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Postpartum maternal exposure to predator odor alters offspring antipredator behavior, basal HPA axis activity and immunoglobulin levels in adult Brandt's voles

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ABSTRACT

Predation risk can program offspring behavior, physiology, and fitness through maternal effect, but most studies have mainly focused on this effect during pregnancy; little is known about the effect of postpartum predation risk on offspring's phenotype. Here, we compared the antipredator behaviors of adult offspring (approximately 90 days old) produced by female Brandt's voles (*Lasiopodomys brandtii*) exposed to one of three treatments: cat odor (CO), rabbit odor (RO), and distilled water (DW) for 60 min daily from postpartum day 1–18. Basal levels of plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT), hypothalamic corticotrophin releasing hormone (CRH), as well as spleen immunoglobulins (IgA, IgM, and IgG) were also measured. Our data showed that the offspring of CO-exposed mothers displayed less head-out behavior to acute 15-min CO exposure, and female offspring showed more freezing behavior. CO offspring showed significantly lower basal ACTH and CORT levels than the RO and DW offspring. Additionally, female but not male CO offspring had higher hypothalamic CRH expression and spleen IgG levels than controls, showing a sex-specific effect. These findings demonstrate that postpartum maternal predator risk exposure promotes a passive-avoidant response to these cues in adult offspring, showing a cross-generational maternal effect of postpartum predation risk. Further, these changes may be associated with alterations in the hypothalamic-pituitary-adrenal axis and immune function.

1. Introduction

A young animal's behavior and physiology are shaped not only by genetic factors but also by a variety of parental and environmental factors during the prenatal and postnatal periods [1]. It is now well established that maternal environmental cues can be transferred to the offspring, consequently altering their phenotypes and potentially increasing their competitiveness in natal environments. For example, water fleas (*Daphnia cucullata*) born of mothers prenatally exposed to predator chemical cues grow larger helmets and survive better than offspring of mothers in a control environment [2]. Offspring of High, Mid, and Low licking/grooming rat dams exhibit levels of licking/grooming highly correlated to the behavior exhibited by their mothers [3–5]. Such maternal effects are of key importance as they can constitute a major source of transgenerational phenotypic plasticity in response to environmental heterogeneity by affecting offspring development and ultimately fitness [6].

Predation risk is one of the most important ecological factors. During reproduction, parents may alter current reproductive efforts in response to the present environmental predation pressure [7]. Predation risk can also trigger transgenerational responses in the prey and ultimately influence the phenotype and fitness prospects of prey offspring. For example, mothers prenatally exposed to predators produce offspring with greater immobility in crickets [8], tighter shoaling behavior in sticklebacks [9], longer tails in common lizards [10], and greater wing length in great tits [11]. In laboratory settings, prenatal predator odor exposure enhances anxiety-related behavior and defensive and stress responses in mice and rats [12,13]. Under high predation pressure, the

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induction of fearfulness, anxiety, and reduced locomotor and exploratory behaviors can promote offspring survival [14]. These prenatal maternal effects may represent a flexible mechanism of developmental programming for antipredator defense [15].

Indeed, maternal effects during the postpartum period, given the length and intricacy of mother-infant interactions, are equally important for mammalian offspring [16]. Maternal separation in rodents, an type of early postpartum stress, can have profound and sex-dependent effects on the adult phenotype of the F1 [17–19], and F2 offspring generations [20]. A low dose of corticosterone (CORT) administered to rat dams through the drinking water reduces anxiety, improves learning and stress coping, and protects the adult offspring against ischemia [21,22]. One study in rats investigated the effect of repeated cat odor (CO) exposure during the early postpartum period on juvenile anxiety-like behavior in the open-field task and some sex-specific effects were found [23]. Ayers and colleges extended this to another predator odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT, derived from the anal secretions of the red fox), to show that TMT-exposed pups emitted more ultrasonic vocalizations and displayed less freezing to TMT at postnatal day (PND) 30 [24]. In these studies, as offspring were directly exposed to predator risk together with the dams, researchers were unable to elucidate whether the behavioral changes in the offspring were caused by postpartum maternal effects induced by predator risk or direct neonatal exposure. Thus, we examined the postpartum maternal effects of predation risk on offspring phenotype with only dams exposed to predator stress.

Brandt's voles (*Lasiopodomys brandtii*) are social herbivorous rodents widely distributed in the Inner Mongolian grasslands of China, Mongolia, and Southeast Baikal region of Russia, where they often dig complex burrow systems [25,26]. They are considered pests because of the serious damage to grassland vegetation and grazing crops [27]. They are an important prey species for a wide range of carnivores, from foxes, polecats (*Mustela eversmanii*), and Pallas' cats (*Otocolobus manul*) to saker falcons (*Falco cherrug*), upland buzzards (*Buteo hemilasius*), and steppe eagles (*Aquila nipalensis*) [28]. A previous study in our laboratory indicated that, among its predators, Brandt's voles exhibit the strongest fear and defensive responses to cat (*Felis catus*) and weasel (*Mustela sibirica*) urine and feces [29]. Repeated CO exposure during pregnancy has been demonstrated to influence the reproductive output, offspring quality, and behavioral phenotypes in Brandt's voles [30,31]. CO exposure during the postpartum period negatively affected the maternal behaviors of Brandt's voles when only the dam was exposed to cat urine and increased locomotor activity of juvenile offspring [32]. Whether the disruption of maternal behavior induced by this postpartum maternal stress affects the offspring's antipredator behavior and this postpartum maternal stress has cross-generational effects are not clear.

Using this model, we compared the postpartum maternal effects of predation risk on offspring behavior using three different olfactory cues: CO, rabbit odor (RO, a non-predator odor) and distilled water (DW, as an unscented control). Adult offspring were behaviorally assayed with exposure to these three cues. We expected that the postpartum cross-generational maternal effect induced by predation risk would increase offspring antipredator responses. Additionally, to analyze its potential mechanism, the long-term programming of the hypothalamic-pituitary-adrenal (HPA) axis was also examined, including plasma adrenocorticotropic hormone (ACTH) and CORT levels, and hypothalamic corticotropin releasing hormone (CRH) expression. As the mammalian immune system is often linked to the endocrine and nervous systems [33], we also examined the levels of antibodies (including IgA, IgM and IgG) in the spleen, as it is crucial for immune function.

2. Materials and methods

2.1. Animals

Brandt's voles used in this experiment were from a laboratory breeding colony at Yangzhou University. Animals were housed in 46 cm × 35 cm × 19 cm polycarbonate cages containing wood shavings as bedding material, under a 12:12 h light: dark cycle (lights on at 7:00 am). Room temperature was maintained at 22 ± 3 °C. Food and water were available *ad libitum*. Before being paired for breeding, animals were housed in same-sex pairs; however, females were housed individually while pregnant. Starting on the estimated expected parturition date, dams were checked daily for litters. The day when pups were found in the nest was designated as postpartum day (PP) 0. Offspring were separated from their mother at PND 21 and housed in same-sex sibling pairs. Experiments were conducted during the light cycle. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China and reviewed by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Yangzhou University (IACUC No, SJXY-1).

2.2. Odor sources

Cat and rabbit urine, but not feces, were used to ensure sufficient odor resources and better quantitation. The basic procedure for urine collection was described previously [30–32]. Cat urine and rabbit urine were taken from an adult male domestic cat and an adult male rabbit (*Oryctolagus cuniculus*), respectively. They were housed in 120 cm × 40 cm × 30 cm wire cages in different rooms and provided with water and food *ad libitum*. In order to eliminate variations among collecting sessions, urine was collected only once from a clean tray placed under the cage for 48 h. It was subsequently filtered out to remove feces or fur and stored at −20 °C for further use. Before exposure, the urine was thawed and diluted with four volumes of DW. DW also served as control, while cat and rabbit urine were used as predator and non-predator odor sources, respectively.

2.3. Experimental procedure

The basic experimental design was identical to that described in our previous study [32]. In brief, 24 postpartum females (n = 8 for each treatment) were randomly assigned to one of three groups: CO, RO and DW groups. These females were individually exposed to cat urine, rabbit urine, and DW, in an exposure apparatus (75 cm × 37 cm × 40 cm), including a hide box (15 cm × 37 cm × 40 cm) constructed using black Plexiglas. At PP1, females were separated from their pups, transported to the exposure room, and placed in the apparatus for 15 min. A dish with approximately 10 g of their diet and another containing a cotton ball (5 cm in diameter) soaked with 1 mL of either diluted urine or DW were then introduced in the apparatus. The females were exposed to these conditions for 60 min before being returned to their home cage. The apparatus was cleaned with a 70 % alcohol solution and dried after each test. These procedures were repeated for 18 consecutive days. Following these procedures, the animals were maintained as normal. Offspring were weaned and separated from dams at PND 21. Experimental naïve offspring (no more than two males and two females; CO: n = 24; RO: n = 24; DW: n = 24) were randomly selected from each litter and used for the behavioral and neuroendocrinological tests in adulthood.

2.4. Antipredator behavioral test

At approximately PND 90, the offspring (12 females and 12 males per group) were each subjected three times to the antipredator behavioral test. The subject was randomly exposed to DW, RO, or CO with a 3-day interval between each test to eliminate interference of the former test to the later test. The basic procedure was identical to that of the maternal exposure to odor. Subjects were placed individually into the apparatus and their behavioral responses were recorded for 15 min using a digital video camera. All behaviors were analyzed using the event-recording program BORIS (www.boris.unito.it) by a trained observer blind to

treatment groups. The following behaviors were defined [34,35]: sniffing odor sources, freezing (complete immobility except for respiration), jumping (the vole stands in its rear legs, raises the forelimbs, and then jumps), avoidance (the vole spends most of the time in the square furthest from the odor sources in front of the hide box), concealing (the voles retreats into the hide box and cannot be seen), head-out (the vole has its head or both the head and shoulders outside the entrance of the hide box with most of its body concealed inside the hide box), vigilant rearing (the vole stands on its rear legs with forelimbs raised, i.e., not placed on anything for support), self-grooming, and others (vole behaviors other than those already mentioned). The apparatus was cleaned and deodorized with a 70 % alcohol solution after each test. To avoid the influence of the female estrus cycle on behavioral performance, the estrus stage of adult females was checked by taking vaginal smears after each behavioral test, and the stage determined by examining cell type composition. Females in estrus or proestrus were excluded from data analysis.

2.5. ELISA for plasma ACTH and CORT concentrations

At approximately PND 102, 3 days after the last behavioral test, the subject voles were sacrificed by rapid decapitation with collection of trunk blood for measurement of plasma hormones. The collection procedure was completed within 3 min of first disturbing the voles to ensure that hormone levels reflected a resting and undisturbed state. Plasma samples were stored at −20 °C until the assay. Plasma ACTH and CORT levels were measured using vole-specific ELISA kits (JL21590 and JL21595, Jianglai Biological Science and Technology, Shanghai, China) according to manufacturer's instructions. The resultant absorbance was measured at 450 nm using a Metertech microplate reader (BioTek Instruments, Winooski, USA) after the reader was zeroed using the blank well. The concentration of each sample was extrapolated from a standard curve. Each determination was performed in duplicate, and the variation between duplicate measurements was less than 5%.

2.6. Western blotting for hypothalamic CRH expression

After blood collection, the brains were quickly removed and frozen in dry ice. Coronal sections were cut in a cryostat and bilateral tissue pinches were taken from the entire 1-mm-thick hypothalamic section. Total proteins were extracted with RIPA lysis buffer containing protease inhibitor, and sample protein concentrations were determined with BCA assays (E112-01/02, Vazyme Biotech Co. Ltd. Nanjing, China). The denatured protein extract was separated using a 10 % SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The membrane was incubated with rabbit polyclonal anti-CRH (1:1000, 10944-1-AP; Proteintech, China) or anti-β-actin antibody (1:5000, bs-0061R; Bioss, China) at 4 °C overnight. Following washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (bs-0295G-HRP, 1: 5000; Bioss, China). Finally, protein bands were visualized using an enhanced ECL kit and the Tanon 5200 Chemiluminescent Imaging System (Tanon, Shanghai, China). Quantification was performed using ImageJ software, and all signals were normalized to the β-actin run on the same membrane.

2.7. ELISA for spleen antibody concentrations

Spleens were homogenized in chilled reverse osmosis pure water. Cell debris was removed by centrifugation at 3000 rpm in an Eppendorf centrifuge for 20 min at 4 °C. The supernatant was collected, and immunoglobulin concentrations measured using vole-specific ELISA kits (JL49309 for IgA, JL49223 for IgM, JL46567 for IgG; Jianglai Biological Science and Technology, Shanghai, China) according to manufacturers' instructions.

2.8. Data analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All data were checked for normality using a one-sample Kolmogorov-Smirnov test. Behavioral and neuroendocrinological data were analyzed using multivariate analysis of variance (ANOVA), including the model's fixed effects (e.g., maternal treatment and sex). When only the significant main effect of treatment or interactions were found in the ANOVA, a Tukey's test of multiple comparisons was conducted for post-hoc analysis. If there were significant main effects of treatment and sex, but no interaction, Tukey's post hoc tests were used for comparisons between each treatment within the same sex, and independent sample t-tests for comparison between sexes within the same treatment. In case only the effect of sex was significant, we used t-tests to make comparisons between males and females. Partial eta square (η^2_p) and Cohen's d were used as measures of the effect size. Pearson correlation analysis was used to examine the relationships among behavioral responses to CO, plasma ACTH and CORT levels, hypothalamic CRH expression, and spleen immunoglobulin levels in adult offspring. All data were presented as mean ± standard error (SEM). Statistical significance was set at $p < 0.05$ (two-tailed tests).

3. Results

3.1. Behavioral responses to DW, RO, and CO in adult offspring

Being exposure to DW, there was a significant main effect of treatment on the duration of head-out [F (2, 66) = 3.472, p = 0.037, η^2_p = 0.095] and avoidance [F (2, 66) = 6.968, p = 0.002, η^2_p = 0.174], a main effect of sex on head-out [F (1, 66) = 4.095, p = 0.047, η^2_p = 0.058], and an interaction between treatment and sex on sniffing [F (2, 66) = 3.262, p = 0.045, η^2_p = 0.090], but not on other behaviors. Adult male offspring of CO-exposed mothers exhibited significantly less head-out behavior than those in the DW group (DW males: 8.3 ± 1.3 s; CO males: 2.6 ± 0.8 s; Turkey's test: p = 0.009, Cohen's d = 1.525) (Fig. 1F). RO offspring showed more avoidance than DW and CO offspring (DW: 3.1 ± 0.7 s; RO: 6.9 ± 1.5 s; CO: 1.9 ± 0.5 s; Turkey's test: RO vs. DW, p = 0.024, Cohen's d = 0.662; RO vs. CO, p = 0.002, Cohen's d = 0.922) (Fig. 1D). RO female offspring also displayed more sniffing than CO females (RO females: 52.9 ± 5.8 s; CO females: 25 ± 3.8 s; Turkey's test: p = 0.014, Cohen's d = 1.655) (Fig. 1A).

Being exposure to RO, there was no significant main effect of treatment, sex and interaction on behavioral performance. No difference was found among these groups ($p > 0.05$ in all cases) (Fig. 2).

Being exposed to CO, there was a significant main effect of treatment on the duration of head-out [F (2, 66) = 9.767, $p < 0.001$, η^2_p = 0.228], freezing [F (2, 66) = 6.732, p = 0.002, η^2_p = 0.169] and avoidance [F (2, 66) = 3.944, p = 0.024, η^2_p = 0.107], and an interaction between treatment and sex on freezing [F (2, 66) = 3.483, p = 0.036, η^2_p = 0.095], but not on other behaviors. Maternal CO experience increased the duration of freezing behavior in female offspring only (DW females: 15.5 ± 4.9 s; RO females: 3.9 ± 1.1 s; CO females: 64.9 ± 23.4 s; Turkey's test: CO females vs. DW females, p = 0.019, Cohen's d = 0.842; CO females vs. RO females, p = 0.002, Cohen's d = 1.061) (Fig. 3B). Adult offspring of CO- and RO-exposed mothers displayed significantly less head-out behavior than offspring of the control group (DW: 17.9 ± 3.3 s; RO: 5.7 ± 0.7 s; CO: 7.8 ± 0.9 s; Turkey's test: DW vs. CO: p = 0.002, Cohen's d = 0.842; DW vs. RO: $p < 0.001$, Cohen's d = 1.035) (Fig. 3F). RO offspring also showed less avoidance than DW and CO offspring (DW: 12.6 ± 1.7 s; RO: 5.6 ± 0.6 s; CO: 12.2 ± 2.8 s; Turkey's test: RO vs. DW, p = 0.037, Cohen's d = 1.094; RO vs. CO, p = 0.05, Cohen's d = 0.655) (Fig. 3D).

3.2. Plasma ACTH and CORT levels in adult offspring

Repeated CO exposure during the postpartum period changed basal

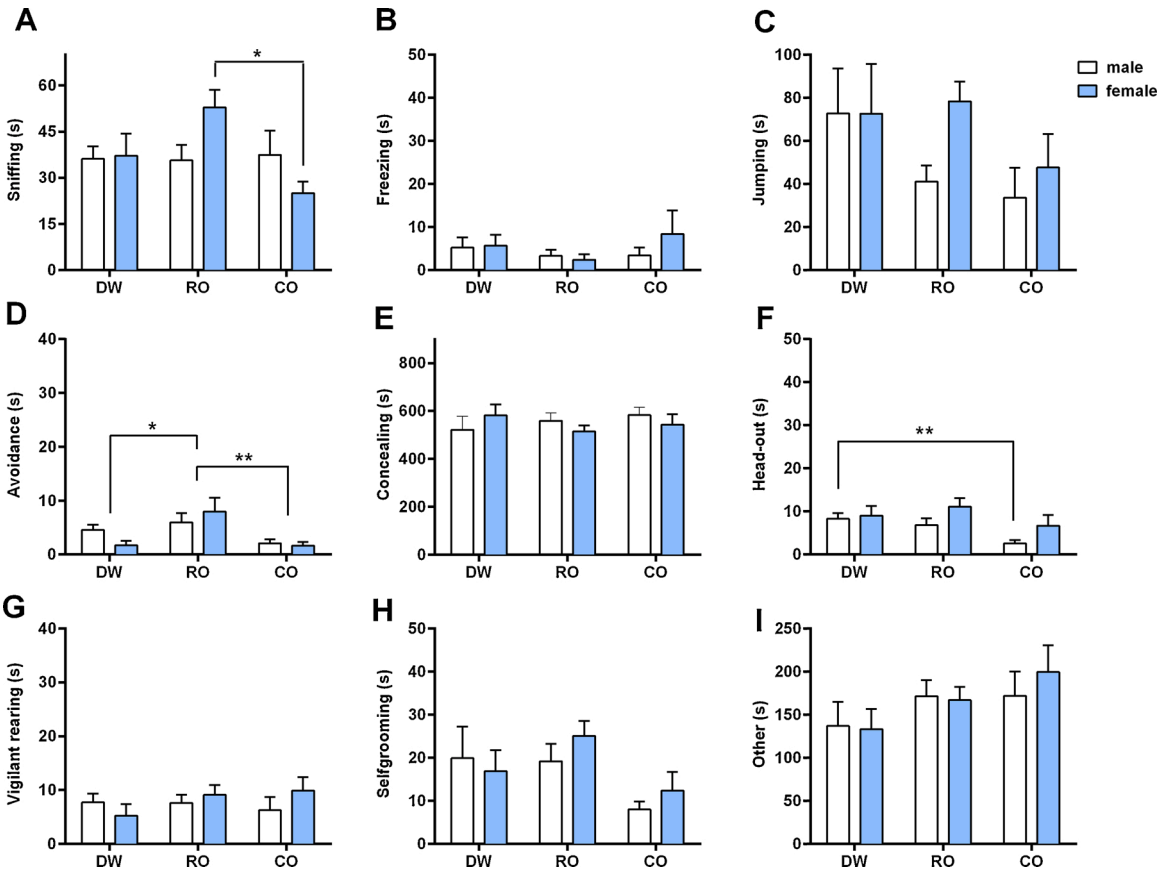

Fig. 1. Effect of postpartum exposure to distilled water (DW), rabbit odor (RO), and cat odor (CO) on offspring behavioral performances to acute DW exposure. **(A)** duration of sniffing (n = 12 in each group; two-way ANOVA with maternal treatment and sex as fixed factors; treatment: $F_{(2, 66)} = 2.541$, $p = 0.086$; sex: $F_{(1,66)} = 0.167$, $p = 0.684$; treatment × sex: $F_{(2, 66)} = 0.262$, $p = 0.045$; Turkey's post-hoc test: RO females vs. CO females, $p = 0.014$); **(B)** duration of freezing; **(C)** duration of jumping; **(D)** duration of avoidance (two-way ANOVA, treatment: $F_{(2, 66)} = 6.968$, $p = 0.002$; sex: $F_{(1,66)} = 0.133$, $p = 0.716$; treatment × sex: $F_{(2, 66)} = 1.457$, $p = 0.241$; Turkey's test: RO vs. DW, $p = 0.024$, RO vs. CO, $p = 0.002$); **(E)** duration of concealing; **(F)** duration of head-out (two-way ANOVA, treatment: $F_{(2, 66)} = 3.472$, $p = 0.037$; sex: $F_{(1,66)} = 4.095$, $p = 0.047$; treatment × sex: $F_{(2, 66)} = 0.612$, $p = 0.545$; Turkey's test: CO males vs. DW males, $p = 0.009$); **(G)** duration of vigilant rearing; **(H)** duration of self-grooming; **(I)** duration of other behaviors. Error bars are ± SEM. $^*p < 0.05$, $^{**}p < 0.01$: significant differences among the three exposure groups.

plasma ACTH and CORT levels in adult offspring. Using two-way ANOVA, we found a significant main effect of treatment on plasma ACTH [F (2, 66) = 3.086, $p = 0.05$, $\eta^2_p = 0.086$] and CORT levels [F (2, 66) = 4.47, $p = 0.015$, $\eta^2_p = 0.119$], with neither a main effect of sex, nor a significant interaction between the main factors. CO offspring showed lower plasma ACTH (DW: 45.9 ± 1.1 pg/mL; CO: 42.5 ± 0.8 pg/mL; Turkey's test: $p = 0.04$, Cohen's $d = 0.704$) and CORT levels (DW: 267.3 ± 5.8 ng/mL; CO: 243.8 ± 4.8 ng/mL; Turkey's test: $p = 0.02$, Cohen's $d = 0.892$) than DW offspring, and the plasma CORT was even lower than that of RO offspring (RO: 264.7 ± 7.1 ng/mL; Turkey's test: $p = 0.043$, Cohen's $d = 0.699$) (Fig. 4 A and B).

3.3. Hypothalamic CRH expression in adult offspring

Two-way ANOVA showed a significant main effect of sex [F (1, 42) = 7.951, $p = 0.007$, $\eta^2_p = 0.159$] and an interaction between sex and treatment [F (2, 42) = 5.827, $p = 0.006$, $\eta^2_p = 0.217$], but no main effect of treatment. Western blotting revealed that repeated CO exposure during the postpartum period changed hypothalamic CRH expression in a sex-specific way. Female offspring of CO-exposed mothers had significantly higher CRH expression than that of DW female offspring (DW females, 0.8 ± 0.1; CO females, 1.5 ± 0.2; Turkey's test: $p = 0.04$, Cohen's $d = 1.461$) (Fig. 4C). In CO offspring, females expressed significantly more CRH in the hypothalamus than males (CO males, 0.5 ± 0.1; Turkey's test: $p = 0.001$, Cohen's $d = 2.313$) (Fig. 4C).

3.4. Spleen index and immunoglobulin concentration in adult offspring

The spleen index was calculated as: 100 % × spleen weight/body weight. There was a significant main effect of treatment on the spleen index [F (2, 66) = 5.787, $p = 0.005$, $\eta^2_p = 0.151$], with no main effect of sex or an interaction between sex and treatment. Male and female offspring of CO-exposed mothers had a higher spleen index than RO offspring (Turkey's test: $p = 0.004$, Cohen's $d = 0.996$, Fig. 5A). As the spleen index is strongly influenced by differences in body weight, we also weighed adult offspring. There was only a significant main effect of sex on body weight [F (1, 66) = 57.467, $p < 0.001$, $\eta^2_p = 0.476$], with male voles being heavier than females (males: 54 ± 0.9 g; females: 43.1 ± 1.1 g; Independent sample t-test: $p < 0.001$, Cohen's $d = 1.706$) (Fig. 5B).

With respect to spleen IgA, ANOVA showed no main effect of treatment or sex, or a significant interaction. No significant difference was found in the spleen IgA of adult offspring ($p > 0.05$ in all cases, Fig. 5C).

However, there was a main effect of treatment [F (2, 66) = 3.751, $p = 0.029$, $\eta^2_p = 0.103$], but no effect of sex or significant interaction for spleen IgM. CO offspring had a significantly higher spleen IgM concentration than DW offspring (DW: 1.1 ± 0.1 mg/mL; CO: 1.3 ± 0.1 mg/mL; Turkey's test: $p = 0.05$, Cohen's $d = 0.632$) (Fig. 5D).

For spleen IgG, the ANOVA showed only an interaction between treatment and sex [F (2, 66) = 16.641, $p < 0.001$, $\eta^2_p = 0.339$], but no main effect of treatment and sex. Maternal CO exposure decreased the

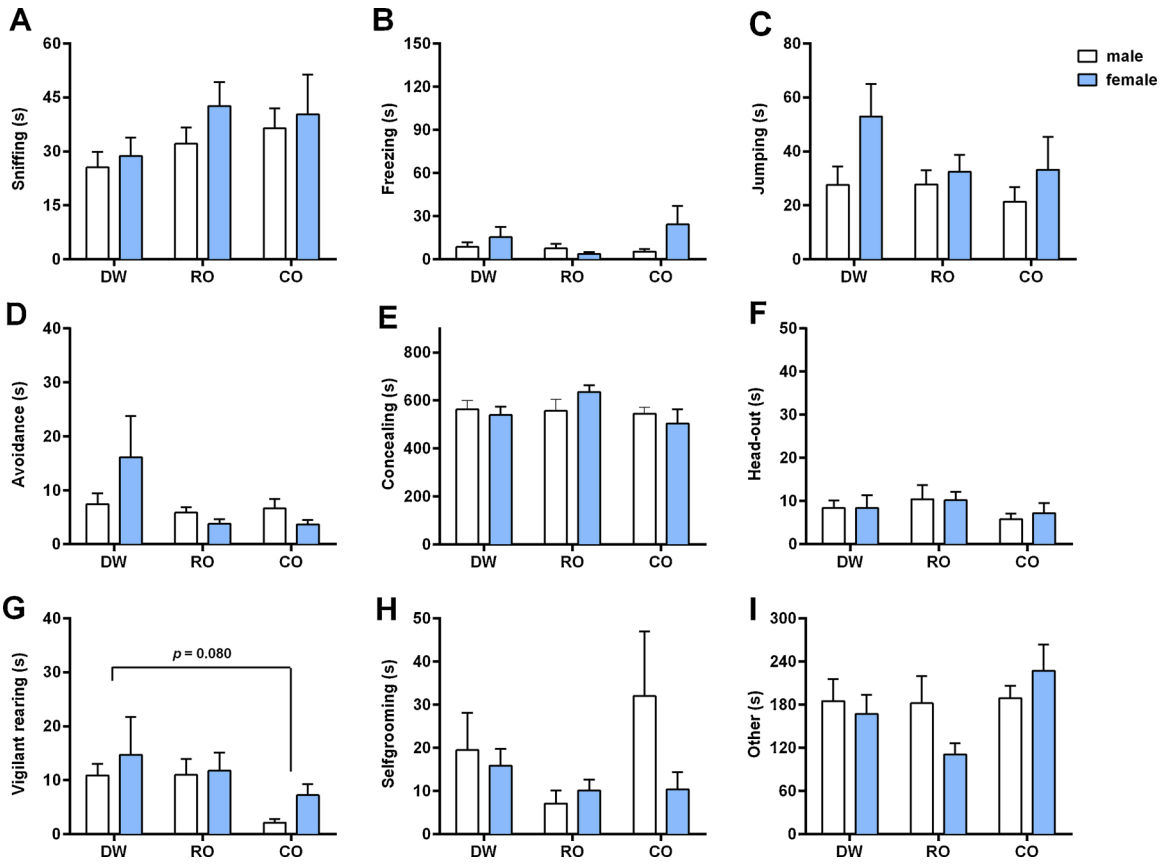

Fig. 2. Effect of postpartum exposure to distilled water (DW), rabbit odor (RO), and cat odor (CO) on offspring behavioral performances to acute RO exposure. **(A)** duration of sniffing; **(B)** duration of freezing; **(C)** duration of jumping; **(D)** duration of avoidance; **(E)** duration of concealing; **(F)** duration of head-out; **(G)** duration of vigilant rearing; **(H)** duration of self-grooming; **(I)** duration of other behaviors. Error bars are ± SEM.

levels of spleen IgG in male offspring (DW males: 9.3 ± 0.4 mg/mL; CO males: 6.5 ± 0.3 mg/mL; Turkey's test: $p < 0.001$, Cohen's $d = 2.088$), but increased them in females (DW females: 7.4 ± 0.4 mg/mL; CO females: 9.2 ± 0.5 mg/mL; Turkey's test: $p = 0.022$, Cohen's $d = 1.282$) (Fig. 5D). DW males showed a higher spleen IgG concentration than females (Turkey's test: $p = 0.013$, Cohen's $d = 1.478$), whereas the opposite was true for CO males (Turkey's test: $p < 0.001$, Cohen's $d = 1.839$) (Fig. 5E).

3.5. *Correlation between behavioral responses to CO, basal levels of HPA axis and spleen immunoglobulins in adult offspring*

To further explore the correlation between behavioral responses to acute CO exposure, plasma ACTH and CORT levels, hypothalamic CRH expression and spleen immunoglobulin levels, a Pearson correlation analysis was performed. The results showed a significant positive correlation of freezing and hypothalamic CRH expression (r = 0.384, $p = 0.007$), and a significant positive relationship between avoidance and plasma ACTH levels (r = 0.261, $p = 0.027$).

4. Discussion

Our recent study showed that postpartum maternal CO exposure disrupted the maternal behavior of Brandt's voles and increased the offspring's locomotor activity [32]. In order to evaluate the effects of maternal disruption induced by postpartum CO exposure on offspring antipredator behavior and to verify the cross-generational effect of this postpartum maternal stress, the present study examined the effects of CO exposure during the postpartum period on behavioral outcomes, HPA axis function, and spleen immunoglobulins in adult offspring. We found the following cross-generational effect: adult offspring of CO-exposed mothers spent less time in risk-assessment, as reflected by the decreased time spent in head-out behavior when exposed to CO; baseline ACTH and CORT levels were lower in the offspring of CO-exposed mothers; CO female, but not male offspring, showed more fear responses (indicated by more freezing behavior) to acute CO exposure. This behavioral response was associated with increased CRH expression in the hypothalamus, suggesting a sex-specific effect of maternal CO exposure. Interestingly, this effect was also shown by spleen antibody levels, with female offspring having increased spleen IgG levels, and males having decreased IgG levels. These results suggest that maternal predator exposure can improve negative avoidance response to subsequent predator cues, and male and female offspring differ in how they cope with these cues. These coping strategies may be associated with sex-specific alterations in the hypothalamic CRH and spleen IgG.

4.1. *Effects of postpartum maternal CO exposure on offspring antipredator behavior in adult Brandt's voles*

In general, prey animals are expected to make use of safe places to reduce their chances of encountering and being attacked by a predator. In our experiment, the hide box represented this safe place similar to the burrow under natural condition, thus the concealing behavior was a dominant reaction. This category accounted for more than 70 % of all behavior scored during odor exposure. However, we did not find any difference in this behavior in adult offspring. This finding is consistent with a previous study in Long-Evans rats, which also report that maternal predation stress on the day of birth has no effect on the amount of time adult offspring spent in the hide box when exposed to a cat collar [23].

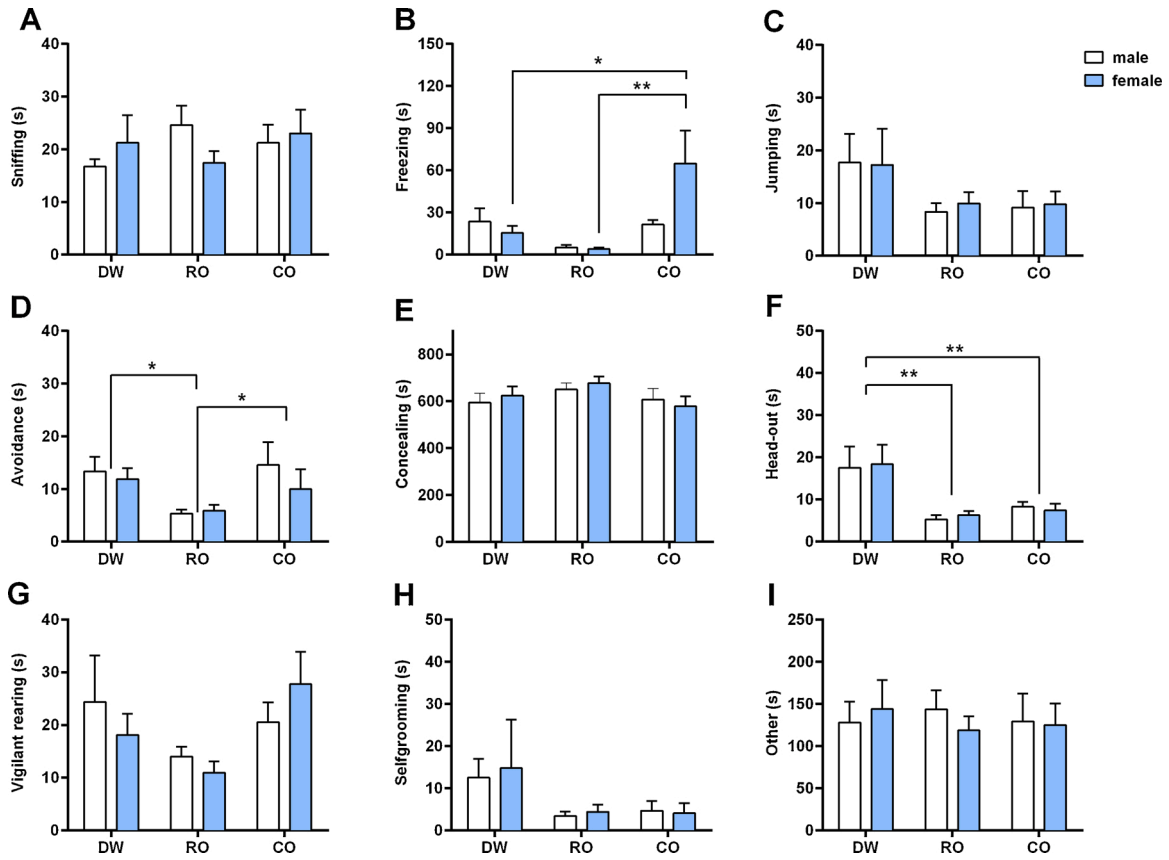

Fig. 3. Effect of postpartum exposure to distilled water (DW), rabbit odor (RO), and cat odor (CO) on offspring behavioral performances to acute CO exposure. **(A)** duration of sniffing; **(B)** duration of freezing (two-way ANOVA, treatment: $F_{(2,\ 66)} = 6.732$, $p = 0.002$; sex: $F_{(1,66)} = 1.728$, $p = 0.193$; treatment × sex: $F_{(2,\ 66)} = 3.483$, $p = 0.036$; Turkey's test: CO females vs. DW females, $p = 0.019$, vs. RO females, $p = 0.002$); **(C)** duration of jumping; **(D)** duration of avoidance (two-way ANOVA, treatment: $F_{(2,\ 66)} = 3.944$, $p = 0.024$; sex: $F_{(1,66)} = 0.629$, $p = 0.431$; treatment × sex: $F_{(2,\ 66)} = 0.427$, $p = 0.654$; Turkey's test: RO vs. DW, $p = 0.037$, RO vs. CO, $p = 0.05$); **(E)** duration of concealing; **(F)** duration of head-out (two-way ANOVA, treatment: $F_{(2,\ 66)} = 9.767$, $p < 0.001$; sex: $F_{(1,66)} = 0.017$, $p = 0.896$; treatment × sex: $F_{(2,\ 66)} = 0.063$, $p = 0.939$; Turkey's test: DW vs. RO, $p < 0.001$, DW vs. CO, $p = 0.002$); **(G)** duration of vigilant rearing; **(H)** duration of self-grooming; **(I)** duration of other behaviors. Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$: significant differences among the three exposure groups.

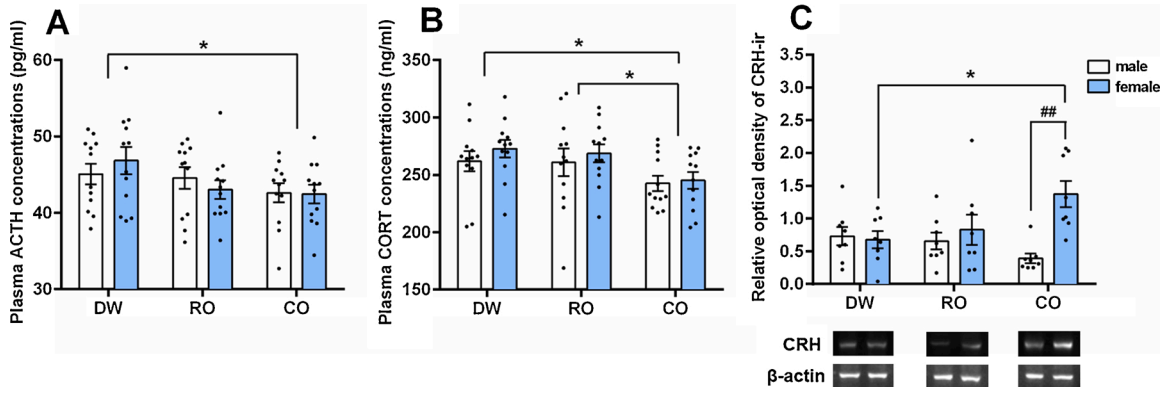

Fig. 4. Effect of postpartum exposure to distilled water (DW), rabbit odor (RO), and cat odor (CO) on basal levels of HPA axis in adult Brandt's voles. **(A)** Plasma ACTH concentrations (n = 12 in each group; two-way ANOVA, treatment: $F_{(2,\ 66)} = 3.086$, $p = 0.05$; sex: $F_{(1,66)} = 0.001$, $p = 0.976$; treatment × sex: $F_{(2,\ 66)} = 0.709$, $p = 0.496$; Turkey's test: CO vs. DW, $p = 0.04$); **(B)** plasma CORT concentrations (two-way ANOVA, treatment: $F_{(2,\ 66)} = 4.470$, $p = 0.015$; sex: $F_{(1,66)} = 1.01$, $p = 0.319$; treatment × sex: $F_{(2,\ 66)} = 0.123$, $p = 0.884$; Turkey's test: CO vs. DW, $p = 0.02$, CO vs. RO, $p = 0.043$); **(C)** hypothalamic CRH expression (n = 8 in each group; two-way ANOVA, treatment: $F_{(2,\ 42)} = 2.801$, $p = 0.072$; sex: $F_{(1,42)} = 7.739$, $p = 0.008$; treatment × sex: $F_{(2,\ 42)} = 10.356$, $p < 0.001$; Turkey's test: CO females vs. DW females, $p = 0.04$, CO females vs. CO males, $p = 0.001$). Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$: significant differences among the three exposure groups. ## $p < 0.01$: significant differences between males and females. *ACTH*, adrenocorticotropic hormone; *CORT*, corticosterone; *CRH*, corticotropin releasing hormone.

Head-out behavior is considered a risk-assessment behavior, allowing information gathering about the possible presence of predators [29, 36]. It seems logical that a vole confronted with a predator odor should retreat to a defensive location (e.g., hide box) from where an assessment of predator risk can be made. In the present study, adult Brandt' voles from CO-exposed mother showed a significant decrease in head-out behavior to CO, indicating that maternal CO exposure significantly inhibited this risk-assessment behavior in adult offspring. In fact, our

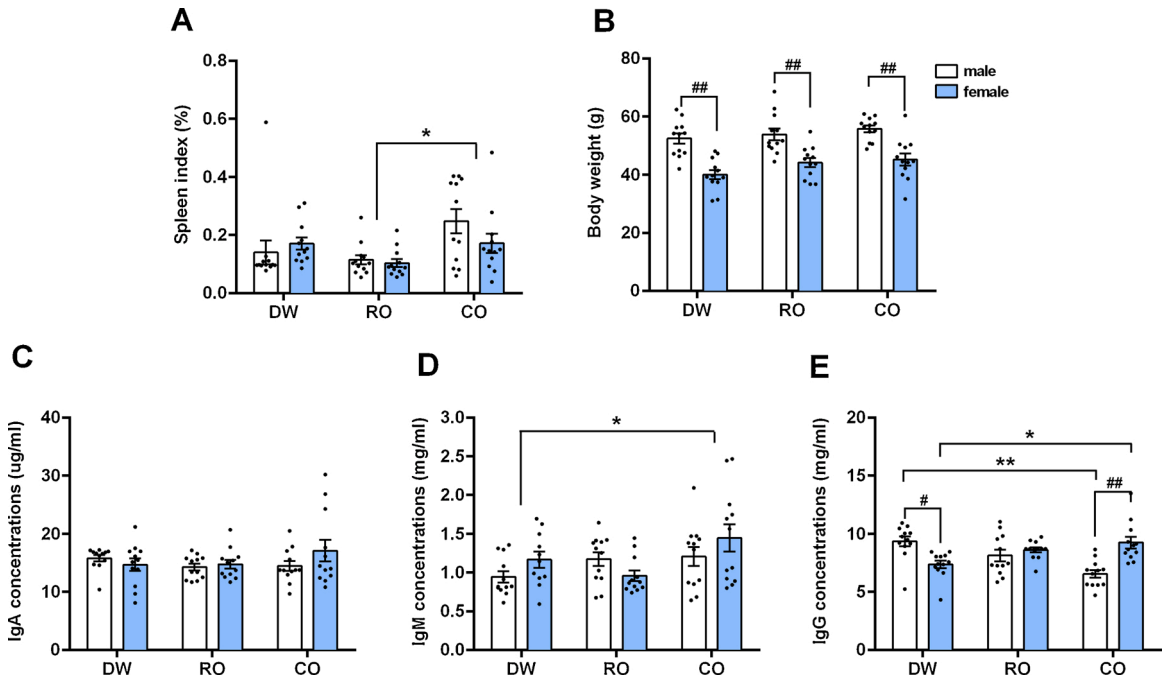

Fig. 5. Effect of postpartum exposure to distilled water (DW), rabbit odor (RO), and cat odor (CO) on **(A)** spleen index (two-way ANOVA, treatment: $F_{(2, 66)} = 5.787$, $p = 0.005$; sex: $F_{(1,66)} = 0.918$, $p = 0.341$; treatment × sex: $F_{(2, 66)} = 1.276$, $p = 0.286$; Turkey's test: CO vs. RO, $p = 0.004$), **(B)** body weight (two-way ANOVA, treatment: $F_{(2, 66)} = 2.503$, $p = 0.090$; sex: $F_{(1,66)} = 57.467$, $p < 0.001$; treatment × sex: $F_{(2, 66)} = 0.143$, $p = 0.867$; Independent sample t-test: males vs. females, $p < 0.001$) and immunoglobulin (Ig) levels in adult Brandt's voles. **(C)** spleen IgA concentrations; **(D)** spleen IgM concentrations (two-way ANOVA, treatment: $F_{(2, 66)} = 3.751$, $p = 0.029$; sex: $F_{(1,66)} = 0.813$, $p = 0.371$; treatment × sex: $F_{(2, 66)} = 2.655$, $p = 0.078$; Turkey's test: CO vs. DW, $p = 0.05$); **(E)** spleen IgG concentrations (two-way ANOVA, treatment: $F_{(2, 66)} = 0.885$, $p = 0.417$; sex: $F_{(1,66)} = 1.415$, $p = 0.238$; treatment × sex: $F_{(2, 66)} = 16.616$, $p < 0.001$; Turkey's test: CO males vs. DW males, $p < 0.001$, CO females vs. DW females, $p = 0.027$, DW males vs. DW females $p = 0.016$, CO males vs. CO females, $p < 0.001$). Error bars are ± SEM. $*p < 0.05$, $**p < 0.01$: significant differences among the three exposure groups. $\#p < 0.05$, $\#\# p < 0.01$: significant differences between males and females.

previous study found that juvenile offspring of CO-exposed dams displayed reduced rearing frequency in the open field task [32], echoing the results here. Rearing on the hind-legs is considered an exploratory behavior [37], and low exploration levels may reduce exposure to predators. However, Mashoodh and colleagues showed that maternal predation threat has no effect on the head-out behavior of offspring in response to predator odor [23]. This may be because of differences in experimental methodology, as offspring were not directly exposed to CO before weaning in our study, and the dams in their study were exposed to CO only on the day of birth, and not for 18 consecutive days, as in our study. There are several possible reasons that may explain the decreased head-out behavior in adult CO offspring. First, adult Brandt's voles from CO-exposed mothers have higher levels of anxiety. This explanation is supported by a previous study in hooded rats that found that changes in risk assessment were not related to activity in the plus maze, but were dependent on total entries and ratio time in the plus maze [38], suggesting that reduced risk assessment may reflect enhanced anxiety. Watt and colleagues also found this relationship between the levels of anxiety and risk assessment in SD rats that were exposed to social defeat stress between PND 35 and 40 [39]. Our previous study also demonstrated that juvenile Brandt's voles from CO-exposed dams displayed an increased anxiety response to a novel environment [32]. Another possibility is that maternal predator risk may increase the cautious defensive behavior in offspring. This hypothesis was supported by our current study, whereby adult offspring of CO-exposed dams exhibited less head-out behavior than the offspring of the DW group. In this case, less head-out behavior may decrease the exposure to predators.

A notable finding in present study is that female offspring of CO-exposed mothers showed more freezing to CO at adulthood. Freezing, defined as the absence of all movement except breathing, is a secondary defense mechanism of prey animals [40]. Previous studies showed that predator odor induces avoidance or freezing behavior depending on the circumstance [41,42]. If a vole is not able to run away from the source of predator odor, the vole freezes (less detectable by predators). If the vole can run away, they will avoid the source rather than freeze. In our study, the hide box provides this location to avoid the odor source. Thus, freezing behavior here may be an index of unconditioned fear [42–44], rather than just a defensive strategy. However, a previous study showed that exposure of mothers and pups to TMT reduces freezing to TMT when re-exposed at PND 30 [24]. This discrepancy may be due to the differences of experimental methodology, as the pups were directly exposed to TMT before weaning in Ayers's study [24]. Indeed, only the pups exposed to TMT at birth with their mother's absence exhibited a decrease of fear-related behavior in adulthood [45]. Our results are supported by studies that reported rats reared to dams exposed to elevated levels of CORT during lactation showed increased behavioral fearfulness and anxiety, as measured in the open field and their resistance to capture, respectively [46,47].

Maternal CO exposure during the postpartum period led to more fear-related behaviors in the female, but not male offspring, showing a sex-specific effect. Previous studies have shown that sex differences exist in risk-taking [48–50], with males being more risk-prone and females being more sensitive to their environment. For example, after classic context fear conditioning, female mice are more likely than males to show a generalized freezing response in a novel context [51]. When exposing rats to the same environment in which they had previously experienced predator odor, females spent more time out of cover and decreased their distance to the stimulus, while males did not [52]. In our study, CO female offspring showed increased freezing to CO exposure. This could reflect the increased sensitization to their environment. Contrarily, males displayed less head-out behavior to CO, which may reflect quick decision-making because of the low-reward and high-risk environment [53].

Several potential pathways through which maternal programming of

offspring behavior during the postpartum period may occur have been suggested. Maternal care was identified as a major influence on offspring behaviors [54,55]. Maternal stress had been shown to decrease rates of maternal care in several different mammalian species including rats [46, 56], savannah baboons [57], gorillas [58], and common marmosets [59]. Our recent study also found this disruption induced by postpartum maternal CO exposure in Brandt's voles [32]. Predator risk possibly activates the dam's HPA axis, and higher levels of maternal−CORT could induce abnormal maternal behavior, indirectly influencing offspring's behavior [46], such as increased fear-related behavior and passive avoidance response in Brandt's voles.

4.2. Effects of postpartum maternal CO exposure on offspring neuroendocrinological parameters in adult Brandt's voles

One potential mediator of stress-induced behaviors is HPA axis activity. In the present study, CO-exposed mothers' offspring showed significantly lower baseline plasma ACTH and CORT levels than DW mothers' offspring. This finding is consistent with several studies, which showed that the male offspring of rat mothers drinking moderate CORT doses (200 μg/mL) during lactation had lower ACTH and CORT levels at basal and stress-induced conditions at 3 months of age [21]. Although basal CORT plasma levels were not modified in adult females, they exhibited a blunted stress response to restraint stress [60]. However, in Macrí and colleagues' study in mice, the male offspring of mothers drinking the highest dose of CORT (100 μg/mL) had a higher CORT response to stress as adults, while offspring of mothers drinking the lowest dose (33 μg/mL) did not differ from controls [61]. A subcutaneous injection study also showed that offspring exposed to low maternal CORT (10 mg/mL) at postpartum had higher baseline CORT levels as young adults [62], whereas high maternal CORT (40 mg/kg) blunted serum CORT concentrations in response to restraint stress in adult male and female offspring [63]. These studies indicate that even small alterations in maternal postpartum CORT may affect the direction of HPA changes in the offspring. Thus, CORT levels in CO-exposed dams should be examined in future studies. The lower baseline CORT levels observed in the offspring of CO-exposed dams may potentiate the development of stress-related symptoms. For example, mice with low morning CORT levels displayed an increased dysphoria-like behavior, as measured by decreases in immobility as well as increases in defecation during a 5-min tail suspension test when compared to high morning CORT mice [64]. Kim and colleagues found that most resilient mice had higher than average basal CORT levels, and many susceptible mice had lower than average basal CORT levels [65]. Basal CORT level is a predictor of stress susceptibility or resilience to subsequent stress exposure. CO offspring with low basal CORT level would be susceptible to acute or chronic stress, which could explain their caution and defensive behavior to acute CO exposure.

In our study, we also found that maternal postpartum CO exposure increased hypothalamic CRH expression in adult female but not male offspring. The direction of the change was not in line with plasma ACTH and CORT changes. However, a previous study showed this inconsistency too, where maternal separation (the separation of pups from their mother for 3 h daily during PND 1–14) mice exposed to 19 days of chronic subordinate colony housing showed increased CRH mRNA expression in the paraventricular nucleus (PVN) and reduced basal plasma CORT levels [66]. Posttraumatic stress disorder is associated with low cortisol levels in the urine and plasma but elevated CRH levels [67,68]. CORT exerts a negative feedback on CRH synthesis and release [69], and the low levels of plasma CORT in CO female offspring might be insufficient to allow for this negative feedback regulation, an idea deserving further investigation. Actually, a recent report suggests that up-regulation of the CRH system in the PVN is related to a stress-susceptible phenotype [70]. Enhanced CRH synthesis may induce stress sensitization in female offspring, thus increasing fear-related behavior to CO exposure, i.e., freezing.

One of the most interesting and unexpected findings was that adult females from CO-exposed mothers had increased spleen IgG levels, while the opposite was true for males, also showing a sex-specific change. IgG is the most abundant immunoglobulin in circulation and a non-specific component of innate immunity [71]. IgG antibodies form the basis of long-term protection against microorganisms, and may represent a state of immunological "readiness." A previous study suggests that high levels of total IgG may reflect better immune functions and overall individual condition [72]. In our study, all voles were apparently healthy and without overt infections. Thus, the results may reflect that maternal CO exposure increased the condition of female offspring but reduced it in males. This might explain why female offspring engage in more sampling of environmental information and are more reactive because they were in better physiological conditions than the males. However, further research is required to prove this sex-difference in overall health with regard to this hypothesis.

Several limitations require consideration when interpreting the current results. First, we do not know what causes the sex-specific effect on the risk-taking behavior and CRH and IgG levels; whether these effects are caused by the different levels of maternal care provided to both sexes require further attention. Second, we observed only basal plasma CORT and ACTH levels and spleen immunoglobulins; whether postpartum maternal predation risk alters the endocrine response to stress is still unknown. Third, odors to which dams had been exposed may linger on their fur, so the offspring may be (in)directly exposed to these odors; this mild exposure may also contribute to their behavioral and physiological development, and thus, caution should be exercised when interpreting this finding.

In summary, the present study indicated that predatory risk, when only the dam is exposed, promotes offspring's passive avoidance and alters the fear-related behavior in a sex-specific fashion. This sex difference also exists for hypothalamic CRH and spleen IgG levels, which may be associated with different coping strategies in response to predator risk. These findings shed light on the importance of postpartum maternal experiences and support the idea that the early maternal environment is a contributing factor shaping adult physiology and behavior. During the postpartum period, CORT and other hormones that reflect the mother's physiological state can be transferred to their offspring via lactation [73,74], having a direct impact on the offspring's glucocorticoid levels and initial phenotype reprograming [75]. Thus, future research is needed to determine this possible mechanism of postpartum maternal predation risk exposure on offspring phenotypes.

Author statement

Ruiyong Wu: Conceptualization, methodology, investigation, validation, funding acquisition, writing - reviewing & editing. **Shan Li** and **Yefeng Huang:** Investigation, data curation, formal analysis, writing - original draft. **Jinyue Pang:** Investigation, data collection. **Yongjian Cai, Xinyue Zhang** and **Tianyi Jiang:** Behavioral analysis, animal breeding. **Shengmei Yang:** Project administration, supervision. **Wanhong Wei:** Funding acquisition, supervision.

Declaration of Competing Interest

All authors declare no conflict of interest.

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