Particle and Fibre Toxicology

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Impact on murine neurodevelopment of early-life exposure to airborne ultrafine carbon nanoparticles

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Abstract

The effects of ultrafine particle (UFP) inhalation on neurodevelopment, especially during critical windows of early life, remain largely unexplored. The specific time windows during which exposure to UFP might be the most detrimental remain poorly understood. Here, we studied early-life exposure to clean ultrafine carbonaceous particles (UFP^C) and neurodevelopment and central nervous system function in offspring.

Pregnant wild-type C57BL/6J mice were either sham-exposed (HEPA-filtered air) or exposed to clean ultrafine carbonaceous particles at a concentration of 438 ± 72 µg/m³ (mean ± SD) and a count median diameter of 49 ± 2 nm (CMD ± GSD) via whole-body exposure for four hours per day. For prenatal exposure, mice were exposed for two consecutive days in two exposure periods, while the postnatal exposure was conducted for four consecutive days in two exposure periods. The mice were divided into four groups: (i) sham, (ii) only prenatal exposure, (iii) only postnatal exposure, and (iv) both prenatal and postnatal exposure. Neurodevelopmental behaviour was assessed throughout the life of the offspring using a functional observation battery.

Early-life UFP^C-exposed offspring exhibited altered anxiety-related behaviour in the open field test, with exclusively postnatally exposed offspring (567 ± 120 s) spending significantly more time within the border zone of the arena compared to the sham group (402 ± 73 s), corresponding to an increase of approximately 41% ($p < 0.05$). The behavioural alterations remained unaffected by olfactory function or maternal behaviour. Mice with both prenatal and postnatal exposure did not show this effect. No discernible impact on developmental behavioural reflexes was evident.

Early life exposure to UFP^C, particularly during the early postnatal period, may lead to developmental neurotoxicity, potentially resulting in complications for the central nervous system later in life. The current data will support future studies investigating the possible effects and characteristics of nanoparticle-based toxicity.

Keywords Ultrafine particulate matter, Early-life exposure, Behavioural development, Anxiety-like behaviour

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Introduction

Air pollution is one of the most significant environmental risks to human health [1], with 99% of the human population living in environments that do not meet the WHO air quality guidelines [2]. Poor air quality can adversely affect our health, thereby increasing the risk of cardiovascular disease [3, 4], exacerbating asthma [5], and contributing to cognitive decline in older people [6]. Traffic-related air pollution, originating from a combination of vehicle exhaust emissions and non-combustion sources like tire and brake wear [7], as well as industrial activities and agricultural practices, contributes to outdoor air pollution. However, ambient air pollution is a complex mixture of gases and particulate matter (PM), with a composition that varies significantly by location [8]. Depending on their emission source and atmospheric processing, PM exists in various sizes, chemical compositions and surface areas [9]. All these physicochemical characteristics play a role in the possible health impacts of PM [10]. Most research has been devoted to micro sized PM, such as PM_{10}, which refers to particles with a diameter of 10 micrometres or less, and $PM_{2.5}$, which includes finer particles with a diameter of 2.5 micrometres or less [2]. However, ultrafine particles (UFP, also known as $PM_{0.1}$, particles with a diameter $\leq$0.1 microns or 100 nm) are expected to pose significant health risks, primarily because of their ability to penetrate the lower respiratory tract and migrate to distant organs [11, 12]. Additionally, due to their high surface area per unit mass, UFPs have a greater capacity to adsorb more toxic compounds (e.g., polycyclic aromatic hydrocarbons [PAHs] and benzene) [13, 14]. UFPs are often produced during the combustion of fossil fuels, which results in an elemental carbon core surrounded by organic carbon and minor fractions of metallic, nitrate, and sulpha compounds [15, 16].

A vulnerable target of UFPs is the brain, as these particles can induce adverse effects either directly by infiltrating the brain or indirectly through systemic inflammation [17–19]. According to a recent study, these particles accumulate in the hippocampus and other memory-related brain areas [20]. Additionally, studies on the translocation of nanomaterials suggest that these particles have the potential to cross the placental barrier and enter the developing foetal brain [21, 22]. Both animal- and population-based studies have linked PM exposure to adverse brain outcomes [23, 24]. Examples of altered performance measures are reduced working memory [25], learning [26, 27], attentiveness [28], and altered behavioural regulation [29]. PM exposure during crucial periods of perinatal life may also affect (neuro)development. An increase in *in utero* exposure to $PM_{2.5}$ has been linked with a decrease in the levels of brain-derived neurotrophic factor (BDNF), a pivotal growth factor essential for brain and nervous system maturation [30]. Long-term exposure to $PM_{2.5}$ has been associated with an increased risk of depression [31, 32], and several studies have suggested that general PM exposure may contribute to the incidence or worsening of neurodegenerative diseases such as dementia [33–35], Parkinson's disease [36], and multiple sclerosis [36]. However, the distinction in central nervous system (CNS) effects between pre- and early postnatal exposure periods is not well understood.

Although UFPs and the carbonaceous fraction present within PM_{10} and $PM_{2.5}$ are considered particularly hazardous [37] due to their ability to cross biological barriers and potentially induce oxidative stress and inflammation, their specific contributions to brain health effects during key developmental windows have not been studied. Therefore, we performed an experimental mouse study to investigate the potential neurodevelopmental effects of exposure to clean ultrafine carbon particles (UFP^C), i.e. in the absence of associated chemical constituents, such as metals and organic species. Pregnant wild-type C57BL/6J mice and their offspring were either sham-exposed (HEPA-filtered air) or exposed to UFP^C (450 $\mu g/m^3$) for four hours per day. The exposure consisted of two sets of two consecutive days for prenatal exposure and two sets of four consecutive days for postnatal exposure, resulting in four groups (control, prenatal, postnatal, and pre- and postnatal exposure). Our aim was to study the impact of early life exposure on offspring's neurological reflexes and brain development. We hypothesise that early life exposure to UFP^C results in adverse neurological impacts on the central nervous system later in life. Neurodevelopmental behaviour was assessed throughout the first three weeks of the life of the offspring using a functional observation battery, including homing and open field tests.

Methods

Animals and breeding

Wild-type C57BL/6J OlaHsd mice were bred and maintained in an individually ventilated caging system (GM500 Mouse IVC Green Line, Tecniplast, UK) in a temperature-controlled environment (21–23 °C). All animals were kept on sterilised commercial softwood bedding with additional nesting material under a 12-hour light/dark cycle (lights on from 4 AM to 4 PM) and had *ad libitum* access to a standard diet and water in their home cage. A radio, which was playing music softly, provided background noise in the room.

Female F0-generation mice were group-housed with six animals per cage for at least two weeks until time-mating, which began at 16 weeks of age, whereas the males were housed separately. To synchronize the oestrous cycles, pairs of females were introduced to bedding from a male's cage containing pheromone secretions for two days. On the third night following pheromone exposure, females

were paired with a single stud male for mating. Mating occurred overnight, and the males were removed from the cages the following morning. Successful mating was confirmed in the morning by the presence of a vaginal plug, which marked gestational day 0 (GD0). Pregnancy progression was subsequently confirmed on GD8, based on a weight gain threshold of 0.7 g to 3.5 g [38]. The F1 offspring were all born within a 24-hour time window and were housed with their dam during lactation. All experimental procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the local ethical committee of Hasselt University for animal experiments (ID 202148B and 201864A1).

Exposure

The pregnant dams were divided based on weight and subsequently exposed whole body over GD 8, 9, 16, and 17 for 4 h/day to either HEPA-filtered clean air or target UFP^C concentration of 450 µg/m^3 (mean particle size ~ 55 nm), generated by spark discharge technology. No food or water was provided during the exposure to avoid oral particle intake. Visual signs of stress were assessed four times per exposure day, and the mice were returned to their home cages after the exposure. Prior to mating, F0 female mice were habituated to the exposure chambers by placing the mice in the exposure chamber for an increasing amount of time for several consecutive days until a four-hour period was reached without signs of stress. Birth was expected on GD 20, and the designated PND was 0. Offspring were randomly divided to receive additional postnatal exposure under the same conditions as previously described over PND 4–7 and 10–13. A total of four offspring groups were established, further referred to as the sham, prenatal, postnatal, and pre- and postnatal groups. A schematic illustration of the experimental timeline is shown in Fig. 1. During the exposure, the mice were held in separate wire mesh cages (26×15.5×18 cm) inside a temperature-controlled 200 L inhalation unit (85×64×37 cm) designed to deliver an evenly distributed exposure [39]. An electric discharge particle generator (VSP-G1, VSPARTICLE, Delft, The Netherlands) was employed to generate an aerosol of UFP^C from carbon electrodes through which 8 L/min nitrogen was flown. The output was mixed with 2.2 L/min oxygen and 10 L/min filtered, moisturized room air, which was directed to the exposure units. More information regarding exposure characterisation and monitoring

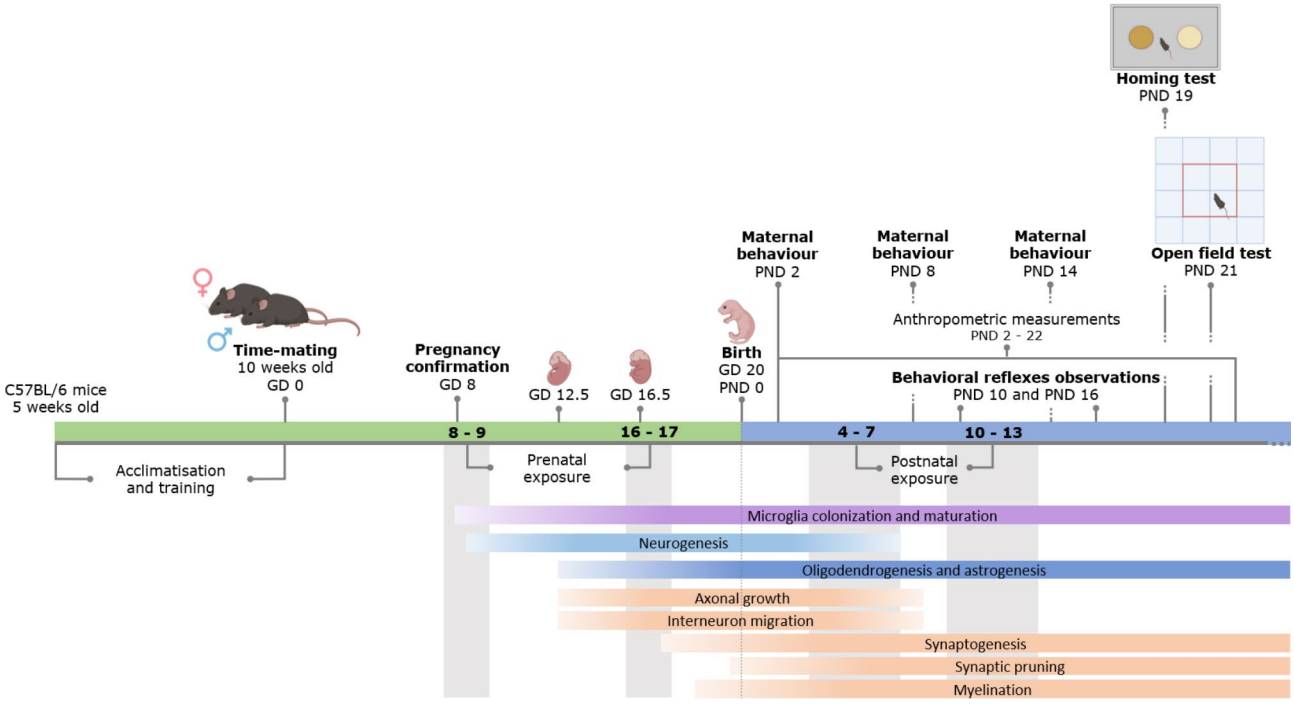

Fig. 1 Experimental timeline. The numbers represent the days, and the day of mating is designated GD 0. On GD 8, 9, 16, and 17, prenatal exposure to either HEPA-filtered air (0 µg/m3) or aerosolised UFP^C (450 µg/m^3) was delivered through maternal whole-body inhalation. Birth was expected at GD 20 and designated PND 0. An additional postnatal exposure was delivered to the offspring at PND 4–7 and 10–13, again to either HEPA-filtered air (0 µg/m^3) or aerosolised UFP^C (450 µg/m^3), resulting in 4 different offspring conditions: sham, prenatal, postnatal, and pre- and postnatally exposed mice. Ten offspring with an equal male-female ratio were subjected to behavioural tests. Anthropometric measurements of the mouse pups were performed between PND 2 and 22. A modified Fox test battery was performed, including righting, negative geotaxis, and cliff aversion on PND 10 and 16. Olfactory function was determined using the homing test at PND 19. The open field test was performed at PND 21. Abbreviations: GD = gestational day; PND = postnatal day; UFP^C = clean ultrafine particles

can be found in the supplementary information (Supplementary Fig. 1 (Additional file 1)).

Behavioural measurements

For all behavioural tests, a maximum of one pup per sex per litter was included to minimise litter effects ($n=10$ mice per group). Animals across different conditions were tested in a random order, and an observer who was blinded to the exposure status performed the tests. Maternal behaviour and behavioural developmental tests were performed during the light periods of the day, whereas the homing test and the open field test were performed during the dark periods when the mice were more active. During the lactation period, weight and length were determined every other day, starting from PND 2 and once more upon reaching adulthood at PND 90.

Developmental behavioural reflexes were evaluated on PND 10 and 16 using an adaptation of the Fox's test battery, which includes tests for ambulation, acoustic startle reflex, righting reflex, negative geotaxis, cliff drop avoidance reflexes, vibrissae placing response, forepaw grasping, and grip strength [40]. Ambulation: Crawling was scored during a 3-minute trial, with 0 indicating no movement, 1 indicating crawling with asymmetric limb movement, 2 indicating slow crawling with symmetric limb movement, and 3 indicating a complete transition to walking. Acoustic startle reflex: while free moving, a clicking sound is delivered approximately 4 cm above the pup's head, and the response to the sound is scored. Grading was performed as follows: 0 when no response was observed, 1 for a startle reaction, 2 if the pinna moved backwards, and 3 if the pup exhibited limb contractions. Righting reflex: pups were placed on their backs on the table surface and held immobile for 3 s. The time required for the pup to right-back onto four paws was recorded. Negative geotaxis: pups were placed face down on an incline of 45° and held immobile for 5 s. Next, the pup was released and the time and direction to face upward were recorded. Scores were given for this response and included 0 for falling down the incline or failing to turn, 1 for turning the head, 2 for turning the body up 90°, 3 for turning the body up 180°, and 4 for climbing up the slope. Cliff drop avoidance reflexes: the pup is placed on the edge of a surface so that the forepaws are dangling over the edge. Scoring was based on the average time needed to remove the snout and paws from the edge, as well as on predetermined scores. These included: 0 for remaining motionless or when it falls from the edge, 1 when it turns the head to the side, 2 when it turns the head aside, pulls back the forepaws, and puts them on the surface, and 3 when it initiates a backward movement. Vibrissae placing response: pups were held by their tail and the response to a lateral touch of the whiskers was scored. A score of 0 corresponds with the body curving to the belly, 1 if the pup raises its head, 2 if it extends one of the forepaws, and 3 if the pup grasps the tip of the stick. Forepaw grasping reflex: the pup was loosely held by the scruff of its neck and a small metal bar was used to stroke the forepaws individually. A score of 1 point was given per paw with which the mouse grasped. Grip strength: each pup was placed on a horizontally placed fiberglass screen wire, which was slowly inverted to 180°. The approximate angle of the screen was recorded when the pup fell off the screen. Mice were max. 20 min removed from their dam and held on heating pillows in between tests as long as possible. All tests were repeated three times and scores and times were averaged.

The pup homing test was performed on PND 19 to evaluate olfactory discrimination performance. Mice were transferred to a clean, empty cage for 30 min prior to testing. In the homing test, the animals were left to choose between 0.5 g of home cage or clean bedding in 90×15 mm petri dishes (one for each bedding material) placed in the left and right zones of a rectangular arena (42.5×26.5 cm). The position of the home cage bedding was alternated for each trial. Trials commenced in the centre of the arena and the animal was video recorded for 5 min. EthoVision XT software (Noldus Information Technology, The Netherlands) was used to track the time spent in the area containing nesting litter and the number of visits to the maternal zone.

The open field test was performed to observe exploratory behaviour and locomotion at PND 21. One mouse at a time was placed in the centre of a square open field arena (50×50 cm) and spontaneous behaviour was recorded for 15 min. The arena was carefully cleaned with an alcohol/water mixture after every test to minimise odour cues. Locomotion was tracked via EthoVision XT software (Noldus Information Technology, The Netherlands) and total distance moved (cm), mean velocity (cm/s), as well as time spent in the periphery or centre of the arena (s) were recorded.

Statistical analysis

Statistical analysis was carried out using commercially available GraphPad Prism software (GraphPad Prism 8, GraphPad Software Inc., USA). P-values <0.05 were considered statistically significant. In general, an ordinary one-way ANOVA was employed to compare means and standard deviation (SD) across conditions, followed by Dunnett's multiple comparison tests with the sham-exposed condition used as a control. Anthropometric data were individually analysed per day unless otherwise described. Statistical analysis of score-based behavioural evaluations and litter size was performed using the Kruskal-Wallis test. To account for variations across different testing days within the adapted Fox battery tests,

we corrected for batch effects (day of measurement) in the analyses. Assumptions for normality and equal variance were assessed by the Shapiro-Wilk normality test and the Brown-Forsythe test for heteroscedasticity. Non-normally distributed data were represented as median ± interquartile range (IQR).

Results

UFPC exposure characterisation

UFP^C exposure, including mass concentration, particle number concentration, particle size distribution, temperature, and humidity per exposure day, is closely monitored; details are provided in Supplementary Table 1 (Additional file 3). In summary, the target mass concentration of UFP^C exposure was set at 450 µg/m^3 on the basis of previously performed exposure studies documented in the literature [17, 41]. The actual mass concentration was, on average, 430 ± 59 µg/m^3 for prenatal exposure on GD 8, 9, 16, and 17 and 442 ± 82 µg/m^3 for postnatal exposure on PND 4–7 and 10–13. The average continuously measured particle number concentration was $5.1\times10^6 \pm 0.4\times10^6$ particles per cc and $4.6\times10^6 \pm 0.4\times10^6$ particles per cc for the prenatal and postnatal exposure periods, respectively. The count median diameter was chosen to be 55 nm to ensure the generation of particles within the ultrafine range (< 100 nm). The actual count median diameter (CMD ± GSD) was 49 ± 2 nm during the prenatal and postnatal exposure. The average size distribution graph of all exposure days is presented in Supplementary Fig. 2 (Additional file 2). The average temperature and relative humidity in the exposure chamber were 21.0 ± 0.4 °C in combination with 58 ± 1% and 27 ± 1 °C in combination with 48 ± 4% for the prenatal and postnatal exposure periods.

Effect of UFPC on anxiety-like behaviour in offspring mice

Postnatal UFP^C exposure induced anxiety-like behaviour in offspring mice. The open field test was conducted to determine general mobility and anxiety-like behaviour of the different exposure groups (n = 10 mice per group, 50% male). The test was performed at PND 21 or 8 days after the last exposure period. No significant changes in distance moved or velocity could be observed across the exposure groups (Fig. 2A & B). Additionally, exploration in the centre and borders of the arena was tracked to further assess anxiety-like behaviour. The results, as shown in Fig. 2C, indicate that the postnatally exposed mice spent significantly less time in the centre and significantly more time exploring the borders of the arena (centre: 318 ± 104 s, p = 0.01; border: 567 ± 120 s, p < 0.01) compared to the sham-exposed conditions (centre: 472 ± 91 s, border: 402 ± 73 s). The prenatal exposed and pre- & postnatal exposed groups did not show any significant altered exploration behaviour in comparison with the sham exposed group.

Effect of UFPC on olfactory function in offspring mice

No significant effect was observed on the olfactory function. The olfactory discrimination performance of offspring was evaluated on PND 19 in the homing test (n = 10 mice per group, 50% male). Overall, the mean time spent at the side of the home cage bedding did not significantly differ across the exposure conditions (Fig. 3A). In addition, the frequency or number of visits to the home cage bedding in the four exposure groups were also comparable (Fig. 3B).

Effects of UFPC on growth and developmental behavioural reflexes

The median number of pups per group was 6 (IQR 4.25) for the sham group, 7.5 (IQR 3.25) for the prenatal group, 8 (IQR 5) for the postnatal group, and 7 (IQR 2.5) for the pre- & postnatal group. No difference in body weight or body height was observed among the different exposure groups throughout the observed period from PND 2 until PND 90. The average pup weights were 1.7 ± 0.2 g (n = 37, 37.8% male), 1.6 ± 0.2 g (n = 44, 45.5% male), 1.7 ± 0.3 g (n = 34, 55.9% male), and 1.7 ± 0.2 g (n = 50, 44% male) at PND 2 for the sham, prenatal, postnatal, and pre- & postnatal exposed groups, respectively. Upon reaching adulthood at PND 90, the average weight was 23.5 ± 3.4 g for the sham exposure group, 23.2 ± 3.5 g for the prenatal exposure group, 23.0 ± 3.3 g for the postnatal exposure group, and 22.8 ± 3.5 g for the pre- & postnatally exposed groups (n = 10 mice per group, 50% male). The average body length increased from 33 ± 2 mm, 33 ± 2 mm, 34 ± 2 mm, and 34 ± 2 mm at PND 2 to 95 ± 3 mm, 95 ± 5 mm, 96 ± 4 mm, and 95 ± 5 mm at PND 90 for the sham exposed, prenatal exposed, postnatal exposed, and pre- & postnatal exposed groups, respectively. Furthermore, for all the exposure groups, the most important developmental milestones were achieved simultaneously, with the unfolding of the pinna at PND 4, followed by the observation of hair development at PND 12, and finally the opening of the eyes at PND 16.

No significant differences in developmental behavioural reflexes were detected between the exposure groups. A combination of nine different tests at two different time points, PND 10 (n = 4 mice per group, 50% male) and PND 16 (n = 10 mice per group, 50% male), were performed to evaluate reflexes. An overview of the results is provided in Fig. 4 and supplementary Table 2 (Additional file 4). While certain reflexes, such as hearing response, ambulation, cliff drop avoidance, and vibrissa placing response were either absent or in early development during the initial examination on PND 10, these

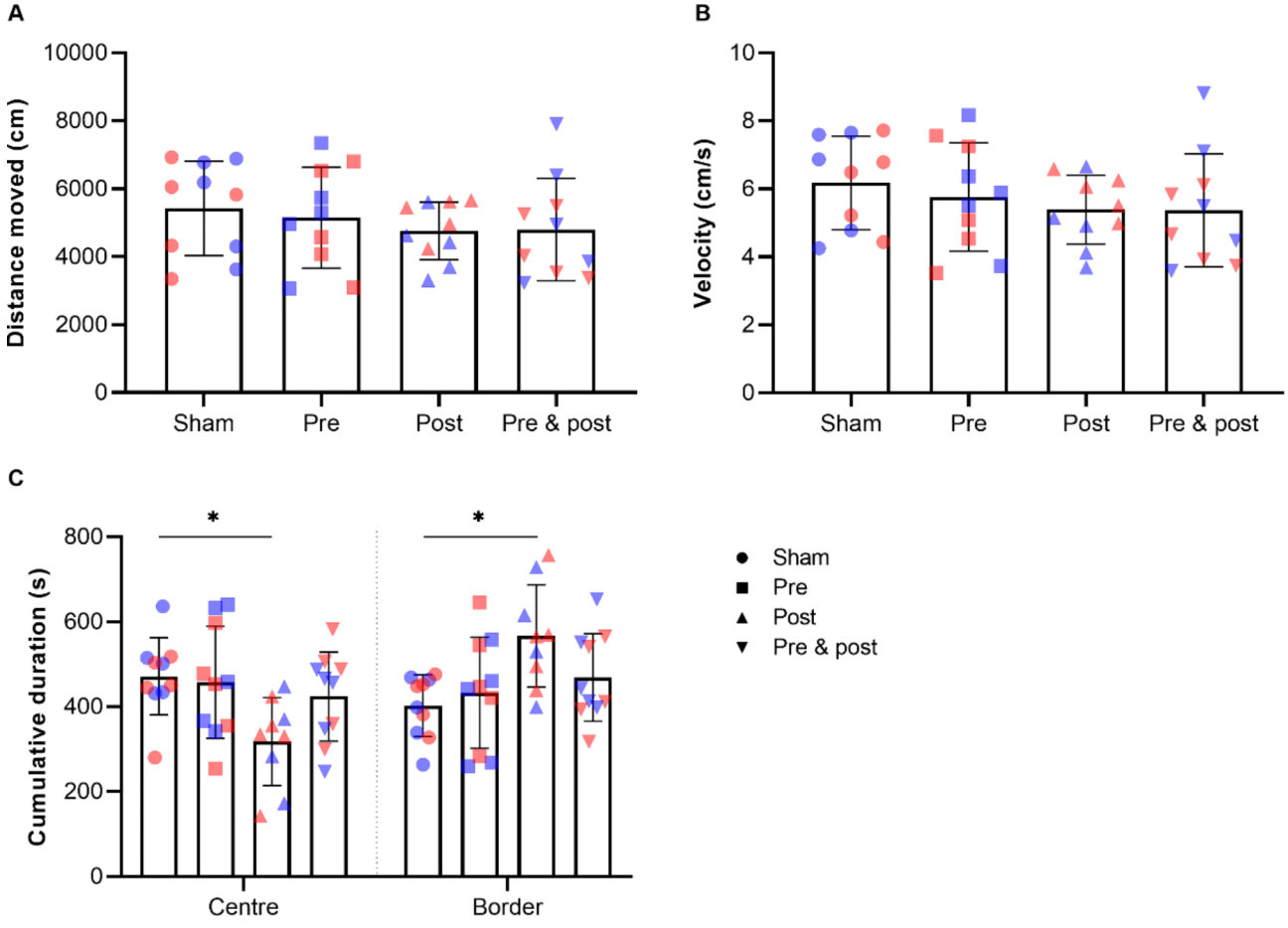

Fig. 2 Analysis of locomotion and anxiety-like behaviour after UFP^{C} exposure. Scatter dot plots showing the performance of offspring in the open field test across the different conditions (circles = sham group, squares = prenatal group, triangles = postnatal group, inverted triangles = pre & postnatal group) at PND 21. **A** – Total distance moved in centimetres during the 15 min test. **B** – Mean velocity of ambulation in centimetres per second. **C** – Time in seconds spent in the different observed zones of the arena including the centre and border. Bars indicate the mean ± SD. Ordinary one-way ANOVA was employed for all data followed by Dunnett's multiple comparison test, except for B where a Brown-Forsythe ANOVA was performed. α = 0.05, *$p < 0.05$. Transparent red symbols represent female individuals and transparent blue symbols represent male individuals ($n = 10$ per condition)

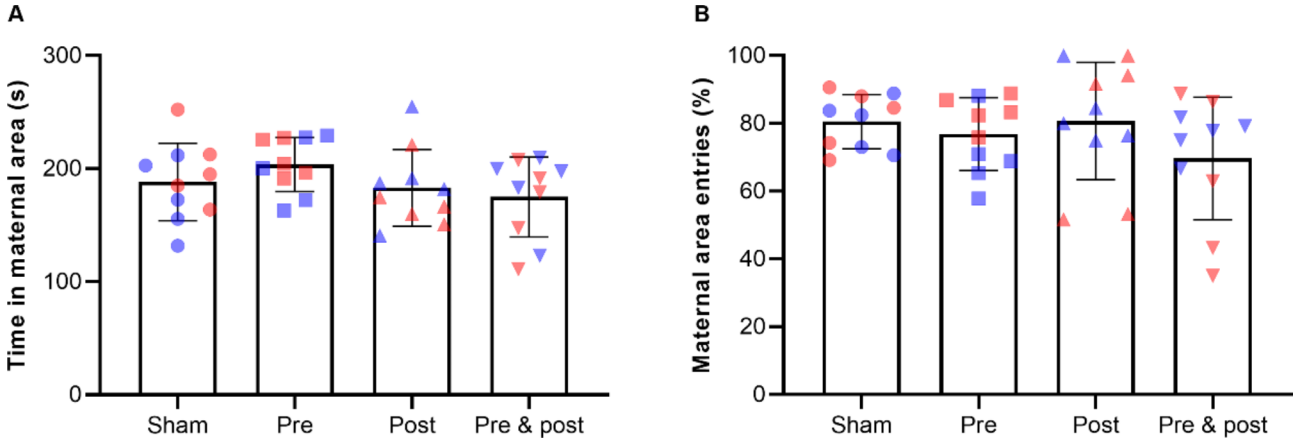

Fig. 3 Olfactory function after UFP^{C} exposure. Scatter dot plots presenting the performance of offspring in the homing test across the different conditions (circles = sham group, squares = prenatal group, triangles = postnatal group, inverted triangles = pre & postnatal group) at PND 19. **A** – Time in seconds spent in the zone with home cage bedding. **B** – The number of visits to the zone with home cage bedding. Bars indicate the mean ± SD. One-way ANOVA (α = 0.05) was employed. Transparent red symbols represent female individuals and transparent blue symbols represent male individuals ($n = 10$ per condition)

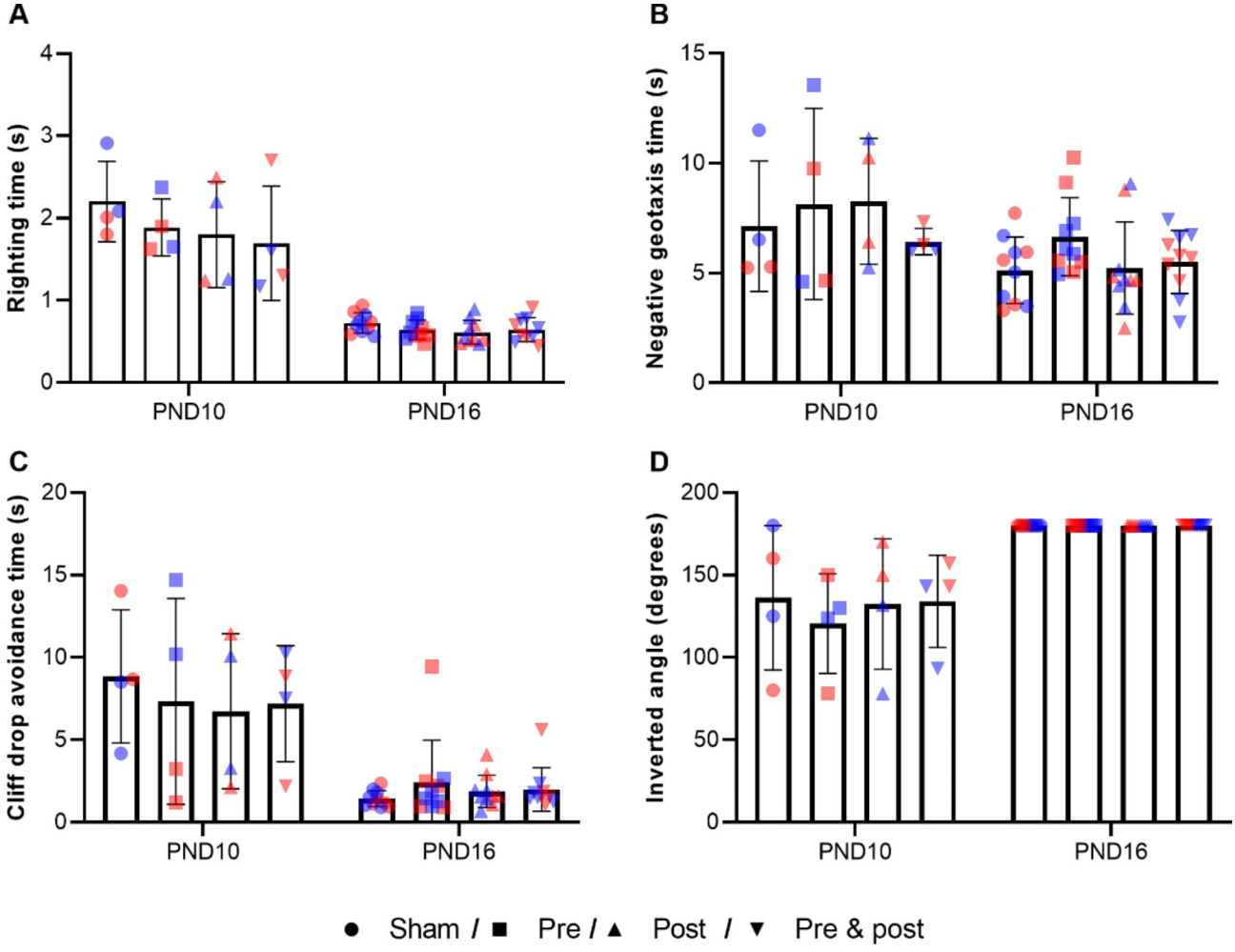

Fig. 4 Developmental behavioural reflexes assessed using adapted Fox battery tests. Scatter dot plots depict the performance of offspring in behavioural developmental tests across different conditions (circles = sham group; squares = prenatal group; triangles = postnatal group; inverted triangles = pre and postnatal group) at PND 10 and PND 16. **A** – Time in seconds required to right through 180°. **B** – Time in seconds to turn upwards in the negative geotaxis test. **C** – Time in seconds required to remove the snout and paws from the edge. **D** – Angle in degrees at which mouse pups fall off the inverted screen. Bars indicate the mean ± SD. One-way ANOVA (α = 0.05) was employed for the data shown. Transparent red symbols represent female individuals and transparent blue symbols represent male individuals ($n = 4$ per group at PND 10; $n = 10$ per group at PND 16)

reflexes further developed equally across all exposure groups by PND 16.

Discussion

Exposure to UFP^C during early postnatal development was associated with altered offspring behaviour in the open field test, whereas no effect of both pre and postnatal early-life UFP^C exposure was observed on the developmental behavioural reflexes of the examined offspring. Male and female mice responded similarly to UFP^C exposure, as indicated by the lack of sex-based variations in the behavioural responses to the various testing conditions. This study investigated the neurodevelopment of mice from birth until adulthood following repeated early-life exposures to mass concentrations of UFP^C (438 ± 72 µg/m³) exceeding average levels in busy cities in Europe. Notably, the concentrations used in our study are significantly higher than typical urban exposures, which in Central Europe range from an average of approximately 3.4 ± 2.3 µg/m³ to a maximum of 5.2 ± 2.8 µg/m³ [42]. Our 4-hour exposure setup approximates a daily average of about 70 µg/m³, which is approximately twenty times greater than the concentrations commonly found in urban settings. This study evaluates vulnerable windows of exposure as a proof of concept. Higher concentrations were intentionally selected to account for the shorter duration of pregnancy.

The exposure in this study spans crucial brain developmental windows, with the prenatal exposure covering neurogenesis, axonal growth, and interneuron migration and the postnatal exposure coinciding with oligodendrogenesis, astrogenesis, synaptogenesis, and synaptic

pruning, as shown in Fig. 1 [43]. The prenatal exposures, ranging from gestational day (GD) 8–9 and 16–17, occurred only partially outside the foetal growth phase, which covers the period from GD 14–17. Normal gestation in humans typically lasts for 38–40 weeks, whereas mice usually have a pregnancy of approximately 20–21 days. Overlapping the major stages of foetal development in both mice and humans results in a reproductive timeline for predicting reproductive and developmental toxicity in animals [44]. In both humans and mice, embryonic development starts with fertilisation at GD day 0, followed by the formation of the blastocyst at GD 4. Placentogenesis occurs between GD 6–14 in mice and GD 28–91 in humans [45]. This period ends with the conclusion of organogenesis at GD 14 in mice and GD 84–98 in humans, encompassing the first trimester in humans. The second trimester in humans lasts from GD 99–196, which is equivalent to GD 14–17 in mice and includes predominantly foetal and placental growth. An accelerated phase of foetal growth initiates at GD 197 in humans and GD 17 in mice, culminating in birth at GD 294 in humans and GD 21 in mice, marking the conclusion of the third trimester [46]. Changes in gestational timing can affect developmental exposure and outcomes, so timing must be carefully considered. However, since the offspring were born within a 24-hour period, indicating uniform growth, the impact is expected to be minimal. The use of a timed breeding model is one of the most reliable methods for obtaining age-matched embryos and pups [47]. Additionally, weight gain confirmed pregnancy progression, indicating successful mating and the health of the dams during gestation [38]. This consistency in birth timing and weight monitoring supports that the observed behavioural changes are linked to UFP^C exposure rather than gestational variability.

In the open field test, early postnatal exposure to UFP^C resulted in decreased activity in the centre and spending the most time in the borders. This behavioural pattern is termed thigmotaxic (i.e., to stay close to walls when exploring an open space), which is a well-established indicator of animal fear [48]. Reduced centre area exploration could potentially also be a sign of poor spatial recognition of mice. Therefore, even while our results point to a possible anxiety-like response, it is important to acknowledge that one behavioural test might not fully capture the variety of anxiety-related behaviours. To provide a more definitive measure of anxiety levels, future studies should use additional behavioural tests, for example the elevated plus maze. Although previous studies did not include whole-body exposure, clean UFP, or the early exposure windows used in our research, they might confirm a potential association between air pollution and anxiety in both rodents and humans. Neonatal rats intranasally exposed to $PM_{2.5}$ (2 or 10 mg/kg body weight, PND 3–15) presented a dose-dependent increase in anxiety-like behaviour in the elevated plus maze at PND 28 (i.e., another animal test to explore anxious behaviour) [49]. In an epidemiological study, exposure to traffic-related air pollution was significantly associated with anxiety symptoms in 12-year-old children [50], supporting the findings of the in vivo animal studies. Despite the ability of black carbon particles to cross the placental barrier and their observed presence in the foetal brain [12], foetal exposure may be mitigated by the toxicological defence mechanisms of the mother and the placenta, which can reduce the bioavailability of harmful substances to the developing foetus. Consequently, the less pronounced effects observed with prenatal exposure may arise from maternal or placental modulation of exposure levels rather than timing alone. Notably, the postnatal exposure duration was twice as long compared to the prenatal exposure, which may explain the observed anxiety effects in the postnatal group but not in the prenatal group, with the difference in dose serving as a potential explanation. Future research should quantify exposure levels during both phases to better understand the effects of timing versus dosage on behavioural outcomes related to air pollution exposure. The mice exposed to UFP^C both pre- and postnatally did not show the altered behaviour observed in the postnatally exposed group during the open-field test. This may suggest that prenatal exposure, particularly regarding its dose or timing, programs adaptive postnatal plasticity, potentially affecting future responses to environmental stressors [51]. Prenatal exposure to UFP^C or stressors may adjust developmental pathways, potentially enhancing resilience or sensitivity to later exposures [52]. For instance, early immunological sensitization may alter future inflammatory responses. Exposure to air pollutants during specific gestational periods has been shown to shift lymphocyte distribution in cord blood: early exposure increases T cells while decreasing B and NK cells, with the reverse effect observed for late exposure [53]. Prenatal UFP^C exposure can also prime CNS cells, like microglia and astrocytes, creating immunological memory [54] and inducing long-term changes in endocrine systems, such as the stress-response axis, which may affect postnatal behaviour and physiology. These adaptations, along with epigenetic modifications of the offspring, could influence neural development related to stress, cognition, and emotion [51]. However, additional research is needed to clarify how early-life UFP^C exposure influences long-term brain function and behaviour, especially regarding how prenatal environmental programming shapes postnatal sensitivity and resilience.

One of the hypothesised ways in which PM can affect brain functioning is through the translocation of the UFP fraction to the brain. Anxiety modulation has been linked

to the hippocampus, which modulates anxiety levels in response to a range of stressful situations by interacting with several brain regions, including the prefrontal cortex, amygdala, hypothalamus, and nucleus accumbens [55]. A recent study revealed increased levels of black carbon in both the prefrontal cortex and hippocampus [20]. The presence of these particles could alter local regulation within the hippocampus and stimulate increased neuronal excitability. Several suggestions have been proposed to explain the link between air pollution and anxiety. Power et al. reported that inflammation and oxidative stress, induced by PM may trigger or exacerbate anxiety symptoms in humans [56]. Moreover, there are simultaneous observation of anxiety-related behaviour and oxidative stress in the brains of rodents [57–59]. Neurotransmitter signalling pathways and neurotrophic factors seem to be potential targets of PM [60, 61]. Early-life exposure to diluted diesel exhaust (171 μg/m³, 8 h/day, GD 2–16) resulted in altered levels of dopamine and noradrenaline [62]. Dysfunction of these neurotransmitters and their receptors is known to result in mood disorders like anxiety [63]. A further study with more focus on neurotransmitter signalling is warranted to further elucidate the underlying mechanisms of UFP^C-induced anxiety.

The lack of altered behaviour during the homing test suggested that the mice's olfactory performance remained intact. Olfactory deficits, a known defect linked with air pollution exposure, can also cause anxiety-like behaviours in mice [64]. It has been postulated by previous research that air pollution can trigger hyposmia (i.e., a reduced ability to detect odours). Hyposmia typically manifests as the loss of olfactory sensory neurons in the olfactory bulb, the brain area that is primarily targeted following inhalation of PM through the nasal route. A case-control study revealed that long-term $PM_{2.5}$ exposure is linked to hyposmia [65]. Similar findings were observed in animal studies, with Hernandez et al. reporting olfaction latency in mice exposed to PM [66]. In addition to olfactory dysfunction, inadequate maternal care may lead to the development of anxiety-like behaviour in offspring. While there is limited research on the relationship between air pollution exposure and maternal care, air pollution exposure and how exposures are conducted contribute to maternal stress, which could affect maternal care in mice [67]. Exposure of the offspring to such early life stress could be a predictor of mental health disorders later in life [68]. Because we did not observe differences in maternal care across the different exposure groups, these results further support the findings that the observed anxiety-like behaviour was caused by postnatal exposure to UFP^C (Supplementary Fig. 3 (Additional file 5)). Moreover, these mice did not receive prenatal exposure, which also rules out the possible effect of maternal stress due to air pollution exposure.

In the current study, we could not directly observe an effect on birth weight. Important to note is that the first weight and length observation in this study was performed two days after birth to prevent nest distribution, which could influence the baseline maternal behaviour observation. This delay in time could be sufficient so that any presence of low birth weight was no longer detectable, especially in mice, which present a high growth rate after birth in comparison to that of humans [46]. Nevertheless, both epidemiological studies, as well as experimental animal studies, showed a link between inhalation of $PM_{2.5}$ exposure during pregnancy, preterm birth, and low birth weight [69, 70]. In addition, a study performed by Blum et al. observed a reduction in birth weight of approximately 10% in mice exposed to $PM_{2.5}$ during any gestational period other than the foetal growth phase [44]. Moreover, prenatal exposure to ultrafine particles does not appear to directly affect maternal health or overall pregnancy results, as seen by the lack of substantial differences in litter size across the exposure groups. This research suggests that while prenatal exposure may cause behavioural issues in offspring, it does not always have noticeable effects on maternal gestational performance. This lack of impact can be further explained by the timing of the first exposure, which occurs after blastocyst implantation at gestational day 4 [71].

Early-life exposure to UFP^C did not result in disturbed behavioural reflex development over time, or at least no significant differences were detected in righting, negative geotaxis, or cliff aversion. Examining behavioural reflex development following exposure to air pollution during critical windows of brain development allows the assessment of potential long-term impacts on cognitive function, learning ability, and emotional regulation. These tests are often employed to investigate early complications of CNS disease and neurodevelopmental disorders, such as autism [72]. One of the neurological responses assessed was the vestibular system of the inner ear. Interestingly, in a recent publication, the risk of developing Menière's disease was significantly correlated with various air pollutants, including PM_{10} [73]. Besides, a substantial body of evidence is available that provides links between early-life PM and neurodevelopmental disorders in childhood or later in adulthood. For example, it has been found that exposure to PM_{10} during the first three years of life is associated with an increased risk of autism spectrum disorders [74]. Similarly, as in humans, Sprague-Dawley rats exposed to $PM_{2.5}$ in the early postnatal period displayed typical behavioural features of autism, including poor social interaction, novelty avoidance, and communication deficits. Interestingly, Li, Kang et al. reported that microglia and astrocytes are activated in the rat brain, further supporting the link discussed earlier between glial activation and neurodevelopmental

defects [75]. In our study, we did not observe behavioural developmental deficits. These findings are in line with the observations by Schaffer et al., confirming that neonatal exposure to UFP^C does not appear to induce neurodevelopmental behavioural changes [27]. It can be argued that the effects of black carbon exposure on neurodevelopment are caused mainly by the surface composition of black carbon, which includes organic compounds, PAHs, heavy metals, and other trace elements. Most of the constituents that can be found on the surface of such a black carbon particle have been proven to induce cellular toxicity, oxidative damage, inflammation, mitochondrial interference, and hormone dysregulation [10, 76]. This study was set up to investigate the exposure effect of the pure carbon core without the additional components bound to its shell. This could contribute to the reduced neurotoxicity of these particles observed in the current study.

Our study assesses sensitive windows of exposure and offers valuable information about how UFP^C exposure affects behaviour, especially the anxiety-like reactions seen in the open field test after postnatal exposure. Its strengths include a robust design with clearly defined exposure windows during critical neurodevelopmental periods and the use of a timed breeding model to ensure uniform developmental stages. However, limitations include reliance on a single behavioural test, which may not fully capture the range of anxiety-like behaviours, and the lack of quantified exposure levels during both prenatal and postnatal phases.

In summary, we detected altered offspring behaviour in the open field test, indicative of anxiety-like behaviour after postnatal exposure to UFP^C (438 ± 72 $\mu g/m^3$; 49 ± 3 nm), whereas no such effects were observed with only prenatal exposure or combined prenatal and postnatal exposure. Our work also revealed that inhalation of UFP^C during early life did not have exposure-related effects on developmental behavioural reflexes or olfactory performance. In conclusion, these findings support the hypothesis that early-life exposure to UFP^C results in developmental neurotoxicity, which makes it plausible that it results in complications for the central nervous system later in life. Possible adaptive responses with respect to anxiety-like behaviour explain the lack of response in mice that were exposed both pre- and postnatally. The present data will aid future investigations examining the potential effects and features of UFP-based toxicity.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions

M. P., K.V., and H.B. conceptualised the study. K.V., L. R., and M. V. performed all the measurements. T. V. provided assistance and feedback with the animal work. F.R.C. and R.P.F.S. UFPC exposure approach. F. R. C., J. B. P. F. and E. D. set up, performed and provided technical support with the exposure. All the authors contributed to the important intellectual content during manuscript drafting or revision. All the authors read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available upon request from the corresponding author.

Declarations

Competing interests

The authors declare no competing interests.

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