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Paternal environmental enrichment transgenerationally alters affective behavioral and neuroendocrine phenotypes

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ABSTRACT

Recent studies have demonstrated that paternal stress in rodents can result in modification of offspring behavior. Environmental enrichment, which enhances cognitive stimulation and physical activity, modifies various behaviors and reduces stress responses in adult rodents. We investigated the transgenerational influence of paternal environmental enrichment on offspring behavior and physiological stress response. Adult C57BL/6J male mice (F0) were exposed to either environmental enrichment or standard housing for four weeks and then pair-mated with naïve females. The F2 generation was generated using F1 male offspring. Male and female F1 and F2 offspring were tested for anxiety using the elevated-plus maze and large open field at 8 weeks of age. Depression-related behavior was assessed using the forced-swim test. Hypothalamic-pituitary-adrenal (HPA) axis function was determined by quantification of serum corticosterone and adrenocorticotropic hormone (ACTH) levels at baseline and after forced-swim stress. Paternal environmental enrichment was associated with increased body weights of male F1 and F2 offspring. There was no significant effect on F1 offspring anxiety and depression-related behaviors. There were no changes in anxiety-related behaviors in the F2 offspring, however these mice displayed a reduced latency to immobility in the forced-swim test. Furthermore, F2 females had significantly higher serum corticosterone levels post-stress, but not ACTH. These results show that paternal environmental enrichment exerts a sex-specific transgenerational impact on the behavioral and physiological response to stress. Our findings have implications for the modelling of psychiatric disorders in rodents.
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1. Introduction

Adverse life events can contribute to a multitude of psychiatric disorders including anxiety, depression and post-traumatic stress disorder (Lupien et al., 2009). It has been demonstrated that rodent models of chronic stress display elevated anxiety-like and depression-like behaviors, as well as hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Russo et al., 2012). Recent studies have revealed that these effects are also present in the progeny of the affected parents. Clinical studies have shown a path for non-genetic transmission of paternal transgenerational effects including those of post-traumatic stress disorder (PTSD) to non-exposed offspring (Schick et al., 2013; Vaage et al., 2011; Yehuda et al., 2014). Genomic DNA sequence is the main carrier of biological information that is passed on to offspring; however, recent studies have demonstrated that epigenetic modifications can also be paternally transmitted to the offspring via the germ cells (Gapp et al., 2014; Rodgers et al., 2013). It is well known that adverse environments can significantly alter the epigenome and such modifications have been associated with several psychiatric disorders (Gräff and Mansuy, 2009; Ptak and Petronis, 2010; Tsankova et al., 2007). Recent work has shown that these environmentally-mediated epigenetic changes can be inherited through the paternal line and this can potentially have consequences on the inheritance of psychiatric disorders (Crews et al., 2012; Gapp et al., 2014). This emphasizes the need to identify not only genetic factors, but also

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environmental influences on the epigenetic mechanisms that may contribute to this inheritance of predisposition to mental illness (Toth, 2014).

Recent studies have shown that stress in male mice affects the behavior of the offspring. Male offspring of stressed fathers displayed heightened anxiety, reduced sociability and increased basal levels of the stress hormone corticosterone (CORT), which also affected female offspring of the two following generations (F2 and F3) via the paternal linage (Saavedra-Rodríguez and Feig, 2013). Furthermore, maternal separation and unpredictable maternal stress (MSUS) of male mice prior to weaning altered offspring behavior for up to three generations (Franklin et al., 2010). Increased anxiety was also reported to develop in male offspring of mice which had experienced social defeat stress prior to mating (Dietz et al., 2011) and in offspring of socially isolated fathers (Pisu et al., 2013). While most studies have described the transgenerational effects of negative environments, such as stress, little is known about the effects of positive environments, such as environmental enrichment.

Environmental enrichment (EE) is an experimental paradigm that has been shown to limit behavioral, cellular and molecular pathologies in various disease models, including models of stress (Chekmareva et al., 2014; Renoir et al., 2013; Rogers et al., 2016). EE rescues the depression-like phenotype and corrects HPA-axis dysregulation in a mouse model of alcohol withdrawal (Pang et al., 2013). EE also has the capacity to influence peripheral stress-response as demonstrated by its correction of adrenal hyperresponsivity pathology in a mouse model of Huntington's disease (Du et al., 2012). In normal rodents, EE has consistently been shown to reduce anxiety-like behavior in the elevated-plus maze and the open-field test (Sztainberg et al., 2010), with recent evidence suggesting the requirement of a minimum 3-week enrichment period (Leger et al., 2015). In contrast, the anti-depressive effects of enrichment remain controversial. While there are reports that even a short 7-day period of environmental enrichment reduces forced-swim test (FST) immobility time in rats (Zanca et al., 2015), other evidence in rats and mice suggest that EE does not alter latency to immobility and total immobility time in the FST (Leger et al., 2015; Possamai et al., 2014).

Recently, it was reported that the male offspring of mice exposed to early life stress through MSUS develop a conflicting behavioral phenotype of reduced anxiety (increased time in the light half side of the light-dark box) with increased depressive behavior (increased immobility time in the FST) (Gapp et al., 2014). Interestingly, environmental enrichment prevented the transmission of paternal traumatic effects to offspring (Gapp et al., 2016). A transgenerational influence of enrichment on hippocampal function is likely to also involve enhancement of long-term potentiation properties as previously reported in a study of 2-week juvenile enrichment (Arai et al., 2009).

In this study, we have investigated the effects of 4 weeks of environmental enrichment on adult male C57Bl/6J mice to determine the transgenerational effects on offspring behavior under non-stress and non-disease conditions. As we recently showed that paternally-mediated effects on offspring anxiety could manifest across two subsequent generations (Short et al., 2016), we broadened our investigation to examination of the F2 generation.

We report that environmental enrichment had no impact on F1 offspring anxiety-related behaviors. In the F2 offspring, however, environmental enrichment did produce a quicker adaptation to floating posture in the forced-swim test of depression-like behavior. Moreover, F2 females showed significantly higher post swim-stress corticosterone levels. Interestingly, female and male F2 progeny of EE males showed elevated body weights corresponding to higher body weights of the male F1 offspring. This is the first evidence for transgenerational effects of environmental enrichment on the behavioral and physiological response to stress.

2. Material and methods

2.1. Mice

32 male C57Bl/6J mice were purchased at 7 weeks of age from the Animal Resources Centre (Murdoch, WA, Australia) and housed in the core animal facility in open-top standard laboratory mouse cages ($15 \times 30 \times 12$ cm) with *ad libitum* food and water. Mice were maintained on a 12-h light/dark cycle (lights on at 0700H). Mice were weighed once a week and the cage bedding was changed weekly. All procedures were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee in accordance with the recommended guidelines set by the National Health and Medical Research Council (NHMRC) of Australia.

2.2. Environmental enrichment paradigm

At 10 weeks of age, male mice were randomly allocated into groups of 4 mice per cage to be housed under standard housing (SH) or environmentally enriched housing (EE) for 4 weeks (16 mice for each group). Enriched mice were housed in larger sized ($25 \times 38 \times 25$ cm) rat boxes with elevated lids, whereas the control mice were housed in standard mouse cages containing only bedding. Housing boxes for the EE group contained a variety of objects (such as cardboard rolls, wire, mesh, wooden and plastic objects and shredded paper), which were changed once a week in order to provide novel and complex stimulation.

2.3. Breeding

After 4 weeks of environmental enrichment, males were pair-mated with 10-week-old naïve C57Bl/6J females in standard cages for 5 days. Females were single housed until they littered down. Females who were not pregnant or lost their litter after birth were removed from the study.

On postnatal day 25, offspring were weaned and divided into new standard housing boxes. Every box comprised 3–5 mice of the same sex and same paternal housing. When offspring were 8 weeks of age, behavioral testing began for all offspring apart from 14 males from both groups (six controls and eight of paternal EE) that were used for generating the F2 generation. These males were mated at the age of 14 weeks with 10-week-old naïve females as described above. F2 offspring were weaned in the same way as F1 and were behaviorally assessed starting from 8 weeks of age.

2.4. Behavioral testing

All tests were performed during the light phase of the light/dark cycle and were completed before 1300H in order to control for time of day effects. Mice were acclimated to the room for at least 1 h before commencement of each test. From 8 weeks of age, offspring were tested on the elevated-plus maze, large open field and forced-swim test for affective behavior. The same mice were used for all the experiments. See Fig. 1A for experimental design diagram.

2.4.1. Maternal behavior

After offspring were born, the maternal behavior towards the pups was observed in two sessions each day (morning and afternoon), from postnatal day 1–5. Observations took place every 5 min for 60 min in every session and were manually scored as previously described (Chourbaji et al., 2011; Short et al., 2016).

2.4.2. Elevated-plus maze

The elevated-plus maze (EPM) is made of light-coloured Perspex and elevated 50 cm from the floor, including two open arms (5 × 30 cm) and two enclosed arms (5 × 30 × 14 cm) extending from a central platform (5 × 5 cm) in a room with dimmed light (25 lx). Mice were placed in the centre of the maze for 5 min and were automatically monitored using TopScanLite 2.00 (CleverSys Inc., VA). Total time spent in the open arms of the maze, number of entries to the open arms and the overall distance moved by each mouse were measured. The maze was cleaned with 70% ethanol and dried with paper towel between each mouse. Mice that jumped off the maze were excluded from the analysis.

2.4.3. Large open field

The large open field (LOF) was made with grey plastic box of 100 × 100 cm with 30 cm high walls, which was highly illuminated at 1600 lx in the centre of the arena, while lighting in the corners and near the walls was lower. Mice were placed in the centre of the arena for 10 min and were automatically monitored using TopScanLite 2.00 (CleverSys Inc., VA). The apparatus was divided into ten virtual fields; four corner fields; four near-wall fields; middle field and a centre field. Distance, duration and number of entries for each field were analysed as well as the overall distance. Duration in the centre was expressed as a percentage of the test duration. The floor and walls were cleaned with 70% ethanol and dried with paper towel after each session.

2.4.4. Forced-swim test

In the forced-swim test (FST), mice were placed in 2.5L glass beakers that were filled with water (23–25 °C) for 5 min. Latency to the first bout of immobility and time spent immobile in the last 240 s were videotaped were automatically calculated (Forced Swim Scan 2.00, CleverSys Inc; VA). Between 12–14 weeks of age, the HPA-axis stress response was assessed, via measurements of serum corticosterone levels after forced-swim stress and mice were sacrificed.

2.5. Serum collection and corticosterone and ACTH quantification

Acute forced-swim stress was performed between 0900–1200H to control for diurnal variations in endogenous corticosterone (CORT) levels. Each mouse was place into a beaker of water (23–25 °C) for 5 min. Mice were killed via cervical dislocation immediately upon removal from the water. Independent separate groups of non-stressed mice were killed to establish baseline CORT levels. Blood was collected from aortic puncture into 1.5 ml tubes, allowed to clot at room temperature for 30 min then centrifuged for 15 min at 1000*g*. Serum was transferred to fresh tubes and stored at −80 °C until further use. CORT levels were determined in triplicates by enzyme immunoassay (EIA) corticosterone kit (Cayman Chemical, MI) according to the manufacturer's instructions. Adrenocorticotropic hormone (ACTH) was measured by enzyme-linked immunosorbent (ELISA) assay (Phoenix Pharmaceuticals, Burlingame, CA) according to the manufacturer's instructions.

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) were used to assess main effects and/or interactions on behavioral and physiological outcomes. Sidak's *post hoc* tests were used to determine specific differences in the event of significant interactions. For maternal nursing behavior assessments and body weight measurements, repeated-measures (RM) 2-way ANOVA was used. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., LA Jolla, CA). The threshold for significance was set at $P < 0.05$.

3. Results

Males that were exposed to environmental enrichment (EE) showed similar body weight to standard housed (SH) littermate controls throughout the four weeks before mating (data not shown).

3.1. Paternal environmental enrichment did not affect maternal nursing behavior or litter size, but did alter offspring body weights

Twice daily observations of female behavior were made to assess whether there were differences depending on paternal EE versus SH housing conditions. Various measures were recorded and combined into three general behavioral types: nurturing, self-maintenance and neglecting. Exposure to enriched environment before mating had no effect on the maternal behaviors towards the offspring for nurturing (EE father: $F_{1,12} = 4.075$, $P > 0.05$; time: $F_{9,108} = 1.438$, $P > 0.05$; interaction: $F_{9,108} = 0.6590$, $P > 0.05$), self-maintenance (EE father: $F_{1,12} = 3.163$, $P > 0.05$; time: $F_{9,108} = 1.686$, $P > 0.05$; interaction: $F_{9,108} = 0.4689$, $P > 0.05$) and neglecting (EE father: $F_{1,12} = 1.354$, $P > 0.05$; time: $F_{9,108} = 1.300$, $P > 0.05$; interaction: $F_{9,108} = 0.9340$, $P > 0.05$) (Fig. 1B). This data indicates that exposure to males who were housed in enriched environment did not change the maternal behaviors of the females towards their litters.

In the F1 offspring, 64 pups were born to control fathers and 74 to EE fathers. In the F2 offspring, 23 pups were born to control grand-fathers and 47 to EE grand-fathers. Litter sizes were not affected due to paternal EE ($F_{1,36} = 0.1335$, $P > 0.05$) and there were no differences in the number of male or female pups ($F_{1,36} = 0.2899$, $P > 24860.05$) and no paternal EE x sex interaction ($F_{1,36} = 0.2486$, $P > 0.05$) (Fig. 1C). F2 litter sizes were also not affected by grand-paternal EE ($F_{1,20} = 0.01297$, $P > 0.05$), yet there were overall more female than male pups ($F_{1,20} = 6.861$, $P < 0.05$), but no grand-paternal EE interaction ($F_{1,20} = 0.1167$, $P > 0.05$) (Fig. 1E).

Female offspring did not differ in body weight due to paternal EE ($F_{1,27} = 1.052$, $P > 0.05$; time: $F_{2,54} = 36.17$, $P < 0.0001$; interaction: $F_{2,54} = 0.1947$, $P > 0.05$). However, male offspring of paternal EE had a significant higher body weights compared with controls ($F_{1,27} = 5.717$, $P < 0.05$; time: $F_{2,54} = 161.7$, $P < 0.0001$; interaction: $F_{2,54} = 0.6260$, $P > 0.05$) (Fig. 1D). Interestingly, the progeny of these mice were also affected, as both females and males displayed significantly higher body weights due to grand-paternal EE (females: $F_{1,26} = 4.327$, $P < 0.05$; males: $F_{1,15} = 12.41$, $P < 0.01$) (Fig. 1F). There were significant effects of time from 8 to 10 weeks of age (females: $F_{2,52} = 14.06$, $P < 0.0001$; males: $F_{2,30} = 43.93$, $P < 0.0001$), but no time x grand-paternal EE effects (females: $F_{2,52} = 0.02278$, $P > 0.05$; males: $F_{2,30} = 0.2499$, $P > 0.05$).

3.2. Paternal environmental enrichment did not impact offspring affective behavior

In order to assess whether paternal environmental enrichment can alter the offspring affective behavior, we exposed both female and male offspring to elevated-plus maze, open-field and forced-swim tests.

In the elevated-plus maze, there was an overall sex effect on time spent in the open arms with females spending more time than males ($F_{1,53} = 10.03$, $P < 0.01$). There was no overall effect of paternal housing ($F_{1,53} = 1.084$, $P > 0.05$) and no sex x paternal housing interaction ($F_{1,53} = 0.8557$, $P > 0.05$) (Fig. 2A). Similarly, there was also an overall sex difference for the number of open arm entries ($F_{1,54} = 5.613$, $P < 0.05$) but no effect of paternal housing ($F_{1,54} = 0.5482$, $P > 0.05$). There was no significant sex x paternal housing interaction ($F_{1,54} = 1.255$, $P > 0.05$) (Fig. 2B). Paternal

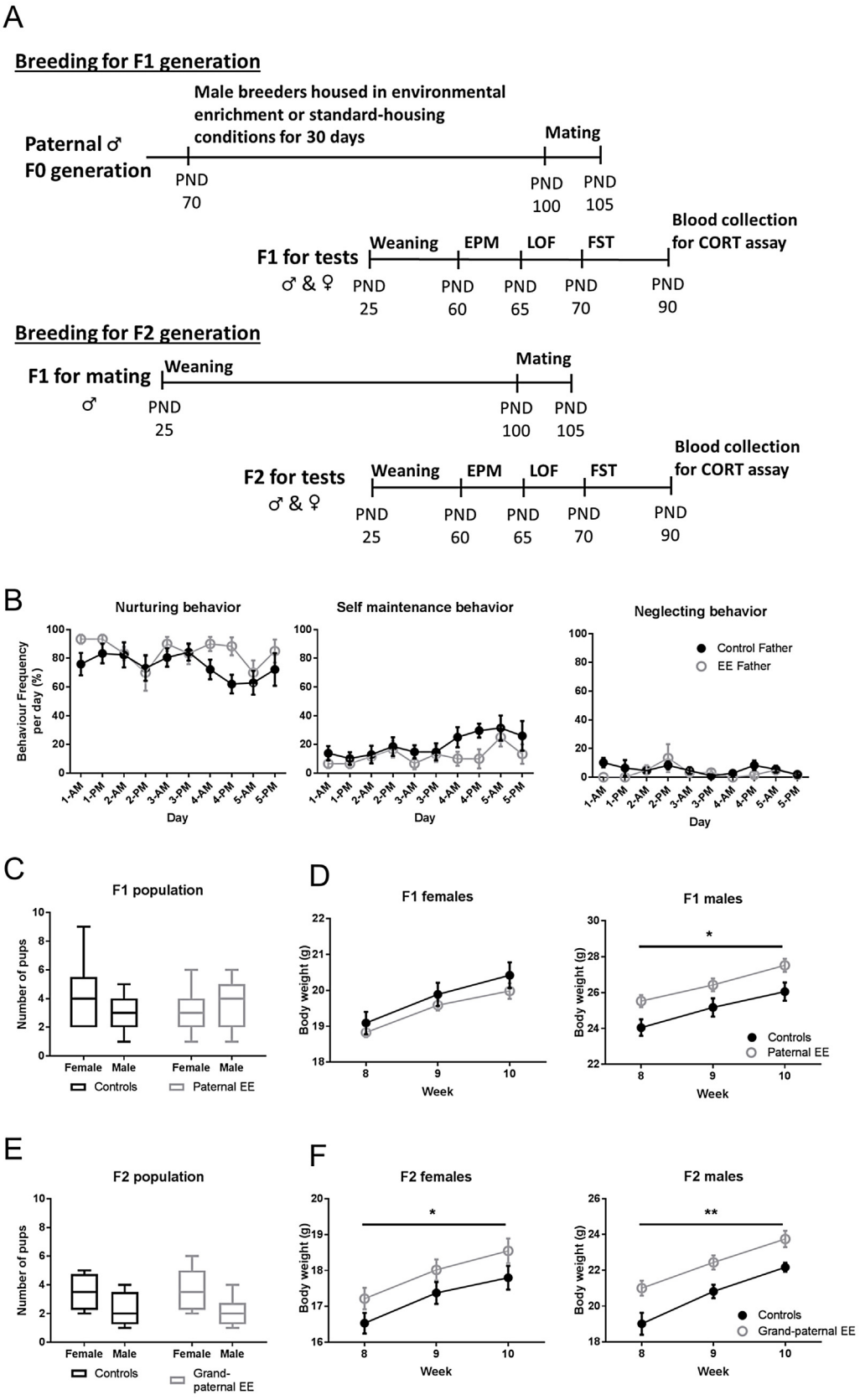


Fig. 1. Study design, nursing behaviors, litter size and progeny body weights. (A) Diagrammatic representation of the experimental design. 8-week-old male C57BL/6 mice (F0 generation) were randomly allocated into standard housing (SH) or environmental enrichment (EE) for four weeks before paired-mating with naïve females. The F1 and F2 generations were tested on the elevated-plus maze (EPM), large open field (LOF) and forced-swim test (FST). Blood assays were conducted to assess HPA axis function. (B) Observations of maternal nursing behavior reveal no impact of exposure to EE males on nurturing, self-maintenance or neglecting behaviors. Data collected from 9 control litters and 5 EE litters, presented as group mean ± SEM. (C, E) Distribution of male and female pups born to each litter for the F1 and F2 generations. Box plot whiskers represent minimum and maximum. (D) Male but not female offspring of paternal EE displayed elevated body weights throughout the study. n = 14 controls and n = 15 paternal EE for

housing ($F_{1,54}$ = 0.01186, P > 0.05) and sex ($F_{1,54}$ = 0. 0.0009257, P > 0.05) did not influence total distance travelled in the maze. However, there was a significant sex x paternal housing interaction ($F_{1,54}$ = 0.5948, P < 0.05) but *post hoc* comparisons did not reveal any significant effects (Fig. 2C).

In the large open field, there was an overall sex effect on the centre time with females spending more time than males ($F_{1,54}$ = 6.484, P < 0.05) (Fig. 2D). There was no effect of paternal housing ($F_{1,54}$ = 0.09183, P > 0.05) and no significant sex x paternal housing interaction ($F_{1,54}$ = 0.2278, P > 0.05) (Fig. 2D). Similarly, sex was a significant factor for the number of centre crossings ($F_{1,54}$ = 6.322, P < 0.05), while paternal housing was not significant ($F_{1,54}$ = 0.05347, P > 0.05) (Fig. 2E). Again, there was no sex x paternal housing interaction ($F_{1,54}$ = 1.487, P > 0.05) (Fig. 2E). The overall distance travelled during the open field test significantly differed by sex ($F_{1,54}$ = 5.219, P < 0.05) but not with paternal housing ($F_{1,54}$ = 0.1878, P > 0.05). There was no significant sex x paternal housing interaction ($F_{1,54}$ = 0.8706, P > 0.05) (Fig. 2F).

Following anxiety testing, female and male offspring were tested in the forced-swim test as a measure of depressive-like behavior. Latency to the first bout of immobility was not influenced by paternal housing ($F_{1,41}$ = 0.2926, P > 0.05) or sex ($F_{1,41}$ = 2.272, P > 0.05), with no significant paternal housing x sex interaction ($F_{1,41}$ = 1.123, P > 0.05) (Fig. 2G). Consistent with that finding, immobility duration was not influenced by paternal housing ($F_{1,41}$ = 0.7889, P > 0.05) or sex ($F_{1,41}$ = 0.1123, P > 0.05). There was no paternal housing x sex interaction: $F_{1,41}$ < 0.0001, P > 0.05) (Fig. 2H).

3.3. Offspring HPA axis stress response was not affected by paternal environmental enrichment

To inspect whether environmentally enriched fathers affect the offspring stress responsivity, we evaluated serum corticosterone and ACTH levels at baseline and, in a separate group of animals, after forced-swim stress.

Two-way ANOVA revealed a significant effect of sex on basal corticosterone levels, with males displaying higher levels than females ($F_{1,20}$ = 4.657, P < 0.05). However, there was no effect of paternal housing ($F_{1,20}$ = 0.1496, P > 0.05) and no significant sex x paternal housing interaction ($F_{1,20}$ = 1.25, P > 0.05) (Fig. 3A). In contrast, there was no overall sex difference for basal ACTH levels ($F_{1,13}$ = 1.325, P > 0.05). There was no effect of paternal housing ($F_{1,13}$ = 0.3139, P > 0.05) and no significant sex x paternal housing interaction ($F_{1,13}$ = 0.6931, P > 0.05) (Fig. 3B).

Post swim-stress serum CORT levels did not significantly differ between the sexes ($F_{1,23}$ = 0.9254, P > 0.05) and were not influenced by paternal housing ($F_{1,23}$ = 0.009041, P > 0.05). There was no sex x paternal housing interaction ($F_{1,23}$ = 0.9673, P > 0.05) (Fig. 3C). Similarly, post swim-stress serum ACTH levels did not significantly differ between the sexes ($F_{1,12}$ = 0.3181, P > 0.05) and were not affected by paternal housing ($F_{1,12}$ = 0.2627, P > 0.05). There was no significant sex x paternal housing interaction ($F_{1,12}$ = 0.6523, P > 0.05) (Fig. 3D).

3.4. Parental exposure to enriched environment induces limited effects on F2 generation depression-like behavior

We mated F1 males of both groups with naïve females in order to generate the F2 generation for behavioral and physiological assessments. Both females and males were tested in the elevated-plus maze, the large open field and the forced-swim test for anxiety and affective behaviors.

In the elevated-plus maze, no differences were observed for time in open arms (grand-paternal housing: $F_{1,39}$ = 0.2514, P > 0.05; sex: $F_{1,39}$ = 0.02455, P > 0.05; interaction: $F_{1,39}$ = 0.2095, P > 0.05) (Fig. 4A), entries to the open arms (grand-paternal housing: $F_{1,39}$ = 0.2999, P > 0.05; sex: $F_{1,39}$ = 0.334, P > 0.05; interaction: $F_{1,39}$ = 0.2999, P > 0.05) (Fig. 4B) and total distance travelled (grand-paternal housing: $F_{1,41}$ = 0.4537, P > 0.05; sex: $F_{1,41}$ = 0.328, P > 0.05; interaction: $F_{1,41}$ = 0.4435, P > 0.05) (Fig. 4C).

Similarly, in the open-field test, no differences were found for time in centre (grand-paternal housing: $F_{1,41}$ = 1.061, P > 0.05; sex: $F_{1,41}$ = 0.0105, P > 0.05; interaction: $F_{1,41}$ = 0.04291, P > 0.05) (Fig. 4D), number of centre crossings (grand-paternal housing: $F_{1,41}$ = 1.129, P > 0.05; sex: $F_{1,41}$ = 0.2832, P > 0.05; interaction: $F_{1,41}$ = 0.3173, P > 0.05) (Fig. 4E) and the total distance travelled (grand-paternal housing: $F_{1,41}$ = 0.0002169, P > 0.05; sex: $F_{1,41}$ = 0.3411, P > 0.05; interaction: $F_{1,41}$ = 0.3029, P > 0.05) (Fig. 4F).

In the forced-swim test, there was an overall effect of grand-paternal housing on latency to immobility as progeny of environmental enrichment were quicker to adapt a floating posture than controls ($F_{1,40}$ = 9.026, P < 0.01). There was also an overall sex difference, with significantly earlier adaptation of floating postures in males compared with females ($F_{1,40}$ = 8.023, P < 0.01). There was no significant grand-paternal housing x sex interaction ($F_{1,40}$ = 0.004023, P > 0.05) (Fig. 4G).

Consistent with this finding, there was an overall sex difference in immobility duration with males floating more than females ($F_{1,41}$ = 15.45, P < 0.001). There was no effect of grand-paternal housing ($F_{1,41}$ = 1.186, P > 0.05) nor a grand-paternal housing x sex interaction ($F_{1,41}$ = 2.023, P > 0.05) (Fig. 4H).

3.5. F2 females of paternal environmental enrichment display increased physiological stress response

Two-way ANOVA revealed a significant overall effect of grand-paternal housing on basal serum corticosterone levels, with lower levels in the mice of grand-paternal EE ($F_{1,11}$ = 5.148, P < 0.05). There was no overall sex difference ($F_{1,11}$ = 0.2883, P > 0.05) and no significant grand-paternal housing x sex interaction ($F_{1,11}$ = 3.861, P > 0.05) (Fig. 5A). Basal serum ACTH levels were not significantly different between the groups (grand-paternal housing: $F_{1,10}$ = 0.0003387, P > 0.05; sex: $F_{1,10}$ = 1.304, P > 0.05; interaction: $F_{1,10}$ = 0.01871, P > 0.05) (Fig. 5B).

There were no overall effects of grand-paternal housing ($F_{1,25}$ = 1.325, P > 0.05) or sex ($F_{1,25}$ = 0.6442, P > 0.05) on serum corticosterone levels post swim-stress. However, there was a significant grand-paternal housing x sex interaction ($F_{1,23}$ = 8.812, P < 0.01) (Fig. 5C). *Post hoc* analysis revealed that female mice of grand-paternal EE showed increased responses to stress compared to controls (P = 0.0021), while there was no effect on males (P = 0.4891). Interestingly, serum ACTH levels post swim-stress were significantly influenced by grand-paternal housing ($F_{1,22}$ = 4.525, P < 0.05) and there was a significant sex x grand-paternal housing interaction ($F_{1,22}$ = 5.243, P < 0.05) but no effect of sex ($F_{1,22}$ = 02938, P > 0.05) (Fig. 5D). *Post hoc* comparisons revealed that male mice of grand-paternal EE had significantly lower serum ACTH levels compared to controls (P = 0.0288), however this was not observed in females (P = 0.9867).

each sex. (F) Grand-paternal EE increased the body weights of both female and male mice. n = 14 mice per group for females and n = 8 control males and n = 9 grand-paternal EE males. Data are shown as group mean ± SEM. *P < 0.05 and **P < 0.01 for overall effect of paternal or grand-paternal housing.

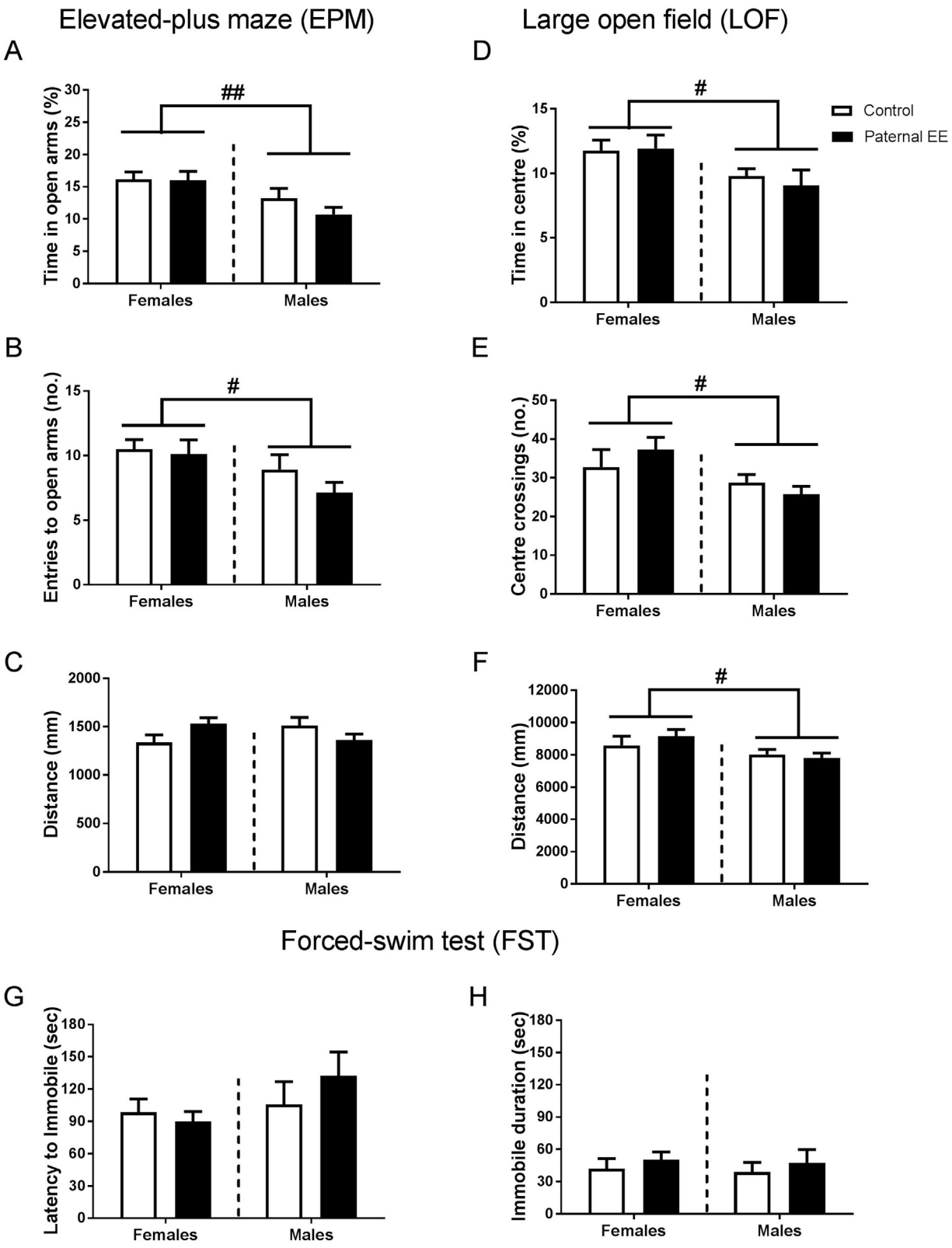


Fig. 2. F1 affective behavior is not altered due to paternal environmental enrichment. (A) Percentage of time spent in the open arms, (B) number of entries to the open arms and (C) total distance travelled in the elevated-plus maze (EPM). (D) Percentage of time spent in the centre, (E) number of centre crossings and (F) total distance travelled in the large open field (LOF). n = 14 controls and n = 15 paternal EE for each sex. Values represent mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for overall effect of sex. (G) Latency to immobility and (H) duration of immobility in the forced-swim test (FST). n = 12 females per group and n = 11 control males and 10 paternal EE males. Values represent mean ± SEM.

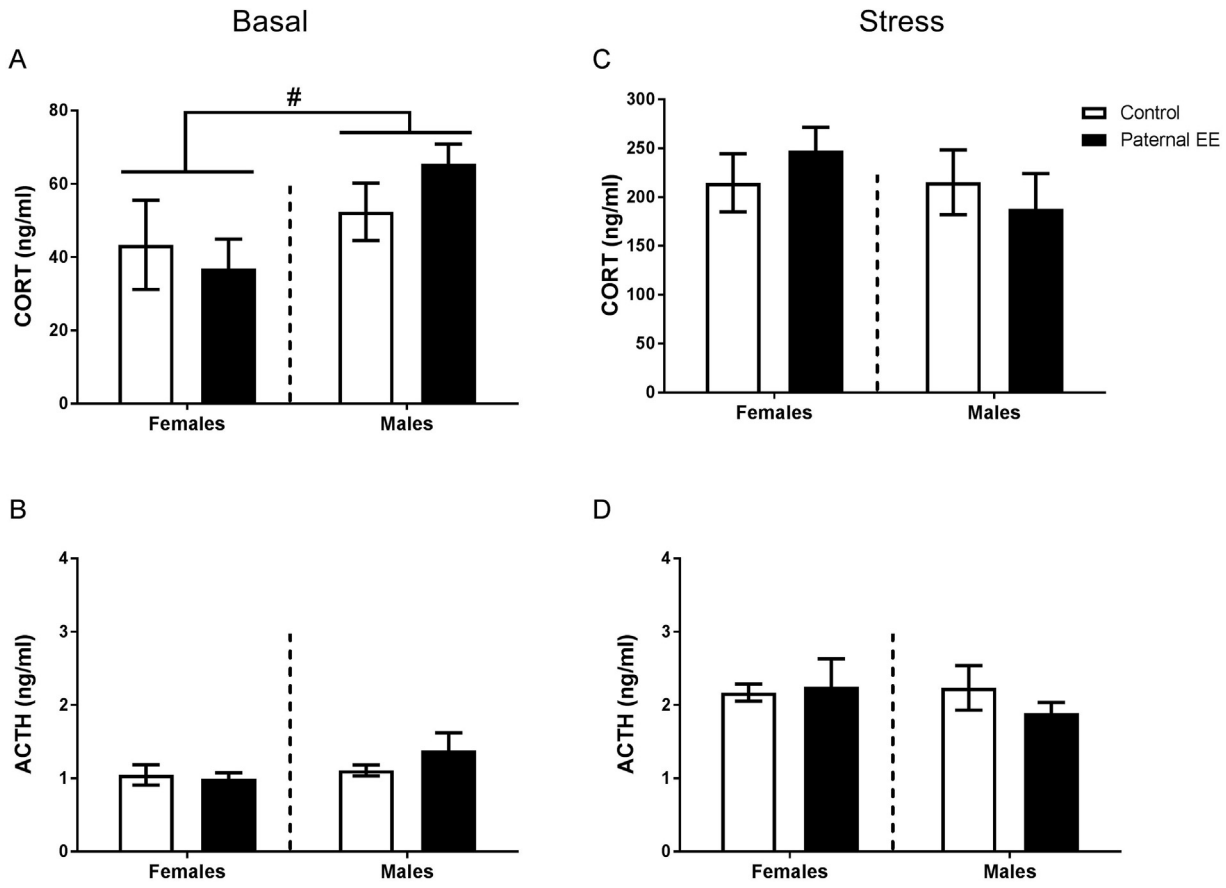


Fig. 3. F1 serum corticosterone and ACTH levels at baseline and after stress. (A) Basal corticosterone (CORT) levels. n = 6 mice for each sex and each group. (B) Basal ACTH levels. n = 3 females and n = 4 males for controls; n = 4 females and n = 6 males for paternal EE. (C) corticosterone (CORT) levels after stress. n = 7 females and n = 6 males for controls; n = 8 females and n = 6 males for paternal EE. (D) ACTH levels after stress. n = 4 mice for each sex and each group. Values represent mean ± SEM. $^{\#}P < 0.05$ for overall effect of sex.

4. Discussion

This study has found that environmental enrichment can influence the physiological and behavioral parameters of offspring across two generations. This is the first evidence for paternal environmental enrichment effects on F1 and F2 offspring adult body weight, which makes an interesting comparison to other transgenerational studies using different environmental influences. There was no observable effect on F1 offspring anxiety behavior, however both male and female F2 offspring had reduced latency to adopting an immobile posture in the FST. Furthermore, F2 females of environmentally enriched grand-fathers responded to stress with increased CORT levels, while the F2 males responded to stress with decreased ACTH levels. These results show sex-specific behavioral and physiological response to a situation of forced-swim stress as compared with control mice. Our data is highly relevant to rodent studies of affective disorders, stress and metabolism, since it is standard practice in many laboratories to provide variable levels of environment enrichment as part of normal rodent colony maintenance. Our present study also provides new insight into environmental influences on transgenerational inheritance through the male linage and has direct relevance to stress reactivity and depressive disorders.

4.1. Paternal environmental enrichment had no influence on F1 offspring affective behavior and stress response

Our laboratory and others have previously demonstrated that environmental enrichment can rescue the affective phenotypes and the abnormal physiological stress response in several disease models (Du et al., 2012; Li et al., 2014; Pang et al., 2013). However, there is growing evidence that the potential benefits of environmental enrichment extend beyond the exposed animal. Environmentally enriched rats in a model of absence epilepsy with co-morbid anxiety not only benefitted from having reduced anxiety and frequency of seizures, but their offspring were found to inherit those anxiolytic and anti-epileptogenic effects (Dezsi et al., 2016). Paternal environmental enrichment also prevented the transgenerational transmission of behavioral changes in offspring born to mice that had experienced early life stress through maternal separation (Gapp et al., 2016). Nevertheless, we found no alterations in the F1 offspring affective behavior and stress response due to paternal EE in the tests we used, suggesting that the positive benefits on behavior might only be observable in the presence of a perturbation to normal behavioral function.

The male-only F1 weight increase coupled to both male and female F2 having greater mean body weights strongly indicate that this particular physical property is heritable via the male germ line. Given that paternal and maternal obesity are both associated with increased offspring body weight (Marco et al., 2013; Ng et al., 2010), it would be interesting to determine whether maternal preconception environmental enrichment would also result in offspring having altered body weights through development into adulthood.

In a recent study, Gapp and colleagues suggested that normalisation of the paternal MSUS-induced offspring hypo-anxiety phenotype was associated with increased glucocorticoid receptor (GR) expression in the hippocampus due to decreased DNA methylation in sperm (Gapp et al., 2016). Conducting a similar gene

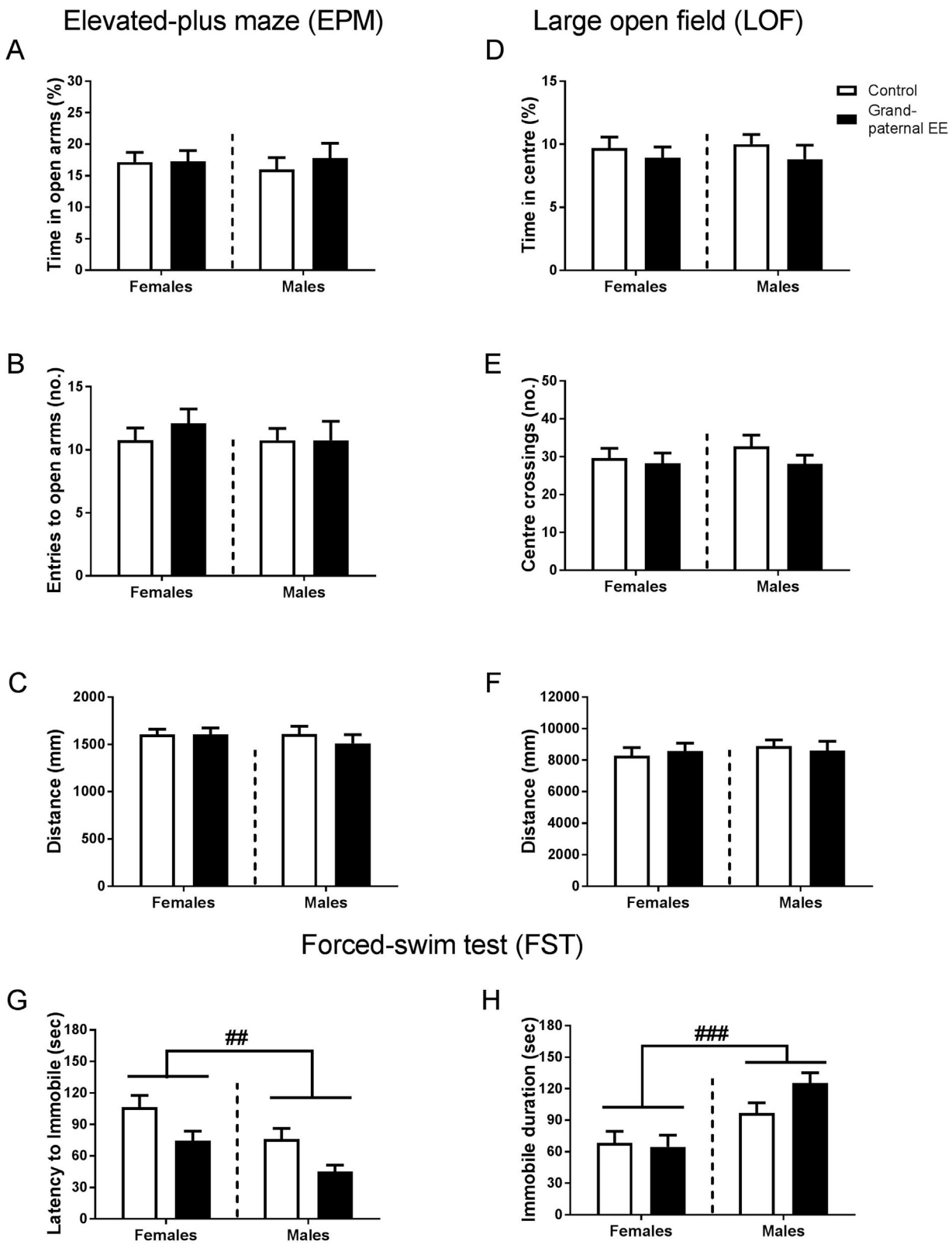


Fig. 4. Grand-paternal environmental enrichment influences F2 performance in the forced-swim test while anxiety-like behavior remains unaltered. (A) Percentage of time spent in the open arms, (B) number of entries to the open arms and (C) total distance travelled in the elevated-plus maze (EPM). (D) Percentage of time spent in the centre, (E) number of centre crossings and (F) total distance travelled in the large open field (LOF). n = 14 females for both groups; n = 9 control males and n = 8 grand-paternal EE males. Values represent mean ± SEM. (G) Latency to immobility and (H) duration of immobility in the forced-swim test (FST). n = 13 controls and 14 grand-paternal EE females; n = 8 control males and n = 9 grand-paternal EE males. Values represent mean ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ for overall effect of sex.

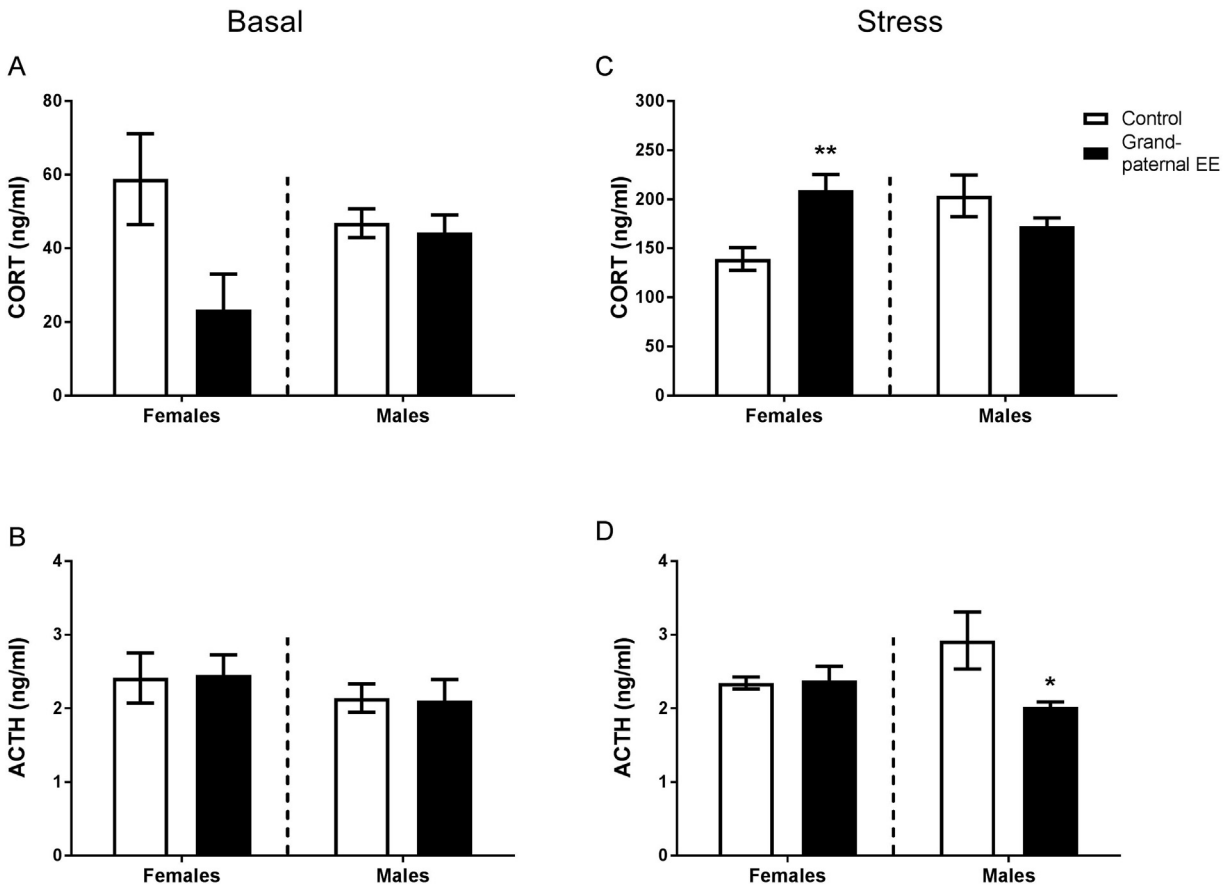


Fig. 5. F2 corticosterone stress response is modified in females of grand-paternally environmentally enriched fathers. (A) Basal corticosterone (CORT) levels. n = 4 controls and n = 3 females for grand-paternal EE; n = 4 males for both controls and grand-paternal EE. (B) Basal ACTH levels. n = 3 females for both controls and grand-paternal EE; n = 4 males for both controls and grand-paternal EE. (C) corticosterone (CORT) levels after stress. n = 10 females for both controls and grand-paternal EE; n = 5 controls males and n = 4 males of grand-paternal EE. (D) ACTH levels after stress. n = 9 females for both controls and grand-paternal EE; n = 4 males for both controls and grand-paternal EE. Values represent mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ for *post hoc* difference between controls and grand-paternal EE.

expression profiling study would be an interesting follow-up to this study since GR expression in the hippocampus is a key regulatory mechanism for behavioral and physiological stress response. However, it would also be important to contrast our environmental enrichment protocol with that of Gapp et al. (2016), which provided running wheels. Our laboratory, and others, have demonstrated dissociable effects of environmental enrichment and running on behavior and hippocampal gene expression (Grégoire et al., 2014; Mustroph et al., 2016; Rogers et al., 2016; Zajac et al., 2010); thus, it would be reasonable to speculate that offspring hippocampal GR expression is not influenced under our experimental conditions.

4.2. Paternal environmental enrichment modifies F2 despair behavior and HPA-axis responsivity in a sex-specific manner

While F1 offspring of EE fathers show no changes in their affective behavior and physiological stress response, our results show that exposure to environmental enrichment has an impact on the F2 offspring phenotypes in a sex-specific manner. There is some evidence that transgenerational inheritance can "skip" a generation and affect the F2 offspring even when the F1 phenotype appears normal. In humans, epidemiological data revealed that grandchildren of males who were undergoing a period of famine had reduced risk for cardiovascular and diabetes mortality in a sex-specific manner, as the effects were transmitted only via the male line (Kaati et al., 2002; Pembrey et al., 2006). Moreover, in rodents, transgenerational inheritance of stress can modify the F2 phenotype without alterations in the F1 phenotype. We previously showed that paternal exposure to CORT had an effect on F2 depression-like behavior but not in F1, an effect that was transmitted through the male lineage (Short et al., 2016). Other studies have also indicated that phenotypes in the F2 or F3 generations differ from those seen in the F1 generation (Bygren et al., 2001; Franklin et al., 2010), although these phenotypes could be affected by the changes in the F1 generation. It is possible that paternal environmental enrichment could specifically affect the behaviors of the F2 generation or, alternatively, that the observed changes in the behaviors are due to the increased male F1 body weight which in turn is caused by an EE paradigm; thus, causing an indirect effect. Elevated body weights in the F1 male offspring of paternal EE, as well as their female and male F2 offspring, suggests sexually dimorphic mechanisms mediating the transgenerational inheritance of acquired traits. However, further investigation is required to fully understand this phenomenon, the biological underpinnings, and to probe other phenotypic aspects of the F1 and F2 offspring.

Environmental enrichment was shown to program the HPA axis differentially in male and female rats (Welberg et al., 2006). Furthermore, in stressful conditions males and females cope differentially (Russo et al., 2012). Our results show that the F2 female progeny from EE grand-fathers responded to stress with elevated corticosterone levels while F2 males responded with reduced ACTH levels. These results are in line with previous transgenerational studies displaying variability in the directionality of the sex-specific effects in both humans (Bygren et al., 2001) and rodents (Dietz et al., 2011; Franklin et al., 2010; Short et al., 2016).

Previous studies have shown that environmental enrichment reduces corticosterone levels in the HPA-axis responsivity test (Sztainberg et al., 2010). Therefore, it may seem surprising, and even paradoxical, that the progeny of EE displays an opposite effect. Many studies have shown that both maternal and paternal manipulations resulted in HPA-axis dysregulation, while in other tested phenotypes only subtle effects were seen, if any (Kapoor et al., 2006; Mueller and Bale, 2008; Weaver et al., 2004; Zaidan and Gaisler-Salomon, 2015). In addition, it was previously demonstrated that, following paternal stress, offspring show reduced physiological stress response (Rodgers et al., 2013), which may also seem surprising as exposure to stress is known to increase the stress response (de Kloet et al., 2005). A possible explanation for these results is that offspring of paternal EE are equipped to thrive in an enriched environment, which may require a more active stress response system. However, when born in a standard-housed environment they respond with HPA-axis alterations that are not optimal for this environment, which may be responsible for the phenotypes described in this study. This concept, that fathers enhance their offspring fitness according to the environment which they are exposed to, has been previously suggested (Danchin et al., 2011), and may implicate an evolutionary role for epigenetic inheritance of acquired traits.

Since corticosterone levels were significantly affected, we also evaluated ACTH levels in the serum in order to further investigate the changes in the HPA axis. We observed that ACTH levels in the F2 females were not modified due to grand-paternal EE; thus, the alterations in the corticosterone levels were likely due to HPA-axis dysregulation. Altered CORT response could be explained by either changes in the adrenal response to ACTH or to altered ACTH release from the pituitary. We previously showed that the HPA-axis pathology is caused by hyper-responsivity of the adrenal cells to ACTH stimulation, but this pathophysiology was rescued when mice were exposed to EE (Du et al., 2012). Therefore, EE not only alters the adrenal response to ACTH, but may also transfer similar effects transgenerationally via epigenetic mechanisms.

4.3. Potential mechanisms mediating the transgenerational inheritance of acquired traits

It is interesting to speculate on the mechanisms of inheritance involved in the transmission of the environmental influence from F0 to F1, which could then differ in the transmission from F1 to F2. Several recent studies have provided evidence that paternal transgenerational inheritance from F0 to F1 is mediated by epigenetic modification including altered expression patterns of small non-coding (snc) RNAs in the sperm (Gapp et al., 2014; Ng et al., 2010; Rodgers et al., 2013; Short et al., 2016). The complex nature of this event is demonstrated by the range of sub-species of sncRNAs which have been implicated including microRNAs, and most recently tRNA-derived small RNAs (Chen et al., 2015; Sharma et al., 2015). There could also be environmental enrichment-induced modifications of the small RNA content in post-meiotic germ cells and this has previously been shown to modify maternal transcripts before zygote activation (Dadoune, 2009). Alternatively, emerging evidence supports a direct influence of the environment on the epigenetic profile of spermatozoa. Environmental enrichment for 28 days, which accounts for the spermatogenic cycle of *Mus musculus* (Oakberg, 1956), could be altering the microRNA content of exosomes secreted by the epididymal epithelial cells which then fuse with the mature sperm in the caudal epididymis (Nixon et al., 2015a, 2015b). Environmental enrichment may be altering the dynamics of exosomal release and the microRNA content of these epididysomes, similar to its recently demonstrated impact on peripheral blood mononuclear cells (Pusic and Kraig, 2014; Pusic et al., 2016). Thus, further investigation is required to confirm that environmental enrichment alters the microRNA content of sperm, as well as determine whether the exposure period to an enriched environment prior to mating modulates the consequential impact on offspring behavior. Nevertheless, as this is an emerging field, the precise mechanisms remain ill-defined. Further investigation of paternal epigenetic inheritance and its impact on offspring acquired traits will have major implications for our understanding of transgenerational gene-environment interactions, and there is much yet to be elucidated regarding potential epigenetic mechanisms of transmission pertaining to specific environmental signals.

5. Conclusions

Paternal environmental enrichment had significant effects on the affective behavior and stress response of their F2 offspring. These results demonstrate the occurrence of transgenerational inheritance throughout the male lineage. Our findings contribute to the emerging field of literature, focusing on the influence of the environment across generations in both rodent model studies and by human epidemiological analyses. Conclusions from the present study will inform future investigation in human cohorts and such transgenerational effects of environmental exposures may have major implications for public health.

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