 31.94 ± 49.04]; indeed 100-ppm females interval was the ones closest to randomness (30 s). As for sniffing duration over the home bedding, Pb-exposed animals were more affected than control animals [Figure 2D; treatment × zone interaction, $F(2, 21) = 3.526$ $p<0.05$; control *vs* 25-ppm: $p<0.01$, control *vs* 100-ppm: $p<0.05$].

3.4.3. Open Field Test (PND 23)

On PND 23, latency to enter the central area was shorter in Pb-exposed (25-ppm and 100-ppm) than in control mice [Figure 2E; main effect of treatment, $F(2, 22) = 3.635$ $p<0.05$; control *vs* 25-ppm: $p< 0.05$, control *vs* 100-ppm: $p<0.05$]. The distance travelled decreased over time for all groups [Figure 2E; main effect of time interval $F(2, 44) = 100.189$ $p<0.01$] and no treatment effect was evident on the habituation profile. The analysis on the other parameters considered did not reveal any effect of either Pb, sex or sex × Pb interactions.

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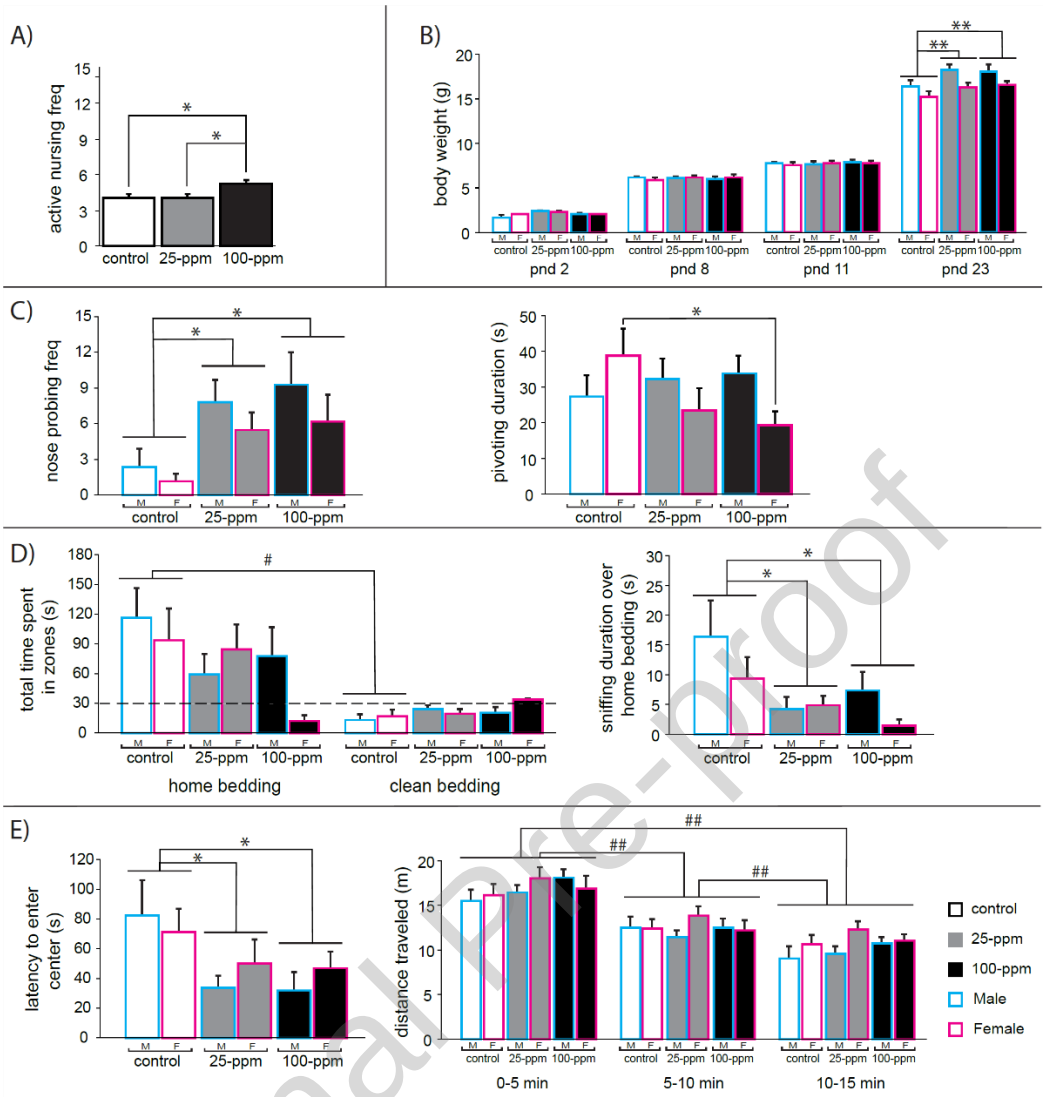

Figure 2. Maternal behavior, offspring body weight and neonatal behavioral tests. A) Maternal active nursing (kyphosis and licking & grooming) frequency scores [mean value of postnatal day (PND) 1-7]. B) Body weight recorded on PND 2, 8, 11, and 23. C) Spontaneous activity (PND 8): nose probing (frequency, number of episodes - left panel) and pivoting (duration - right panel). D) Homing test (PND 11): total time spent in home bedding area *vs* clean bedding area (left panel, dashed line indicates absence of preference), sniffing duration over home bedding (right panel). E) Open field test (PND 23): latency to enter the central area (left panel), total distance traveled over 5-minute (min) periods during a 15-min test (0-5, 5-10, 10-15 min; right panel). Number of litters/pups per sex (n): A), B) control= 9, 25-ppm= 10, 100-ppm= 8; C) n: control= 6, 25-ppm= 10, 100-ppm= 6; D) n: control= 7, 25-ppm= 10, 100-ppm= 7; E) n: control= 8, 25-ppm= 10, 100-ppm= 7. Data are mean values + SE; *, **, $p<0.05$ and $p<0.01$ respectively for comparisons between different treatment groups; $^{\#}$, $p<0.05$ for comparisons between home and clean bedding within control group; $^{\#\#}$, $p<0.01$ for comparisons between time intervals during open field test.

3.4.4 Three Chamber Social Interaction Test

In S1 and S2, no treatment effect was evident. In S3, all groups spent more time sniffing the cage containing the social partner than the empty cage and, similarly, spent comparably more time in the social compartment than in the one containing the empty cage [Figure 3A; main effect of compartment, $F(1, 22) = 91.97$ $p< 0.01$ and $F(1, 22) = 19.07$ $p<0.01$, respectively]. In S4, time spent in novel compartment was longer for all groups than time spent in the familiar one [Figure 3B; main effect of compartment, $F(1, 22) = 23.81$, $p<0.01$] and all

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treatment groups sniffed the novel partner significantly longer than the familiar partner [F (1, 22) = 83.36, p<0.01]. However, sniffing of novel partner by 25-ppm animals was significantly lower than controls (p<0.01).

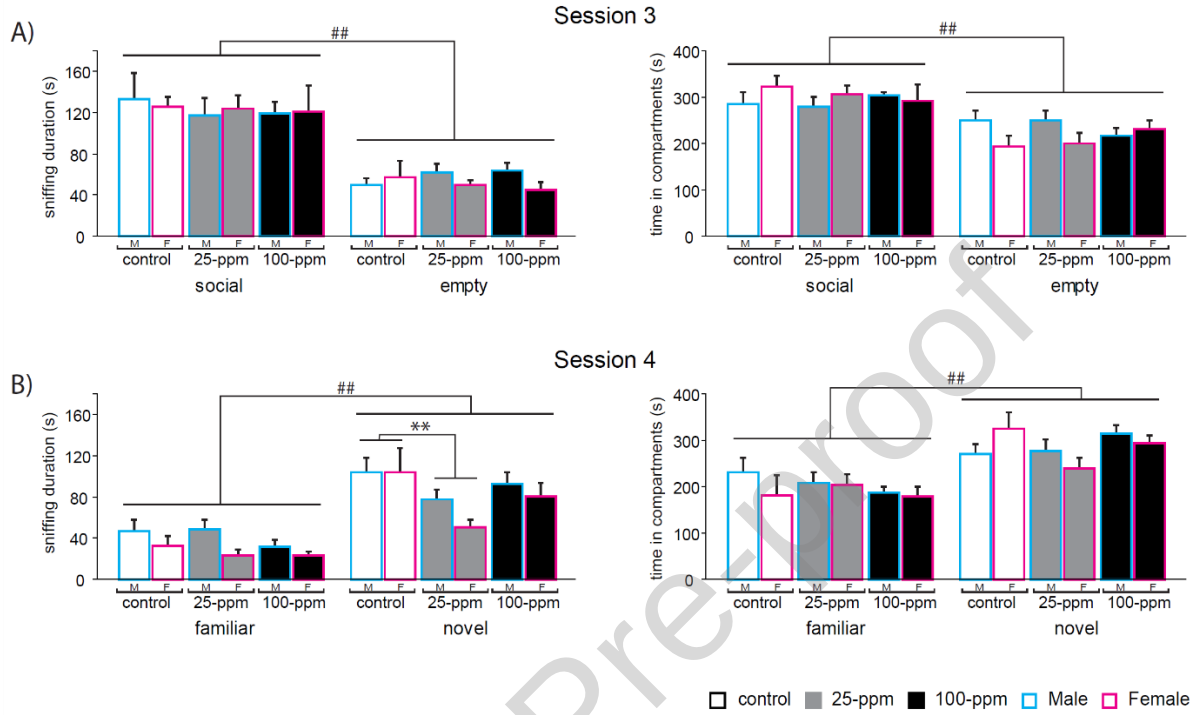

Figure 3. Three-chamber social interaction test. A) duration of sniffing of the social partner cage and empty cage (left panel) and time spent in the two compartments (right panel) in session 3; B) duration of sniffing of the familiar partner cage and novel partner cage (left panel) and time spent in the two compartments (right panel) in session 4. For each sex, n: A), B) control= 8, 25-ppm= 10, 100-ppm= 8. Data are mean values + SE; **, p<0.01 for comparison between 25-ppm group and control group; ##, p<0.01 for comparisons between social/empty or familiar/novel conditions.

3.4.5. Olfactory Habituation/Dishabituation Test

Olfactory responsiveness was evaluated by measuring time spent sniffing or chewing the cotton swab by each subject (Figure 4). Duration of interaction with the scented cotton swab did not differ in the three treatment groups for neutral (water), non-social (vanilla, almond), and social (same sex, opposite sex) odors [treatment × odor interaction; F (8, 68) = 0.36 p=0.93]. To evaluate habituation to each odor throughout the three trials, we compared the time spent in investigation of each odor in in t1 and t3; all treatment groups showed habituation to non-social odors [vanilla: F (2, 34) = 21.79, p<0.01; almond: F (2, 34) = 38.60 p<0.01]; all treatment groups also habituate to same-sex odor, except for 25-ppm males and 100-ppm females [treatment × sex x trial interaction; F (4, 34) = 2.011 p= 0.11; t1 vs t3 control males p < 0.05, control females p < 0.01, 25-ppm females p < 0.05, 100-ppm males p < 0.01; t1 vs t3 25-ppm males and 100-ppm females ns].

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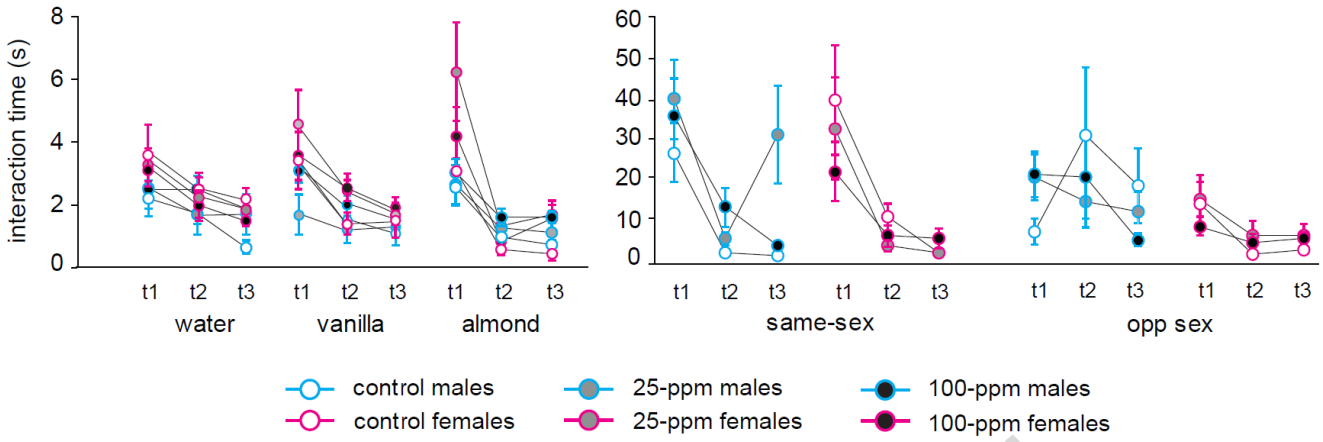

Figure 4. Responsiveness to non-social (vanilla and almond) and social [same sex and opposite (opp) sex] olfactory cues. For each sex, n: control= 5, 25-ppm= 10, 100-ppm= 6. Data are mean interaction time (sniffing ± chewing) ± SE.

3.4.6. Water Maze Test

Throughout training sessions (S1-S4), all animals, except 100-ppm males, exhibited decreased latency to reach hidden escape platform over sessions. Specifically, 100-ppm males required more time to locate the platform, showing significantly longer latency than controls during S2 [Figure 5A; treatment × training days × sex interaction, $F(6, 48) = 3.89$ $p<0.01$; control males *vs* 100-ppm males, $p<0.01$]. A similar profile was evident for path length data in S2 [Figure 5B; treatment × session × sex interaction, $F(6, 48) = 1.53$ $p=0.18$; control males *vs* 100-ppm males: $p<0.05$]; average speed data in S2 and S3 are higher in Pb treated males [Figure 5C; treatment × session × sex interaction, $F(6, 48) = 1.92$ $p=0.09$, S2 control males *vs* S2 100-ppm males: $p<0.01$, S3 control males *vs* S3 25-ppm males: $p<0.05$].

Since we observed significant alterations during training in Pb-exposed males only, further statistical analysis was performed considering only male mice (for females see Figure S1). Evaluation of time spent in the quadrant containing the platform throughout S2-S4 confirmed the altered spatial learning in Pb exposed male groups (Figure 5D-F). Indeed, both 25- and 100-ppm males spent less time than control males in the platform quadrant [Figure 5D S2: main effect of treatment, $F(2, 16) = 7.67$ $p<0.01$; control males vs 100-ppm males: $p<0.01$; Figure 5E S3: main effect of treatment, $F(2, 16) = 13.44$ $p<0.01$; control males vs 25-ppm males: $p<0.01$, control males vs 100-ppm males: $p<0.01$]. No significant differences were evident in S4 (Figure 5F). During the probe trial, whereas control males spent significantly more time in platform quadrant, time spent by Pb-exposed males in platform and opposite quadrants did not differ [Figure 5G; treatment × quadrant interaction, $F(2, 16) = 3.81$ $p<0.05$; control platform quadrant *vs* control opposite quadrant: $p<0.05$]

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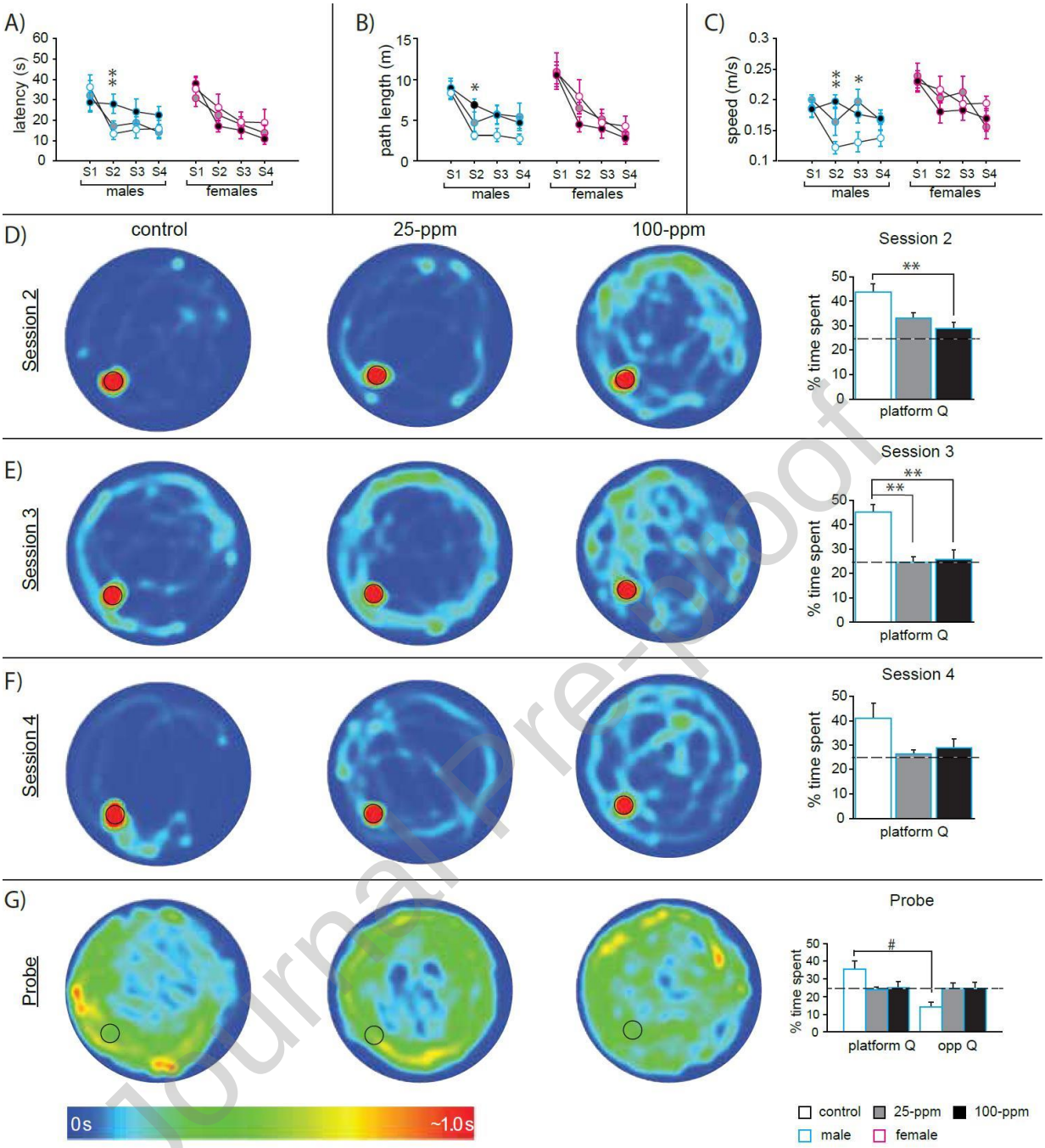

Figure 5. Effects of developmental Pb exposure on spatial learning and memory in the water maze test. Training parameters considered were: A) latencies to reach the platform; B) length of swimming path; C) swimming speed; heat map of male positions during 4th trial (left panel) and percentage of time spent in the platform quadrant (Q) (right panel) in Session 2 (D), 3 (E), 4 (F); probe session (G). Dashed line indicates absence of preference for quadrant. For each sex, n: control= 7, 25-ppm= 10, 100-ppm= 7. Data are mean values + SE; *, **, $p<0.05$ and $p<0.01$ respectively for comparisons between control and Pb treatment groups; $^{\#}$, $p<0.05$ for comparisons within the same treatment group.

3.5. Lead Biomonitoring

As shown in Figure 6, Pb levels were measured in blood and brain (in 2- and 7-month-old mice), and bone (in 7-month-old mice only).

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At two months of age, blood lead levels (BLLs) of 100-ppm animals were significantly higher than control and 25-ppm animals [Figure 6A and Table S2: main effect of treatment, F (2, 23) = 20.081 p<0.01; control *vs* 100-ppm: p<0.01, 25-ppm *vs* 100-ppm: p<0.01]. At seven months of age, values were much lower, yet 100-ppm animals maintained significantly higher BLLs comparing to control and 25-ppm animals; likewise, 25-ppm animals showed significantly higher BLLs than controls [Figure 6B; main effect of treatment, F (2, 35) = 14.254 p<0.01; control *vs* 25-ppm: p<0.05, control *vs* 100-ppm: p<0.01, 25-ppm *vs* 100-ppm: p<0.05]. Treatment × sex interaction-based post-hoc comparisons indicated that 100-ppm female BBL values resulted significantly higher than corresponding male values (p<0.05).

Data from brain samples illustrate a similar yet stronger scenario. At two months of age, 100-ppm brain Pb levels were significantly higher than corresponding values in either control or 25-ppm groups [Figure 6A and Table S3; main effect of treatment, F (2, 30) = 47.837 p<0.01; control *vs* 100-ppm: p<0.01, 25-ppm *vs* 100-ppm: p<0.01]. At seven months of age, although Pb levels in brain were lower than at 2-months, 100-ppm animals still had higher values than control and 25-ppm animals [Figure 6B; main effect of treatment, F (2, 33) = 15.153 p<0.01; control *vs* 100-ppm: p<0.01, 25-ppm *vs* 100-ppm: p<0.01].

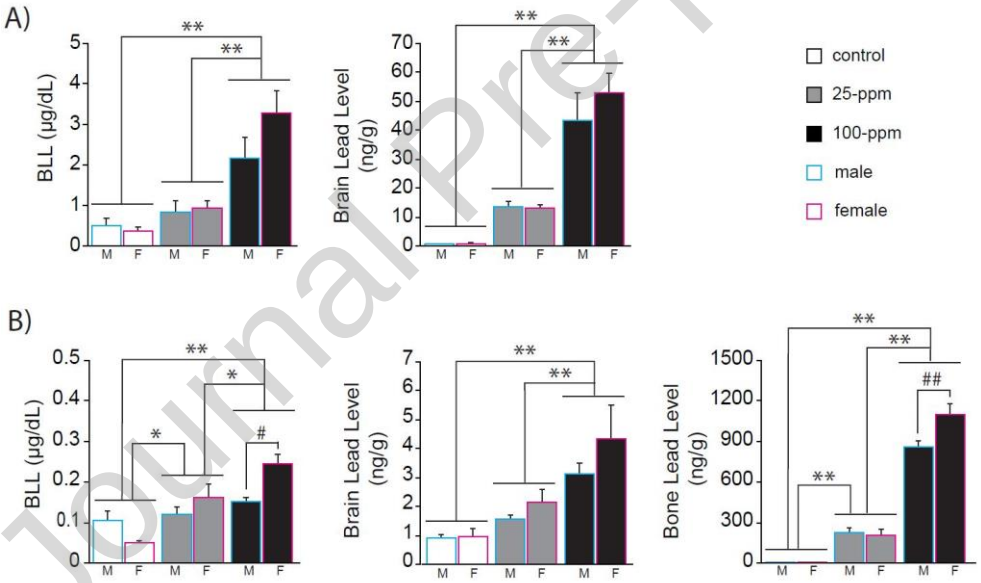

Figure 6. Pb levels in young adulthood and adulthood stage. (A) Average Pb levels in blood (n: control = 6M/4F, 25-ppm = 4M/4F, 100-ppm = 6M/5F) and brain (n: control = 6M/6F, 25-ppm = 5M/6F, 100-ppm = 6M/7F) at 2-months of age; (B) average Pb levels in blood (n: control = 7M/6F, 25-ppm = 7M/7F, 100-ppm = 7M/7F), brain (n: control = 7M/7F, 25-ppm = 6M/7F, 100-ppm = 6M/6F), and bone (n: control = 7M/6F, 25-ppm = 7M/6F, 100-ppm = 7M/7F) at 7-months of age. M and F indicate males and females, respectively. Data are mean values + SE; *, **, p<0.05 and p<0.01 respectively for comparisons between treatment groups; $^{\#}$, $^{\#\#}$, p<0.05 and p<0.01 respectively for comparisons within 100-ppm group.

In 7-month-old mice, long-term Pb accumulation was also monitored in bone matrix. Bone Pb levels of 100-ppm animals were significantly higher than corresponding control and 25-ppm values; likewise, 25-ppm bone Pb levels were significantly higher than the corresponding control values [Figure 6B and Table S4; main effect of treatment, F (2, 34) = 288.617 p<0.01; control *vs* 25-ppm: p<0.01, control *vs* 100-ppm: p<0.01, 25-ppm *vs*

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100-ppm: p<0.01]. Treatment × sex interaction-based post-hoc comparisons indicated that 100-ppm female bone Pb values were significantly higher than the male ones (p<0.01).

4. DISCUSSION

Lead exposure is still a matter of concern worldwide, as it has becoming increasingly clear that there is no safe level of this contaminant in the environment for children's neurobehavioral development. In this study, we intended to fill a gap in knowledge concerning the longitudinal effects in a mouse model of developmental exposure to low doses of Pb comparable to those reported in infants/children in urban scenarios (Polanska et al., 2018). Indeed, in our experiment, 100-ppm BLL levels at young adulthood (two months of age, five/six weeks after Pb exposure) is lower (2.6 μg/dL) than the last reference value proposed by CDC for children (3.5 μg/dL) (CDC, 2021). We also know from our previous studies in rats, by applying an exposure schedule similar to the one of the present study that BLLs at the end of the treatment at weaning ranged from a median of 6 to 25 μg/dL in 25- or 100-ppm doses respectively (Tartaglione et al., 2020).

Assessment of neonatal and infant behavior is a very important step in follow-up of child neurodevelopment, and it is routinely used in longitudinal human studies through validated scales [such as The Neonatal Behavioral Assessment Scale, NBAS and Bayley Scales of Infant Development, BSID (Dórea, 2019)] that in many cases allowed to evidence early effects of perinatal exposure to Pb on motor and cognitive development (Ortiz et al., 2017; Polanska et al., 2018). Thus, we assessed neonatal mice through a neurodevelopmental test battery designed as to acquire information on the effects of Pb on maturation of early sensorimotor capabilities, including orientation toward relevant stimuli and maturation of motor coordination in the early stages. We observed even at these low doses mild but significant changes in neonatal behaviors of male and female offspring. More in detail, we found alterations in specific neonatal behavioral responses, including increased nose probing and impaired recognition of the nest odor for animals exposed to both doses of Pb and decreased pivoting for 100-ppm Pb females only. Nose probing behavior in developing mouse is an important component of the suckling behavior consisting of various orientation movements of the snout aimed at nipple localization. Suckling is considered as an odor-mediated behavior itself, regulated by the main olfactory epithelium, and the initiation of the process is linked with odor experience and recall (Logan et al., 2012). Odor preference may be established with attenuated fear learning during the early stage of life; a rodent study showed that presence of mother odor can suppress the infant level of cortisol and amygdala activity, especially between PND 10-15 (Moriceau et al., 2010). Interestingly, Pb-exposed pups failed to discriminate the scent of their nest from clean bedding in the homing test at PND 11, and particularly they were much less engaged in sniffing behavior once reaching the nest-scented home bedding compared to control mice. The enhanced frequency of nose probing behavior and the reduced preference for familiar cues exhibited by Pb-exposed pups may possibly reflect a deficient recognition of socially relevant olfactory cues. Given the role of familiar odors in pups emotional development, even subtle impairment in olfactory orientation in critical periods might

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impact on behaviors later on (Landers and Sullivan, 2012). Our hypothesis was supported by the slight impairment in discrimination/habituation for same-sex olfactory cues seen at adulthood (see below). We failed to evidence any significant effect on motor development, with the exception of a small decrease in pivoting shown by 100-ppm females. Pivoting is an immature pattern of locomotor activity that is progressively substituted by onward quadrupedal locomotion, and it may be considered analogous to human infants' crawling behavior (Rachwani et al., 2019). In our study, decreased pivoting is mirrored by a trend to increased immobility, suggesting 100-ppm female pups are hypoactive (as also shown by USV rate). In most of the studies, decreased locomotor activity has not been associated directly with Pb-exposure so far; however, association with iron deficiency is supported by the literature (Angulo-Kinzler et al., 2002; Shafir et al., 2008) through the well-known interference of Pb with to heme synthesis pathway (Hsieh et al., 2017).

Consideration of maternal behavior is relevant in studies exploring the effects of prenatal exposure to chemicals to properly include any modification attributable to indirect toxicity via the exposed dam. Maternal care is one of the most important element in the early ecological niche of the newborn: much evidence showed that alterations in maternal care (e.g., licking frequency) may influence developmental programming by modifying expression levels of key genes in the offspring via changes in epigenetic regulations (Lauby et al., 2019; Lauby et al., 2021; Maud et al., 2018). Interestingly, we found increased active nursing behavior in 100-ppm dams, which however did not correlate with either early- or late- behavioral outcomes of the offspring. These modifications of maternal care are worthy of being further investigated with experimental designs more focused on maternal behavior of the exposed dams.

As early-adolescent (PND 23), mice were tested for the behavioral response to a novel environment (OPF). Pb-exposed mice travel a similar distance compared to control mice, exhibiting the expected profile of habituation, defined by a decrease in locomotor activity over time. The effect of Pb on locomotor activity varies in the literature, as reported by both human/children meta-analyses and animal studies. Some studies found Pb-induced hyperactivity mostly in children (Nilsen and Tulve, 2020) and rodents (Bouyatas et al., 2019; Duan et al., 2017; Luo et al., 2014; Moreira et al., 2001; Tang et al., 1994), and also in a zebrafish model (Wang et al., 2016); on the contrary, other showed reduced locomotor activity in rodents (Basha and Reddy, 2015; Chintapanti et al., 2018; Leão et al., 2020). Rodent studies with BLLs comprised between 5 and 20 µg/dL however did not evidence marked effects on locomotion or habituation (Rocha and Trujillo, 2019). Further to measures of locomotion, we also considered the latency to enter the central area of the OPF as an index of anxiety or impulsivity. We found that, despite the comparable time spent in the central area by all experimental groups, animals of both Pb-exposed groups reached the unsafe central area significantly faster than the control animals, suggesting a disinhibited profile in novelty response. This specific aspect at this low-dose levels is of interest when considering the suggested association between early chronic Pb exposure, increased impulsivity and higher risk of conduct disorders in children/adolescents (Braun et al., 2008).

Since the impairment in nest-odor recognition detected at very early stages of life could be predictive of deficits in sociability and olfactory discrimination later in life, animals were assessed in 3-chamber social interaction

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test and olfactory habituation/dishabituation test to evaluate at either juvenile or adult stage the behavioral response to social- and olfactory-relevant cues, respectively. In these behavioral domains the effects of Pb were evident at the 25-ppm dose only: 25-ppm animals showed diminished sniffing response to social novelty and weakened habituation to odor of same-sex conspecific. These findings are consistent with data in rats indicating that Pb exposure elicits an adverse effect on olfactory discrimination (Lim et al., 2005), in mice where non-linear decrease in odor discrimination was observed at lower but not higher levels of lead exposure for BLLs ranging from 0.02 to 20.31 ug/dL (Flores-Montoya et al., 2015) as well as in humans showing an association between elevated BLL and decreased olfactory memory in children (Zhang et al., 2017).

At adulthood, in Pb-exposed mice, we found marked sex-related impairment in spatial learning and memory in water maze, with males being the most affected. All animals acquired the task over sessions; however, Pb-exposed males, especially 100-ppm males, showed slower learning acquisition throughout the training sessions and memory retention deficit in the probe trial. Notably, in comparison with previous studies, selectivity of Pb effects on males is here strengthened by our efforts to minimize effects of estrus cycle on behavioral performance of females (choice of “one-day” water maze paradigm, testing of females in metestrous or diestrous phases only) and by the statistical analyses considering each male and female coming from the same litter as paired.

The cognitive effects of developmental Pb-exposure are among the most robust reported in the literature; BLLs are inversely associated with IQ scores (Mazumdar et al., 2011; Schnaas et al., 2006; Skerfving et al., 2015) and meta-analyses of Pb-exposure studies, both in humans and experimental animals, indicate sex-specific neurotoxic effects affecting males more than females. Throughout 1988-2014, NHANES study showed that males have greater Pb body burden than females (Brody et al., 1994; Tsoi et al., 2016). Polanska et al. showed an interaction between cord BLLs and lowered scores for cognitive functions of infant boys (Polanska et al., 2018). These findings were strengthened by other studies (Joo et al., 2018; Skerfving et al., 2015), further supporting a higher vulnerability of males to Pb neurotoxicity (Gade et al., 2021).

In human biomonitoring, blood is the most used matrix to detect Pb-levels; yet the half-life of Pb in blood and soft tissues is roughly a month, and BLL is primarily a measure for acute exposure, while the half-life in bones is about 30 years and bone lead level is thus a measure for chronic exposure (Maret, 2017). In this study, Pb-levels in blood and brain were traced at young adult- and adult-ages, while long-term accumulation was traced in bone samples.

Interestingly, present Pb data together with those from our previous experiments in rats (Tartaglione et al., 2020), using comparable exposures, or with those using higher doses [(Moreira et al., 2001), 500-ppm], suggest that: i) BLL rapidly decreases after the end of exposure and ends up to BLL<5 µg/dL five weeks after; ii) brain levels of PB decrease not so rapidly and are about 48.5 µg/dL five weeks after the end of exposure and 3.7 µg/dL 20 weeks later. Bone (tibia) lead concentrations are likely the most reflective of cumulative lifetime levels of Pb; in our study, tibia Pb levels were higher for 100-ppm animals, with females showing higher levels than males. This might be due to calcium retention in bone mass during developmental bone formation

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(2011); bones are indeed not only the main and final accumulation points of Pb in the body [90% of Pb is stored in bones (Maret, 2017)] but also source of internal exposure, because of bone calcium turnover throughout pregnancy and lactation (Silbergeld, 1991).

Behavioral alterations found in the present study indicate that males are more susceptible, although females seem to have relatively higher levels of Pb in blood and bone several months after the end of exposure. As stated by (Singh et al., 2018), much remains unclear regarding the sex-specific responses of the brain to Pb. There are contrasting results showing that either males or females are more vulnerable; our present results confirm these discrepancies. In addition they also suggest that higher internal levels of Pb do not necessarily correspond to more significant impact on behavior. It is hard to say whether differential efficacy of Pb detoxification could explain sex-dependent behavioral profiles.

5. CONCLUSION

This study mimicked developmental, low-level Pb exposure and illustrated short- and long-term effects of Pb with a longitudinal experimental design. Pb exposure caused mild but diffused effects in early-life behaviors, concerning reactivity to olfactory stimuli and selective motor responses. At adulthood, performance of Pb-exposed males is impaired in a spatial learning and memory task. Interestingly adult Pb blood and brain levels follow a different profile after developmental exposure: brain levels appear to decrease much slower than blood ones. As a whole, for Pb levels, a clear dose-response profile is evident in tissues from 2- and 7-month-old mice exposed to Pb till weaning (blood, brain and bone levels). By contrast, both Pb doses similarly affected behavioral development in most of the test selected, confirming also in experimental models the absence of threshold values for Pb neurotoxicity.

These data in laboratory mice allow a different perspective to evaluate the Pb reference value for children proposed by CDC: to those, low blood levels could actually correspond higher levels in different, not accessible organs such as the brain.

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Acknowledgments

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Authorship Contribution Statement

Öykü Dinçkol: Conceptualization, Methodology, Investigation, Data analysis, Writing – Original Draft; Byron Fuentes: Investigation; Anna Maria Tartaglione: Investigation, Writing – Review & Editing; Anna Pino: Writing – Review & Editing; Laura Ricceri: Conceptualization, Data analysis, Writing – Review & Editing; Gemma Calamandrei: Conceptualization, Resources, Writing – Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

Öykü Dinçkol: Conceptualization, Methodology, Investigation, Data analysis, Writing – Original Draft; Byron Fuentes: Investigation; Anna Maria Tartaglione: Investigation, Writing – Review & Editing; Anna Pino: Writing – Review & Editing; Laura Ricceri: Conceptualization, Data analysis, Writing – Review & Editing; Gemma Calamandrei: Conceptualization, Resources, Writing – Review & Editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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HIGHLIGHTS

- Low-dose lead (Pb) developmental exposure has short- and long-term effects on mouse behavior.
- Pb differentially affects selected behavioral responses in female and male mice.
- Blood Pb levels in 2-month-old mice (100-ppm dose) are comparable to the most recent CDC reference value for Pb blood in children.
- Pb monitoring data indicate that Pb is still detectable in brain and bone tissues six months after the end of exposure

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