

# Maternal postpartum corticosterone and fluoxetine differentially affect adult male and female offspring on anxiety-like behavior, stress reactivity, and hippocampal neurogenesis

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## ABSTRACT

Postpartum depression (PPD) affects approximately 15% of mothers, disrupts maternal care, and can represent a form of early life adversity for the developing offspring. Intriguingly, male and female offspring are differentially vulnerable to the effects of PPD. Antidepressants, such as fluoxetine, are commonly prescribed for treating PPD. However, fluoxetine can reach offspring via breast milk, raising serious concerns regarding the long-term consequences of infant exposure to fluoxetine. The goal of this study was to examine the long-term effects of maternal postpartum corticosterone (CORT, a model of postpartum stress/depression) and concurrent maternal postpartum fluoxetine on behavioral, endocrine, and neural measures in adult male and female offspring. Female Sprague–Dawley dams were treated daily with either CORT or oil and fluoxetine or saline from postnatal days 2–23, and offspring were weaned and left undisturbed until adulthood. Here we show that maternal postpartum fluoxetine increased anxiety-like behavior and impaired hypothalamic–pituitary–adrenal (HPA) axis negative feedback in adult male, but not female, offspring. Furthermore, maternal postpartum fluoxetine increased the density of immature neurons (doublecortin-expressing) in the hippocampus of adult male offspring but decreased the density of immature neurons in adult female offspring. Maternal postpartum CORT blunted HPA axis negative feedback in males and tended to increase density of immature neurons in males but decreased it in females. These results indicate that maternal postpartum CORT and fluoxetine can have long-lasting effects on anxiety-like behavior, HPA axis negative feedback, and adult hippocampal neurogenesis and that adult male and female offspring are differentially affected by these maternal manipulations.

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## 1. Introduction

According to the DSM-5, perinatal depression is defined as depression during pregnancy and the early postpartum. As with major depression, one of the most common treatments for perinatal depression is pharmacological antidepressants, such as selective serotonin reuptake inhibitors (SSRIs; Oberlander et al., 2006; Kim et al., 2014). As more women receive antidepressants

to treat perinatal depression, the population of children who have been exposed to antidepressants during the perinatal period also increases (Oberlander et al., 2006). However, maternal SSRI use may be problematic as SSRIs such as fluoxetine (Prozac) can cross the placental barrier (Hendrick et al., 2003) and pass into breast milk (Wisner et al., 1996; Weissman et al., 2004), potentially affecting the developing offspring. Indeed, perinatal SSRI exposure is associated with adverse outcomes in the infant such as reduced weight gain (Chambers et al., 1999), levels of reelin required for normal brain development (Brummelte et al., 2013), psychomotor scores during the first year (Santucci et al., 2014), increased hypertension (Chambers et al., 2006), cardiac defects (Malm et al., 2011), and risk for autism (Croen et al., 2011). However, the

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negative effects of perinatal fluoxetine may outweigh the detrimental effects of untreated maternal depression on child development. Specifically, children of mothers with postpartum depression (PPD) are more likely to develop depression, anxiety, and attention deficits even long after the mother's depression has remitted (Pilowsky et al., 2006; Murray et al., 2011). Thus, the potential therapeutic effect of maternal SSRIs may mitigate these negative effects on child development. In fact, maternal SSRI use is associated with enhanced infant readiness to interact with their mother (3 mo infants; Weikum et al., 2013b), accelerated perceptual development (6 mo and 10 mo infants; Weikum et al., 2012), and improved executive function (6 yo; Weikum et al., 2013a). However, it is unclear whether the effects of maternal fluoxetine are advantageous in the long term or precede negative behavioral outcomes that emerge later in life. This study aims to fill this gap.

Preclinical research investigating the long term effects of perinatal fluoxetine on emotional behavior has yielded mixed results, likely due to methodological differences including timing and method of administration. For example, direct administration of fluoxetine to pups during the postnatal period increased anxiety-like behavior (Yu et al., 2014), while maternal exposure to fluoxetine (gestation and lactation) resulted in no significant effect on anxiety-like behavior in adult offspring (Lisboa et al., 2007; Francis-Oliveira et al., 2013). Additionally, direct administration of fluoxetine to pups during the postnatal period decreased depressive-like behavior in adult rats (Mendes-da-Silva et al., 2002) whereas maternal fluoxetine (gestation and postpartum) increased depressive-like behavior in adult female but not male mice offspring (Lisboa et al., 2007). In addition, the current state of research examining neonatal fluoxetine exposure is hindered by a general lack of preclinical research investigating maternal fluoxetine exposure within a model of depression or PPD. Because mothers typically use SSRIs to treat depression, there is a need for preclinical research to address how maternal fluoxetine influences offspring within a concurrent model of depression or stress in order to contribute valid conclusions regarding the use of SSRIs to treat PPD. To this end, there are a few studies examining how gestational stress followed by maternal postpartum fluoxetine normalizes immobility in the forced swim test in adolescent male and female offspring (Rayen et al., 2011) as well as blunts serum corticosterone (CORT; primary glucocorticoid in rats) levels in adolescent male, but not female, offspring (Pawluski et al., 2012c). However, gestational stress did not result in a depressive phenotype in the dam in this study (Pawluski et al., 2012b), so it is unclear whether these results can be interpreted as modeling maternal depression. Moreover, it is unknown how modeling depression and antidepressant treatment occurring exclusively in the postpartum affect offspring development. This is an important problem to investigate because approximately 40% of perinatal depression arises solely in the postpartum period (Wisner et al., 2013) and treatment and outcome for mother and child differ depending on the timing of depression onset (Cooper and Murray, 1995). Thus, there is a need to study postpartum antidepressant treatment in animal models of depression based on postpartum and antenatal depression, respectively.

The hippocampus exhibits morphological alterations long after exposure to developmental stress (reviewed in Korosi et al., 2012; reviewed in Loi et al., 2014). Although maternal depression does not predict significant changes in hippocampal volume in children (Lupien et al., 2011), childhood maltreatment (Chaney et al., 2014) and low maternal bonding (Buss et al., 2007) are associated with reduced hippocampal volume in adulthood, which both may be present in PPD. Reduction in hippocampal volume can be attributed to a number of factors such as lower levels of hippocampal neurogenesis. Broadly speaking, stress reduces adult hippocampal

neurogenesis depending on age at the time of stress exposure and sex of the subject (reviewed in Gobinath et al., 2014). For example, maternal deprivation diminished expression of doublecortin (an endogenous protein expressed in immature neurons) in adult male but not female rat offspring (Oomen et al., 2010, 2011). Furthermore, adult hippocampal neurogenesis may play an important role in the etiology of mood-related disorders such as depression (reviewed in DeCarolis and Eisch, 2010; reviewed in Eisch and Petrik, 2012), as well as regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Snyder et al., 2011). Despite evidence that antidepressants can normalize HPA axis activity (Ising et al., 2007) and increase hippocampal neurogenesis (Malberg et al., 2000; Santarelli et al., 2003; Boldrini et al., 2009; Epp et al., 2013), little is known about how maternal fluoxetine affects HPA axis and adult neurogenesis in the hippocampus of offspring beyond the time they are exposed to the drug. Maternal postpartum fluoxetine reversed the detrimental effects of prenatal stress on hippocampal doublecortin expression in both male and female adolescent rat offspring (Rayen et al., 2011). However, by adulthood, maternal postpartum fluoxetine only diminished doublecortin expression after prenatal stress exposure, particularly in adult male offspring (Rayen et al., 2015). Thus, hippocampal neurogenesis represents a neurobiological intersection of developmental exposure to stress, antidepressants, and adult behavioral outcomes and will be investigated in the present study.

We have previously shown that chronic CORT administered to the dam postpartum increases maternal depressive-like behavior and diminishes maternal care (Brummelte et al., 2006, 2010; Brummelte and Galea, 2010; Workman et al., 2013b, submitted). Interestingly, maternal postpartum CORT decreases hippocampal cell proliferation in male offspring at weaning (Brummelte et al., 2006) and increases anxiety-like behavior in adolescent male, but not female, offspring (Brummelte et al., 2012). However, it is unclear whether these sex differences or effects on offspring brain and behavior persist when the dam is exposed to concurrent maternal antidepressant exposure. The present study investigates whether high levels of maternal postpartum CORT and concurrent fluoxetine administered to dams differentially affect adult male and female offspring outcome at the behavioral (anxiety- and depression-like behavior, locomotion), endocrine (HPA axis dysregulation), and neural (doublecortin expression) levels. We hypothesized that maternal postpartum fluoxetine would negatively affect behavior, HPA axis regulation, and hippocampal neurogenesis in the affected adult offspring. Further, we expect that both sexes will be differentially affected by maternal postpartum fluoxetine and CORT.

## 2. Materials and methods

### 2.1. Animals

Thirty-two adult female Sprague–Dawley rats (2–3 months old) and 16 adult male Sprague–Dawley rats (2–3 months old, Charles River) were initially housed in same-sex pairs in opaque polyurethane bins (24 × 16 × 46 cm) with aspen chip bedding. Rats were maintained in a 12 h: 12 h light/dark cycle (lights on at 7:00 a.m.) and given rat chow (Jamieson's Pet Food Distributors Ltd, Delta, BC, Canada) and tap water ad libitum. All protocols were in accordance with ethical guidelines set by Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee.

### 2.2. Breeding procedures

For breeding, males were single housed and two females and one male were paired daily between 5:00 and 7:00 P.M. Females

were vaginally lavaged each morning between 7:30 and 9:30 A.M. and samples were assessed for the presence of sperm. Upon identification of sperm, females were considered pregnant, weighed, and single housed into clean cages with autoclaved paper towels and an enrichment tube.

One day after birth (birth day = postnatal day 0), all litters were culled to 5 males and 5 females. If there were not enough males or females in one litter, pups were cross-fostered from a dam that gave birth the same day. If there were not enough pups available to support a 5 male and 5 female litter, then dams maintained a sex-skewed or smaller litters (this happened twice with both being in the CORT/saline group). Dams were randomly assigned to one of four treatment groups: 1) CORT/fluoxetine; 2) CORT/saline; 3) Oil/fluoxetine; 4) Oil/saline. Beginning on postpartum day 2, dams received two daily injections of either subcutaneous CORT (40 mg/kg) or sesame oil (1 ml/kg) and intraperitoneal fluoxetine (10 mg/kg) or saline (1 ml/kg) for 22 consecutive days. The effects of maternal postpartum CORT/saline on depressive-like behavior were verified in the dam (Workman et al., 2013b, submitted), and data investigating maternal outcome will be published separately (Workman et al., 2013b, submitted). Dams received both injections in succession between 11 A.M. and 2 P.M. Pups were weaned on postpartum day 24 and pair-housed with an unrelated, same-sex cage mate whose mother received the same treatment. No more than 2 males and 2 females were taken from each litter for the behavioral tests. Besides weekly cage changing, offspring remained undisturbed until behavioral testing.

### 2.3. Drug preparation

An emulsion of CORT (Sigma–Aldrich, St. Louis, MO, USA) was prepared every 2–3 days by mixing CORT with ethanol and then adjusting with sesame oil to yield a final concentration of 40 mg/ml of CORT in oil with 10% ethanol. The dose was chosen because it reliably induces a depressive-like phenotype in dams, impairs maternal care, and affects offspring development (Brummelte et al., 2006, 2010; Brummelte and Galea, 2010; Brummelte et al., 2012; Workman et al., 2013a). Fluoxetine (Sequoia Research Products, Pangbourne, UK) was prepared every 2–3 days by dissolving in dimethyl sulfoxide (DMSO; Sigma Aldrich) and adjusting with 0.9% saline to yield a final concentration of 10 mg/ml fluoxetine in saline with 10% DMSO. This dose of fluoxetine was chosen based on work illustrating that this dose increased brain derived neurotrophic factor and cell proliferation in the hippocampus and amygdala after 21 days of injections in both male and female rodents (Hodes et al., 2010). Control dams were given two vehicle injections: “oil” consisted of 10% ethanol in sesame oil to control for the CORT injections, and “saline” consisted of 10% DMSO in 0.9% saline to control for the fluoxetine injections.

For the dexamethasone suppression test, a solution of dexamethasone (Sigma Aldrich) was prepared 1–2 days prior to the test by dissolving dexamethasone in propylene glycol and adjusted to yield a final dose of 50 ug/kg dexamethasone in propylene glycol. This dose and timing of dexamethasone injection were chosen

based on previous studies (Cole et al., 2000).

### 2.4. Behavioral testing

Beginning at postnatal day  $65 \pm 2$ , 6–10 male and female rats per group underwent behavioral testing (elevated plus maze, open field test, forced swim test, and novelty suppressed feeding). Based on the four maternal treatments described above, rats from each of the following groups (60 rats total) were utilized: Adult male Oil/Saline offspring,  $n = 8$ ; Adult male Oil/Fluoxetine offspring,  $n = 6$ ; Adult male CORT/Saline offspring,  $n = 6$ ; Adult male CORT/Fluoxetine offspring,  $n = 10$ ; Adult female Oil/Saline offspring,  $n = 9$ ; Adult female Oil/Fluoxetine offspring,  $n = 6$ ; Adult female CORT/Saline offspring,  $n = 6$ ; Adult female CORT/Fluoxetine offspring,  $n = 9$ . Behavioral tests were conducted in the same order for all the animals with 48 h between each test. Behavioral testing occurred at 9:00 A.M. each day under dim light conditions (approximately 12 lux). Twenty-four h after the final behavioral test, rats underwent a dexamethasone-suppression test under standard bright light conditions (approximately 180 lux). Seventy-two h after dexamethasone suppression test, all rats were perfused and brain tissue was collected. For an overview of experimental procedures, refer to Fig. 1.

#### 2.4.1. Elevated plus maze

The elevated plus maze was used to evaluate anxiety-like behavior in male and female offspring. Briefly, the apparatus consists of two open arms bisected by two closed arms (arm length: 50 cm; arm width: 10 cm; arm wall height: 40 cm). Rats were placed into the center of the apparatus, facing the open arm. Each test session lasted 5 min and was video recorded. The apparatus was cleaned using a 15% vinegar solution between each testing session to remove any odors or waste. The numbers of entries (all four paws entering an arm) into the open arm and closed arm as well as time (in seconds) spent in the open arm and closed arm were analyzed. Ratio of time spent in the closed arm versus the open arms and center was used as an index of anxiety as previously described (Brummelte et al., 2012).

#### 2.4.2. Open field test

The open field test was used to assess general locomotor activity as previously described (Brummelte et al., 2006). The apparatus, a  $90 \times 90 \times 40$  cm square arena divided into 16 squares of equal dimension, was placed in a dimly lit room. Rats were placed in the apparatus facing the same corner and video recorded for 10 min. The apparatus was cleaned using a 15% vinegar solution between each testing session to remove any odors or waste. A line crossing was defined as all four paws crossing a gridline (Brummelte et al., 2006). Total number of line crossings was used as an index of general locomotion.

#### 2.4.3. Forced swim test

Approximately 48 h after open field test, rats were tested in the forced swim test to assess depressive-like behavior. A glass

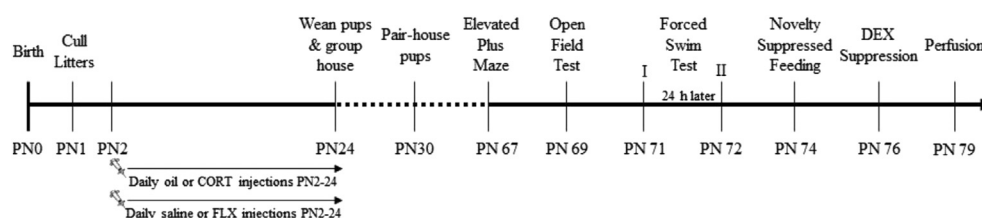


Fig. 1. Timeline. CORT: corticosterone; DEX: dexamethasone; FLX: fluoxetine; PN: postnatal day.

cylindrical tank (45 × 28 cm) filled to a depth of approximately 30 cm of tap water  $25 \pm 1$  °C. For the first session, rats were placed into the water for 15 min. The second session took place 24 h later and rats were placed into the water for 5 min. Water was replaced between each rat. An observer blind to treatment conditions scored the sessions for percent time spent swimming, climbing, or immobile using BEST Collection Software (Educational Consulting, Inc., Hobe Sound, FL, USA).

#### 2.4.4. Novelty suppressed feeding

Approximately 48 h after forced swim test, rats were tested for anxiety-like behavior in the novelty suppressed feeding paradigm. In this test, rats must resolve an anxiogenic conflict of entering the center of arena to access a morsel of chow after being food deprived (Bodnoff et al., 1989; Santarelli et al., 2003; Bessa et al., 2009; Leuner et al., 2010). Food was removed from rats' cages 16 h prior to testing to incite motivation to consume food during the test. Each rat was placed in a square arena (60 × 60 cm) facing the right corner. Latency to feed was recorded in seconds as an index of anxiety-like behavior. The trial was terminated either after the rat began to eat or after 10 min if the rat did not eat. Lab chow was added to the cages after testing, and food consumption was measured in each cage 1 h after test to assess whether feeding behavior was altered by maternal postpartum CORT or fluoxetine.

#### 2.4.5. Dexamethasone suppression test

Approximately 48 h after novelty suppressed feeding, rats were tested for HPA axis negative feedback using the dexamethasone suppression test. Dexamethasone was administered to all rats subcutaneously 90 min prior to a 30 min restraint stressor. Tail blood samples were collected at the beginning of restraint ( $t = 0$ ), the end of restraint ( $t = 30$ ), and 1 h after cessation of restraint ( $t = 90$ ).

#### 2.5. Tissue collection

Approximately 72 h after dexamethasone suppression test, rats were weighed and then given an overdose of Euthanyl. Rats were perfused with 60 ml cold 0.9% saline followed by 120 ml cold 4% paraformaldehyde. Brains were extracted and postfixed using 4% paraformaldehyde overnight at 4 °C. Brains were then transferred to 30% sucrose in phosphate buffer at 4 °C until they sank to the

bottom. Brains were rapidly frozen with dry ice and sectioned using a freezing microtome (Leica, Richmond Hill, ON, Canada) at 40  $\mu$ m and collected in series of 10. Sections were stored in antifreeze (ethylene glycol/glycerol; Sigma) and stored at  $-20$  °C until processing.

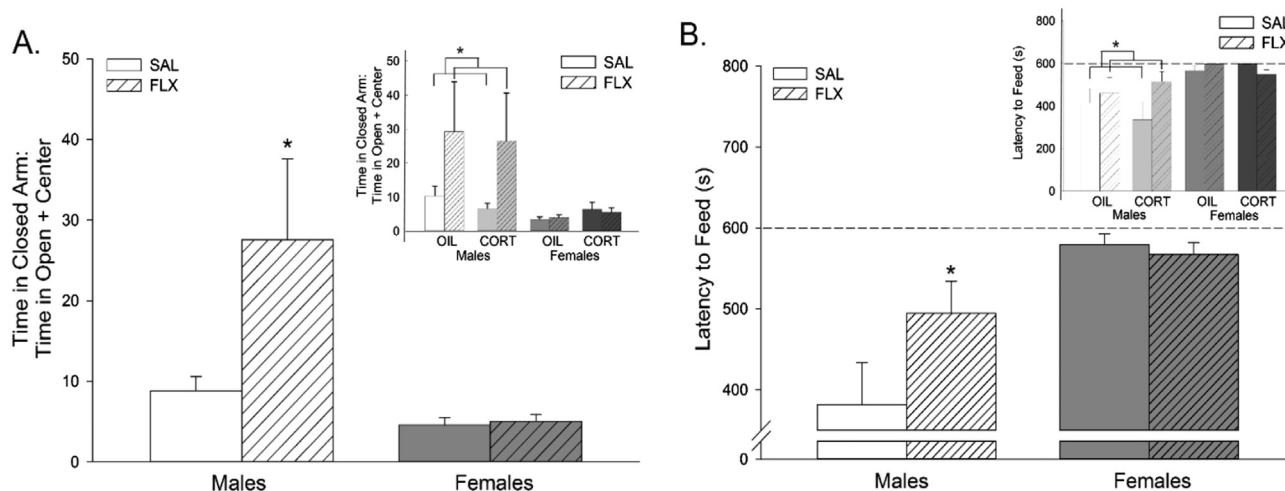
#### 2.6. Corticosterone assay

Blood samples were stored overnight at 4 °C to allow blood to clot completely. Blood was then centrifuged at 10,000 g for 15 min. The serum was collected and stored at  $-20$  °C until radioimmunoassay. Total CORT (bound and free) was measured using the ImmChem Double Antibody 125I radioimmunoassay Kit (MP Biomedicals, Solon, OH, USA). The antiserum cross-reacts 100% with CORT, 0.34% with deoxycorticosterone, 0.05% with cortisol, and does not cross-react with dexamethasone ( $<0.01\%$ ). All reagents were halved and samples run in duplicate.

#### 2.7. Doublecortin immunohistochemistry

Sections were rinsed  $5 \times 10$  min in 0.1 M phosphate buffered saline (PBS), treated with 0.3% hydrogen peroxide in dH<sub>2</sub>O for 30 min, and incubated at 4 °C in primary antibody solution: 1:1000, goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 0.04% Triton-X in PBS and 3% normal rabbit serum for 24 h. Sections were then rinsed  $5 \times 10$  min in 0.1 M PBS and transferred to a secondary antibody solution with 1:500, rabbit anti-goat (Vector Laboratories, Burlington, ON, Canada) in 0.1 M PBS for 24 h at 4 °C. Then, sections were washed  $5 \times 10$  min in 0.1 M PBS and incubated in ABC complex (ABC Elite Kit; 1:1000; Vector) for 4 h. Sections were then washed in 0.175 M sodium acetate buffer  $2 \times 2$  min. Finally, sections were developed using diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate Kit, Vector), mounted on slides, and dried. Sections were then counterstained with cresyl violet, dehydrated, and coverslipped with Permount (Fisher).

Doublecortin-expressing cells were quantified in 3 dorsal sections ( $-2.76$  mm to  $-4.68$  mm below bregma) and 3 ventral sections ( $-5.52$  mm to  $-6.60$  mm below bregma) using the 40 $\times$  objective using an Olympus CX22LED brightfield microscope. Areas of these sections were quantified using ImageJ (NIH, Bethesda, MD, USA) and used for density calculations (number of cells per mm<sup>2</sup>).



**Fig. 2.** Anxiety-like behavior as measured by (A) ratio of time spent in closed arms compared to center and open arms (mean + SEM) in elevated plus maze and (B) latency to feed (mean + SEM) in novelty suppressed feeding task. Maternal postpartum FLX increased ratio of time spent in the closed arms versus open arms and center of elevated plus maze and increased latency to feed in novelty suppressed feeding task in comparison to maternal postpartum saline in adult male offspring only. Dashed line in (B) represents end of test session (600 s). \* denotes  $p < 0.05$ .  $n = 6-10$ /group/sex. CORT: corticosterone. FLX: fluoxetine. SAL: saline.



Two hundred cells positively labeled for doublecortin (100 dorsal and 100 ventral) were randomly selected and categorized as either proliferative (no process or short process), intermediate (medium process with no branching), or post-mitotic (strong dendrite branching in the molecular layer or delicate dendritic tree branching present in the granule cell layer) based on previously published criteria (Plümpe et al., 2006; Workman et al., 2015a,b; see Fig. 7A–C).

## 2.8. Data analyses

Data collected from the elevated plus maze test, open field test, and novelty suppressed feeding task were analyzed using ANOVA with sex, maternal postpartum CORT, and maternal postpartum fluoxetine as between-subjects factors. Behavior in the elevated plus maze was analyzed using repeated measures ANOVA with arm of maze (closed and open arm) as the within-subjects factor. Behavior in open field test was analyzed using repeated measures ANOVA with area of maze (center, periphery) as within-subjects factor. Behavior in the forced swim test was analyzed using repeated measures ANOVA with behavior (percent time climbing, swimming, and immobile) as the within-subjects factor. CORT concentrations from the dexamethasone suppression test were analyzed using repeated measures ANOVA with time ( $t = 0$ , beginning of restraint;  $t = 30$ , end of restraint;  $t = 90$ , 1 h after restraint ended) as the within-subjects factor. The density of doublecortin-expressing cells was analyzed using repeated measures ANOVA with region (dorsal, ventral) as the within-subjects factor. Morphology of doublecortin-expressing cells was analyzed using repeated measures ANOVA with region (dorsal, ventral) and type of cell (proliferative, intermediate, post-mitotic) as the within-subjects factor. Post hoc comparisons used Newman–Keuls. Because we had hypotheses that there would be interactions between sex, CORT, and fluoxetine, *a priori* comparisons were subjected to Bonferroni corrections. All data were analyzed using Statistica software (v. 9, StatSoft, Inc., Tulsa, OK, USA). All effects were considered statistically significant if  $p \leq 0.05$ , trends are discussed if  $p \leq 0.10$ .

## 3. Results

### 3.1. Maternal postpartum fluoxetine increased anxiety-like behavior in the elevated plus and novelty suppressed feeding task in adult male, but not female, offspring

In the elevated plus maze, maternal postpartum fluoxetine increased the ratio of time spent in the closed arms versus open arms + center in comparison to maternal postpartum saline in adult male (*a priori*;  $p = 0.023$ ), but not female offspring ( $p = 0.946$ ; Fig. 2A). Overall males had a higher ratio of time spent in the closed arms versus open arms + center compared to females (main effect

**Table 2**

Mean ( $\pm$ SEM) food consumption per cage 1 h after novelty suppressed feeding task. Males overall ate more than females within an hour of being returned to their home cage (\* $p < 0.001$ ). CORT: corticosterone; FLX: fluoxetine; SAL: saline.

	Males*	Females
Maternal OIL/SAL	23.00 $\pm$ 3.65	8.40 $\pm$ 1.97
Maternal CORT/SAL	22.00 $\pm$ 5.69	7.67 $\pm$ 0.67
Maternal OIL/FLX	17.33 $\pm$ 1.45	4.33 $\pm$ 2.33
Maternal CORT/FLX	18.50 $\pm$ 0.65	8.40 $\pm$ 0.93

of sex;  $p = 0.027$ ). There was a trend for maternal postpartum fluoxetine to increase ratio of time spent in closed arms versus open arms + center compared to maternal saline in adult male, but not female, offspring (interaction between sex and fluoxetine;  $F(1, 52) = 2.78$ ;  $p = 0.099$ ) but no other significant main or interaction effects (all  $p$ 's  $> 0.10$ ). Males spent more time in the closed arms in comparison to females (interaction between arm of maze and sex;  $F(1, 52) = 867.7$ ;  $p = 0.02$ ; Table 1). There were no other significant main or interaction effects for time in open and closed arms (all  $p$ 's  $> 0.14$ ). Females had more arm entries into closed arms in comparison to males (interaction between arm of maze and sex;  $F(1, 52) = 13.21$ ;  $p < 0.001$ ) regardless of maternal postpartum CORT or fluoxetine. Maternal postpartum CORT increased closed arm entries compared to maternal postpartum oil (interaction between arm, maternal postpartum CORT, maternal postpartum FLX;  $F(1, 52) = 4.76$ ;  $p = 0.034$ ; Table 1) within saline exposed offspring ( $p = 0.05$ ) but not fluoxetine exposed offspring ( $p = 0.83$ ). There were no other significant main or interaction effects for arm entries (all  $p$ 's  $> 0.11$ ).

In the novelty suppressed feeding task, maternal postpartum fluoxetine increased latency to feed compared with maternal postpartum saline in adult male (*a priori*;  $p = 0.023$ ), but not female offspring ( $p = 0.801$ ; Fig. 2B). Females had longer latencies to feed than males (main effect of sex;  $p < 0.001$ ) and there was a trend for maternal postpartum fluoxetine to increase latency to feed in comparison to maternal postpartum saline in adult males only (interaction between maternal fluoxetine and sex;  $F(1, 52) = 3.08$ ;  $p = 0.085$ ). There were no other significant main or interaction effects ( $p$ 's  $> 0.086$ ). Lastly, males ate more than females within an hour of returning to their home cage (main effect of sex;  $p < 0.001$ ; Table 2).

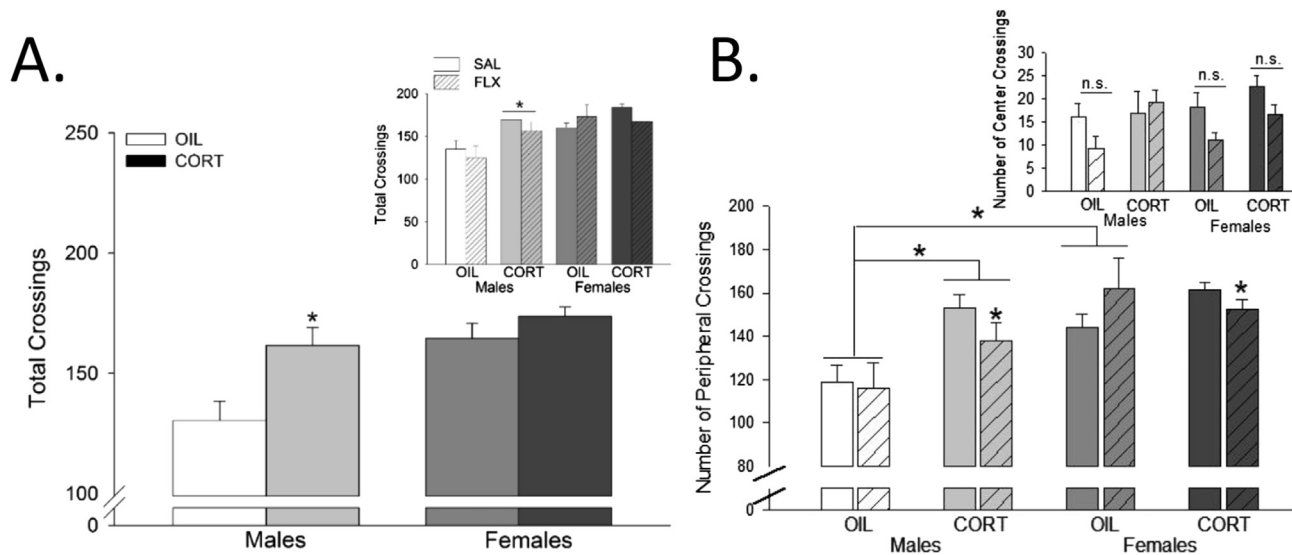
### 3.2. Maternal postpartum CORT increased total locomotor activity and peripheral crossings in adult male, but not female, offspring in the open field test. Maternal CORT/fluoxetine decreased peripheral crossings

Maternal postpartum CORT increased total crossings in the open field test compared to maternal postpartum oil in adult male (*a priori*;  $p = 0.002$ ), but not female offspring ( $p = 0.383$ ; Fig. 3A). Females made more total crossings than males (main effect of sex;

**Table 1**

Mean  $\pm$  SEM of additional variables in the elevated plus maze. Males overall spent more time in the closed arms and made fewer closed arm entries ( $p < 0.05$ ). Maternal CORT/saline increased closed arm entries in comparison to maternal oil/saline ( $p < 0.05$ ). \* denotes  $p < 0.05$ . CORT: corticosterone; FLX: fluoxetine; SAL: saline.

		Mean percent time in the open arms $\pm$ SEM	Mean percent time in the closed arms $\pm$ SEM	Mean open arm entries $\pm$ SEM	Mean closed arm entries $\pm$ SEM
Male	Maternal OIL/SAL	3.04 $\pm$ 0.95	86.92 $\pm$ 2.73*	2.50 $\pm$ 0.73	8.63 $\pm$ 0.68
	Maternal CORT/SAL	3.94 $\pm$ 2.31	83.44 $\pm$ 4.22*	1.83 $\pm$ 0.40	10.67 $\pm$ 1.38*
	Maternal OIL/FLX	3.22 $\pm$ 1.42	92.44 $\pm$ 2.37*	1.17 $\pm$ 0.48	8.33 $\pm$ 0.56
	Maternal CORT/FLX	6.47 $\pm$ 3.11	86.80 $\pm$ 3.95*	1.60 $\pm$ 0.40	9.30 $\pm$ 0.79
Female	Maternal OIL/SAL	9.33 $\pm$ 3.23	72.26 $\pm$ 4.50	2.00 $\pm$ 0.55	10.44 $\pm$ 0.85
	Maternal CORT/SAL	4.50 $\pm$ 1.61	82.83 $\pm$ 3.13	1.17 $\pm$ 0.40	12.17 $\pm$ 0.95*
	Maternal OIL/FLX	4.06 $\pm$ 1.33	78.94 $\pm$ 2.51	1.33 $\pm$ 0.49	12.17 $\pm$ 1.35
	Maternal CORT/FLX	6.89 $\pm$ 3.11	79.70 $\pm$ 4.56	1.44 $\pm$ 0.34	11.00 $\pm$ 0.41



**Fig. 3.** Locomotor behavior as measured by total crossings (mean + SEM) in open field test ( $n = 6-10/\text{group}/\text{sex}$ ). Maternal postpartum CORT increased ambulation in adult male offspring only (A). Maternal postpartum oil-exposed males had fewer peripheral crossings in comparison to maternal postpartum CORT-exposed males and oil-exposed females. Maternal postpartum CORT/fluoxetine diminished peripheral crossings in comparison maternal postpartum CORT/saline (B). There were no significant effects on center crossings (see inset in B). \* denotes  $p < 0.05$ . CORT: corticosterone. FLX: fluoxetine. SAL: Saline.

**Table 3**

All animals spent more time in the periphery than in the center of the open field (\* $p < 0.001$ ). CORT: corticosterone; FLX: fluoxetine; SAL: saline.

		Mean percent time in the periphery $\pm$ SEM (*)	Mean percent time in the center $\pm$ SEM
Male	Maternal OIL/SAL	96.17 $\pm$ 1.34	3.83 $\pm$ 0.47
	Maternal CORT/SAL	95.29 $\pm$ 3.29	4.71 $\pm$ 1.34
	Maternal OIL/FLX	97.45 $\pm$ 0.66	2.55 $\pm$ 0.66
	Maternal CORT/FLX	94.30 $\pm$ 0.86	5.69 $\pm$ 0.86
Female	Maternal OIL/SAL	94.79 $\pm$ 0.91	5.21 $\pm$ 0.91
	Maternal CORT/SAL	94.86 $\pm$ 0.64	5.14 $\pm$ 0.64
	Maternal OIL/FLX	96.01 $\pm$ 1.09	3.99 $\pm$ 1.09
	Maternal CORT/FLX	96.20 $\pm$ 0.62	3.80 $\pm$ 0.62

**Table 4**

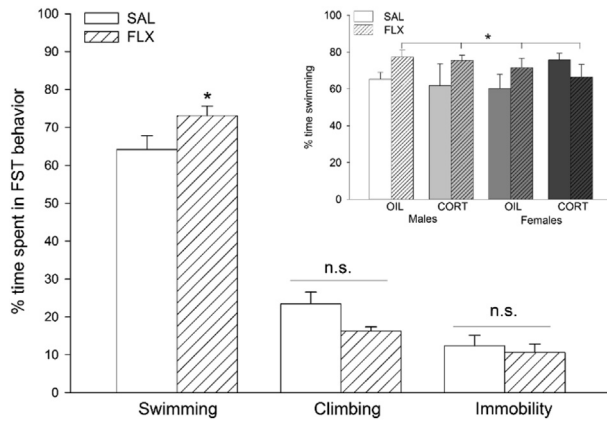
Maternal postpartum fluoxetine increased percent time swimming in comparison to maternal postpartum saline during day 1 of the forced swim test (\* $p < 0.001$ ). CORT: corticosterone; FLX: fluoxetine; SAL: saline.

		Mean percent time climbing $\pm$ SEM	Mean percent time immobility $\pm$ SEM	Mean percent time swimming $\pm$ SEM
Male	Maternal OIL/SAL	29.22 $\pm$ 2.09	34.26 $\pm$ 4.00	36.52 $\pm$ 3.82
	Maternal CORT/SAL	30.63 $\pm$ 4.26	36.54 $\pm$ 3.98	32.84 $\pm$ 5.02
	Maternal OIL/FLX	22.99 $\pm$ 3.21	43.63 $\pm$ 4.01	33.38 $\pm$ 5.08*
	Maternal CORT/FLX	19.33 $\pm$ 1.90	33.70 $\pm$ 4.90	46.97 $\pm$ 5.28*
Female	Maternal OIL/SAL	25.16 $\pm$ 3.46	33.72 $\pm$ 5.31	44.12 $\pm$ 5.88
	Maternal CORT/SAL	34.66 $\pm$ 7.55	27.65 $\pm$ 3.88	37.69 $\pm$ 7.18
	Maternal OIL/FLX	31.10 $\pm$ 2.00	18.14 $\pm$ 4.98	50.75 $\pm$ 4.31*
	Maternal CORT/FLX	28.51 $\pm$ 1.82	28.85 $\pm$ 5.37	42.65 $\pm$ 5.53*

$p < 0.001$ ) and maternal postpartum CORT increased total crossings in comparison to maternal postpartum oil controls (main effect of maternal postpartum CORT;  $p = 0.003$ ). There were no other significant main or interaction effects were present for total crossings (all  $p$ 's  $> 0.077$ ).

Maternal postpartum CORT increased peripheral crossings in comparison to maternal postpartum oil in adult males ( $p < 0.0001$ ) but not adult females ( $p = 0.40$ ; interaction between area, sex, and maternal CORT;  $F(1, 52) = 4.28$ ;  $p = 0.04$ ; Fig. 3B). Furthermore maternal postpartum fluoxetine decreased peripheral crossings in comparison to maternal postpartum saline (interaction between

area, maternal CORT and maternal fluoxetine;  $F(1, 52) = 4.73$ ;  $p = 0.034$ ; Fig. 3B) only within the CORT-exposed offspring ( $p = 0.032$ ) but not oil-exposed offspring ( $p = 0.24$ ). There were no significant differences in center crossings ( $p$ 's  $> 0.22$ ). Animals spent a higher percent time in the periphery of the open field than in the center (main effect of area;  $p < 0.0001$ ). There were no other statistically significant main or interaction effects for percent time in periphery or center (all  $p$ 's  $> 0.09$ ; Table 3).



**Fig. 4.** Percent time spent swimming, climbing, and immobile (mean  $\pm$  SEM) in forced swim test in both males and females during day 2 of forced swim test ( $n = 6-10$ /group/sex). \* denotes  $p < 0.05$ . CORT: corticosterone. FLX: fluoxetine. FST: forced swim test. N.S.: non-significant effect. SAL: saline.

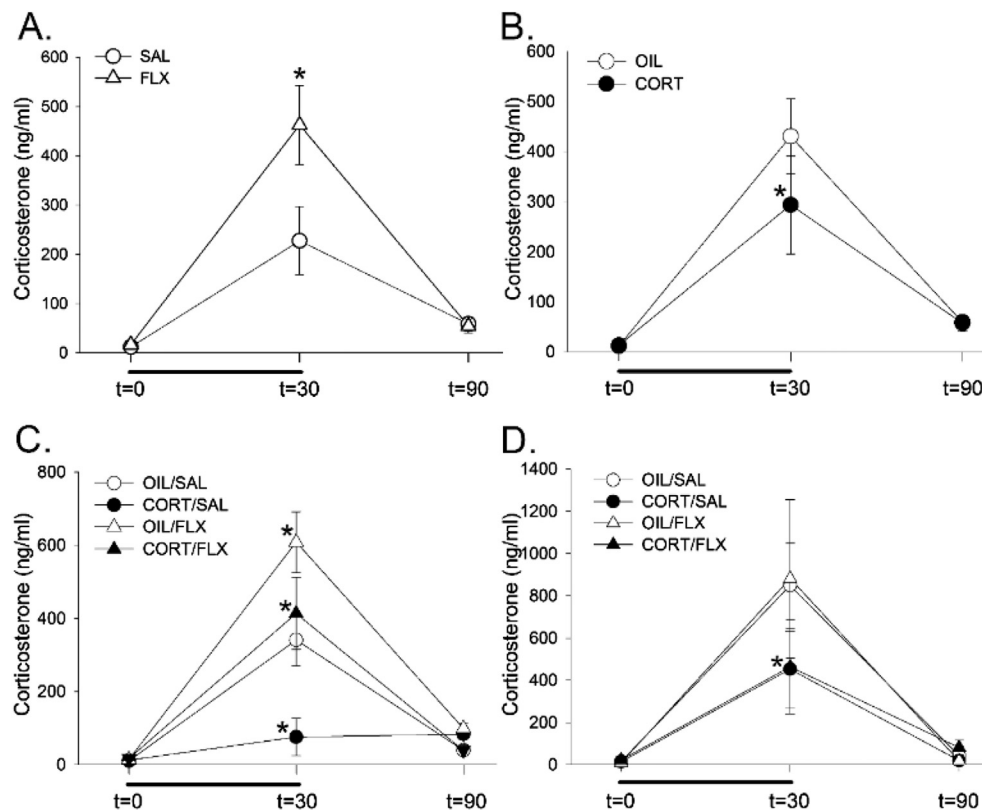
### 3.3. Maternal postpartum fluoxetine increased time spent swimming in the forced swim test in both adult male and female offspring

Maternal postpartum fluoxetine increased time spent swimming compared with maternal postpartum saline, regardless of maternal postpartum CORT during day 2 of the forced swim test (interaction between maternal fluoxetine and behavior type;  $F(2,$

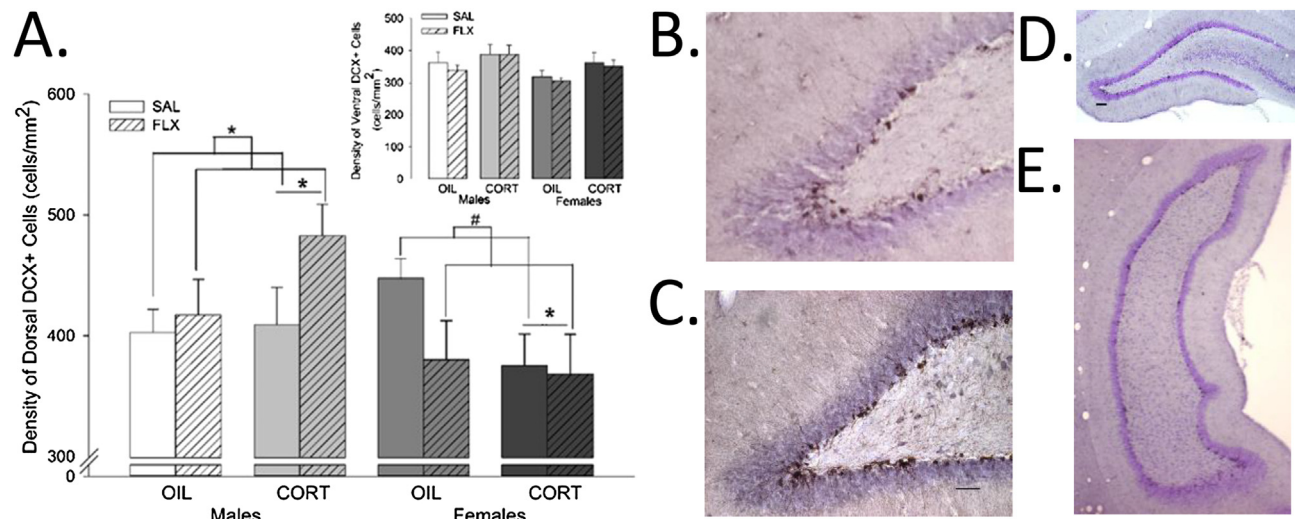
104) = 4.497;  $p = 0.013$ ; Fig. 4). There were no other significant main or interaction effects on any other forced swim test behaviors (all  $p$ 's  $> 0.146$ ). To determine if this effect on swimming behavior was affected by day, we further analyzed percent time swimming with a repeated measures ANOVA using day (day 1, day 2) as a within factor. Maternal postpartum fluoxetine increased percent time spent swimming regardless of day, sex or maternal postpartum CORT (main effect of fluoxetine;  $F(1,52) = 5.721$ ,  $p = 0.02$ ; Table 4, Fig. 4). Additionally, animals had a higher percent swimming on day 2 than day 1 (main effect of day;  $F(1, 52) = 176.75$ ,  $p < 0.0001$ ; Fig. 4 versus Table 4).

### 3.4. Maternal postpartum fluoxetine impaired HPA axis negative feedback only in adult male offspring in the dexamethasone suppression test. Maternal postpartum CORT enhanced HPA axis negative feedback in both adult male and female offspring

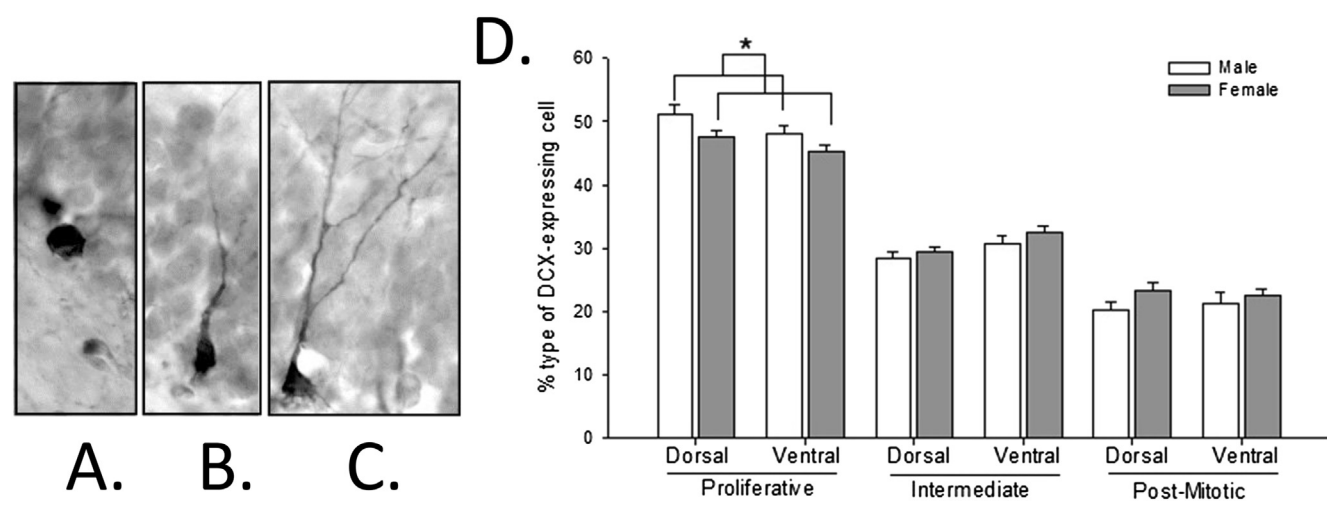
Male and female offspring were analyzed separately, due to the well-established sex differences in HPA axis regulation (reviewed in Viau, 2002). In adult male offspring, maternal postpartum fluoxetine exaggerated male offspring CORT release at  $t = 30$  in comparison to maternal saline male controls (interaction between time and maternal fluoxetine;  $F(2,22) = 8.05$ ;  $p = 0.002$ ; Fig. 5A). Furthermore, in adult male offspring, maternal postpartum CORT blunted serum CORT release at  $t = 30$  in comparison to maternal postpartum oil male controls (interaction between time and maternal CORT;  $F(2,22) = 4.74$ ;  $p = 0.019$ ; Fig. 5B). Similarly, in adult female offspring, *a priori* comparisons revealed that maternal postpartum CORT blunted serum CORT release at  $t = 30$  in



**Fig. 5.** Serum CORT (mean  $\pm$  SEM) during dexamethasone suppression tests in males (A–C) and females (D). Maternal postpartum FLX exaggerated CORT release after restraint stress in comparison to maternal postpartum saline in adult male offspring (A). Maternal postpartum CORT blunted serum CORT release after restraint stress in comparison to maternal postpartum oil in adult male offspring (B). All four maternal experimental groups are displayed for the male offspring (C) and female offspring (D). Only  $t = 30$  was greater than all other time points. Solid black line represents 30 min of restraint stress. \* denotes  $p < 0.05$ .  $n = 6-10$ /group/sex. CORT: corticosterone. FLX: fluoxetine. SAL: saline.



**Fig. 6.** Maternal postpartum FLX increased density of dorsal doublecortin-expressing cells (mean + SEM) in the adult male offspring compared to maternal saline in the maternal CORT group only ( $p < 0.01$ ). However maternal postpartum FLX tended to decrease the density of doublecortin-expressing cells in the adult female offspring in the dorsal hippocampus compared to controls ( $p < 0.07$ ; A, Maternal postpartum CORT increased the density of doublecortin expression in males ( $p = 0.02$ ) but decreased it in females ( $p = 0.017$ ) in comparison to maternal postpartum oil. There was no significant effect of either sex or maternal postpartum FLX in the ventral hippocampus (see inset). \* denotes  $p < 0.05$ , # denotes  $p < 0.10$ . B, representative photomicrograph of adult male offspring exposed to maternal postpartum saline; C, representative photomicrograph of adult male offspring exposed to maternal postpartum fluoxetine, scale bar = 100 µm; D, representative photomicrographs of dorsal hippocampus, scale bar = 100 µm; E, representative photomicrographs of ventral hippocampus CORT: corticosterone. DCX+: doublecortin-expressing; SAL: saline. FLX: fluoxetine.



**Fig. 7.** Examples of doublecortin-expressing cells at the proliferative (A), intermediate (B), and post-mitotic stage (C). All offspring expressed a greater proportion of proliferative doublecortin-expressing cells in comparison to intermediate or post-mitotic cells (D). Males express more proliferative doublecortin-expressing cells than females regardless of region (D). \* denotes  $p < 0.05$ ; DCX: doublecortin.

**Table 5**  
Mean ( $\pm$ SEM) body mass (g). Maternal postpartum FLX alone increased body mass in comparison to maternal postpartum saline in the adult male offspring. Additionally, maternal postpartum CORT/FLX significantly diminished body mass in adult male offspring in comparison to maternal postpartum CORT or FLX alone.\* denotes  $p < 0.05$ . n = 6–10/group/sex. CORT: corticosterone; FLX: fluoxetine; SAL: saline.

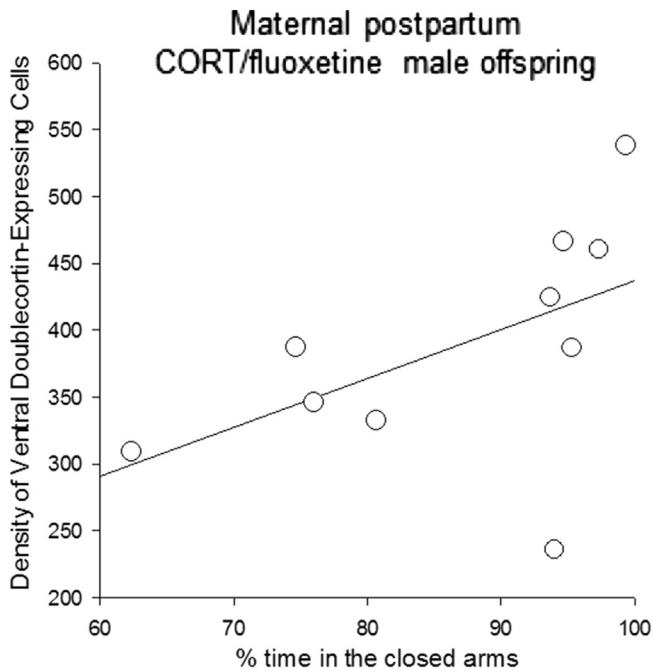
	Males	Females
Maternal OIL/SAL	503.75 $\pm$ 10.95	309.89 $\pm$ 9.38
Maternal CORT/SAL	533.50 $\pm$ 11.05	283.50 $\pm$ 17.23
Maternal OIL/FLX	554.17 $\pm$ 18.30*	298.33 $\pm$ 11.20
Maternal CORT/FLX	486.20 $\pm$ 15.63*	283.56 $\pm$ 3.88

comparison to maternal postpartum oil in the adult female offspring ( $p = 0.014$ ). No other significant main or interaction effects were present in the female offspring (all  $p$ 's  $> 0.10$ ).

**3.5. Maternal postpartum fluoxetine and maternal postpartum CORT increased the density of doublecortin-expressing cells in dorsal hippocampus but not ventral hippocampus in adult male offspring. Males had a higher proportion of proliferative doublecortin-expressing cells in comparison to females**

Maternal postpartum fluoxetine increased the density of dorsal, but not ventral, doublecortin-expressing neurons compared to maternal saline in adult males (interaction between region, sex,





**Fig. 8.** Density of doublecortin-expressing cells in ventral hippocampus was positively associated with percent time in the closed arm of the elevated plus maze in male offspring of CORT/fluoxetine-treated dams. CORT: corticosterone.

and maternal postpartum fluoxetine;  $F(1, 51) = 3.97$ ;  $p = 0.05$ ; Fig. 6A). However this effect was driven by the male offspring also exposed to maternal postpartum CORT/fluoxetine (*a priori*;  $p = 0.01$ ) but not in maternal oil/fluoxetine group ( $p = 0.79$ ). Intriguingly, the opposite effect was seen in females such that maternal postpartum fluoxetine tended to decrease the density of dorsal doublecortin-expressing immature neurons in adult females compared to maternal postpartum saline controls ( $p = 0.07$ ; Fig. 6A). Maternal postpartum CORT increased density of dorsal doublecortin-expressing cells in male offspring in comparison to maternal CORT-exposed female offspring ( $p < 0.001$ ) and to maternal postpartum oil control males (*a priori*;  $p = 0.023$ , interaction between region, sex, and maternal CORT;  $F(1,51) = 4.367$ ;  $p = 0.042$ ; Fig. 6B). Maternal postpartum CORT diminished the density of doublecortin-expressing cells in the dorsal hippocampus in the adult females in comparison to maternal postpartum oil ( $p = 0.017$ ). There was also significant interaction between region and maternal CORT ( $p = 0.033$ ), main effects of region ( $p < 0.001$ ) and sex ( $p = 0.033$ ) but no other significant main or interaction effects (all  $p$ 's  $> 0.21$ ).

We also examined the phenotype of the doublecortin-expressing cells in both the dorsal and ventral dentate gyrus. Males had significantly more proliferative doublecortin-expressing cells in comparison to females, regardless of region ( $p = 0.01$ ; interaction between sex and type of cell;  $F(2, 100) = 274.3$ ;  $p = 0.05$ ; Fig. 7D). There was a main effect of doublecortin morphology such that there were more proliferative cells compared to the other two types of cells and more intermediate cells than post-mitotic cells (all  $p < 0.0002$ ) but no other significant effects ( $p > 0.4$ ).

### 3.6. Maternal postpartum CORT/fluoxetine diminished body mass of adult male offspring

In adult male offspring only, maternal postpartum oil/fluoxetine increased body mass in comparison to maternal postpartum oil/

saline whereas maternal postpartum CORT/fluoxetine diminished mass in comparison to maternal postpartum CORT alone or fluoxetine alone (interaction between sex, CORT, and fluoxetine;  $F(1, 52) = 5.120$ ;  $p = 0.028$ ; Table 5). As expected, adult males weighed more than the adult females (main effect of sex;  $p < 0.001$ ). No other significant main or interaction effects were present (all  $p$ 's  $> 0.081$ ).

### 3.7. Density of doublecortin expression in ventral hippocampus was positively correlated with percent time spent in closed arms of the elevated plus maze in maternal postpartum CORT/fluoxetine male offspring

Among the maternal postpartum CORT/fluoxetine-exposed male offspring, time spent in the closed arms of the elevated plus maze was positively associated with density of ventral hippocampus doublecortin expression ( $r = 0.832$ ;  $p = 0.01$ ; Fig. 8). All other variables were either not significant after correcting for multiple correlations or when outliers in correlations were removed.

## 4. Discussion

Here we show that maternal exposure to fluoxetine during the postpartum period can have long-lasting effects on anxiety-like behavior, HPA axis negative feedback, and hippocampal neurogenesis in adult offspring depending on sex. In adult male offspring, maternal postpartum fluoxetine increased anxiety-like behavior in the elevated plus maze and novelty suppressed feeding test and density of doublecortin-expressing cells in the dorsal hippocampus. Maternal postpartum fluoxetine also impaired HPA axis negative feedback in males. Perhaps not surprising, both adult male and female offspring from maternal postpartum fluoxetine-treated dams exhibited increased swimming behavior in the forced swim test, indicative of enhanced serotonergic tone (Detke et al., 1995). Maternal postpartum CORT enhanced HPA axis negative feedback in both males and females, but increased locomotor behavior and hippocampal doublecortin-expressing cells in adult male offspring only. Perhaps the most striking finding in our study is that the majority of effects of maternal postpartum CORT and fluoxetine were seen in adult male offspring. This is consistent with many studies that indicate that males may be more susceptible to perturbations during early development (Stevenson et al., 2000; Kent et al., 2012). Collectively, these data reveal that maternal postpartum fluoxetine has long-lasting effects on anxiety-like behaviors, the HPA axis, and neuroplasticity in male offspring.

### 4.1. Maternal postpartum fluoxetine increased anxiety-like behavior in adult male offspring, but not female offspring, regardless of maternal postpartum CORT exposure

Maternal postpartum fluoxetine increased anxiety-like behavior in adult male but not female offspring in the elevated plus maze and the novelty suppressed feeding tests. This increase in anxiety-like behavior was not due to differences in locomotor activity as maternal postpartum fluoxetine did not affect total crossings in the open field test in either adult male or female offspring. This is in line with similar studies showing that either prenatal fluoxetine exposure (Olivier et al., 2011) or direct administration of fluoxetine to mice pups (postnatal days 2–21; Yu et al., 2014) increases latency to feed in the novelty suppressed feeding test. However, our results are the first to show that fluoxetine increases anxiety-like behavior in adult male offspring when administered to nursing dams, even with concurrent CORT exposure (a model of postpartum stress/depression). In women, maternal postpartum fluoxetine increases breast milk concentration of both fluoxetine and its active

metabolite norfluoxetine (Wisner et al., 1996). Therefore, it is possible that in nursing offspring, maternal fluoxetine exposes the developing brain to high levels of serotonin and subsequently disturbs development of the serotonin system. Indeed, developmental disturbances to the serotonin system, such as genetically knocking out the serotonin transporter or the 5HT-1a receptor, are associated with increased anxiety-like behavior (Lira et al., 2003; Lo Iacono and Gross, 2008; respectively), which is consistent with our findings. The relationship between perinatal exposure to fluoxetine and anxiety-like behavior may be related to abnormal activity of the serotonin reuptake transporter and 5-HT1a receptor, both of which are implicated in the etiology of anxiety (SERT: Sen et al., 2004; 5-HT1a: Heisler et al., 1998; Ramboz et al., 1998). Although we did not find an effect of maternal postpartum fluoxetine in adult female offspring in elevated plus maze, possible effects of maternal postpartum fluoxetine on anxiety-like behavior in the novelty suppressed feeding test could have been obscured by a ceiling effect, as most females did not feed in the 10 min trial. Further studies need to optimize this test for female rats by food depriving for longer, extending the length of the trial, or offering more palatable food (Machado et al., 2013). Moreover, in adult mice, females metabolize fluoxetine faster than males (Hodes et al., 2010; McNamara et al., 2010). Given this sex difference in fluoxetine exposure due to metabolism, it is likely that developmental fluoxetine exposure had a more potent effect on the males than in the females, resulting in larger effects of developmental fluoxetine in males than females. Finally, our results are also consistent with previous work with this model of PPD that have found that maternal postpartum CORT does not increase anxiety-like behavior in adult male or female offspring (Brummelte et al., 2006).

It should be noted that lower doses of maternal fluoxetine do not significantly affect anxiety-like behavior in either male or female offspring (7.5 mg/kg/day: Lisboa et al., 2007; 5 mg/kg/day: Francis-Oliveira et al., 2013). Additionally, differences in timing of fluoxetine administration may contribute to differences in anxiety-related outcomes as both Lisboa et al., 2007 and Francis-Oliveira et al., 2013 exposed dams during gestation and postpartum whereas the current study exposed dams only in the postpartum. These lower doses of fluoxetine may not be sufficient to alter offspring development, or there may be differences in offspring outcome if dams are treated with fluoxetine throughout gestation as well as postpartum. Furthermore, higher doses of maternal fluoxetine (25 mg/kg/day) during mid-gestation (prenatal day 15) through postpartum (postnatal day 12) decreased anxiety-like behavior in adult male (Kiryanova and Dyck, 2014) and female mice (McAllister et al., 2012). Together, this highlights the importance of dose and timing of fluoxetine as crucial methodological factors when evaluating effects of maternal fluoxetine on offspring outcome (reviewed in Kiryanova et al., 2013).

#### 4.2. Maternal postpartum fluoxetine increased serotonin-mediated behavior (swimming) in the forced swim test in both adult male and female offspring

In the present study, maternal postpartum fluoxetine increased percent time spent swimming, but not percent time spent immobile or climbing, in the forced swim test in both adult male and female offspring. Increased swimming behavior is indicative of increased serotonin activity (Detke et al., 1995). Thus, our findings suggest that maternal postpartum fluoxetine increased serotonin-mediated behavior in both adult male and female offspring. This may not be surprising given the aforementioned evidence that maternal postpartum fluoxetine increases milk concentration of fluoxetine (Wisner et al., 1996). It should be noted that another study did not find a significant effect on swimming behavior after

maternal postpartum fluoxetine (Rayen et al., 2011). However, there were differences between studies in terms of dose and administration (Rayen et al., 2011: 5 mg/kg via osmotic mini-pump) as well as age at testing (Rayen et al., 2011: adolescence). Maternal postpartum fluoxetine did not significantly alter immobility, which is inconsistent with studies showing that maternal fluoxetine (7.5 mg/kg/day) increased immobility in adult female but not male mice offspring (Lisboa et al., 2007). However, dose and species differences could account for this discrepancy, as forced swim test outcomes differ between mice and rats (Slattery and Cryan, 2012). Our results confirm previous work with this model of PPD in which maternal postpartum CORT did not significantly affect depressive-like behavior of adult offspring in the forced swim test (Brummelte et al., 2006, 2012). Our findings show that maternal postpartum fluoxetine exerts enduring changes in serotonin-related behavior, which may manifest from disturbances to the developing serotonin system following developmental exposure to fluoxetine.

#### 4.3. Maternal postpartum fluoxetine impaired HPA negative feedback whereas maternal postpartum CORT enhanced HPA negative feedback in adult male offspring

In adult male offspring, maternal postpartum fluoxetine exaggerated stress-induced increase in serum CORT concentrations whereas maternal postpartum CORT blunted stress-induced increase in serum CORT concentrations in the dexamethasone suppression test. To our knowledge, no studies have examined the effects of maternal fluoxetine on HPA axis negative feedback in offspring. One study found maternal postpartum fluoxetine blunted serum CORT in adolescent male but not female rat offspring although samples were collected at the time of perfusion (Pawluski et al., 2012c), complicating whether this reflects a basal or stress-induced measure as anesthetics can rapidly increase CORT levels (Wu et al., 2015). Clinical findings indicate that prenatal fluoxetine increased corticosteroid-binding globulin levels in neonates (Pawluski et al., 2012a) and blunted evening levels of serum cortisol in 3 month old infants (Oberlander et al., 2008). Developmental fluoxetine also may alter HPA axis negative feedback by affecting limbic structures that regulate HPA axis activity. For instance, maternal postpartum fluoxetine diminished hippocampal glucocorticoid receptor density in adolescent male but not female rat offspring (Pawluski et al., 2012c). Additionally, maternal fluoxetine during gestation and lactation enhanced activation (Fos expression) in the basolateral amygdala and medial amygdala after restraint stress in adult female but not male rat offspring (Francis-Oliveira et al., 2013). Both the hippocampus and amygdala are sources of limbic control over the HPA axis (reviewed in Herman and Cullinan, 1997) and could therefore contribute to differences in HPA axis negative feedback. Sex differences in stress circuits may underlie these effects of maternal fluoxetine on HPA axis in males. Although we did not find an effect of maternal postpartum fluoxetine on adult female HPA axis activity, it is possible that our dose of dexamethasone was not sufficient to elicit group differences in CORT concentrations. Basal and stress-induced activity of the HPA axis are generally higher in females compared with males and as seen in our data (compare Fig. 5C with 5D; reviewed in Goel et al., 2014). Thus, a higher dose of dexamethasone for females may be necessary to optimally assess HPA axis negative feedback (Osborn et al., 1996).

Interestingly, maternal postpartum CORT blunted serum CORT concentrations in adult male and female offspring. Previous work with using this model showed that after 1 h of restraint, maternal postpartum CORT did not significantly alter serum CORT concentrations in either adult male or female offspring (Brummelte et al.,

2006, 2012). This suggests that maternal postpartum CORT results in developmental disturbance specific to negative feedback of the HPA axis. This may be related to the fact the maternal postpartum CORT results in increased brain and serum CORT content in the offspring (Brummelte et al., 2010). Alternatively, maternal postpartum CORT could indirectly affect the developing HPA axis via diminished quality of maternal care (Brummelte et al., 2006, 2012). Indeed, maternal separation (a similar model of maternal stress/neglect) blunted HPA axis activity in juvenile (Litvin et al., 2010) and adolescent male rats (Ogawa et al., 1994). Additionally, clinical evidence suggests that children under conditions of extreme parental neglect in Romanian orphanages exhibit blunted diurnal cortisol release (Carlson and Earls, 1997). Thus, early life adversity, such as maternal postpartum CORT, can induce permanent disruptions to the HPA axis of both male and female offspring.

#### 4.4. Maternal postpartum fluoxetine increased density of doublecortin-expressing cells in the dorsal hippocampus of adult male offspring

Maternal postpartum fluoxetine increased density of doublecortin-expressing cells in the dorsal dentate gyrus in adult male offspring. This is at least partially consistent with a prior study which showed that maternal postpartum fluoxetine slightly increased density of doublecortin-expressing cells in adult male offspring and decreased it in the adult female offspring (Rayen et al., 2015). However, our results suggest that after behavioral testing, maternal postpartum fluoxetine stimulates doublecortin expression in the dorsal (but not ventral) dentate gyrus, and only in the adult male offspring. This study used a higher dose of fluoxetine (10 mg/kg) than Rayen et al., 2015 (5 mg/kg). Regardless, maternal fluoxetine appears to increase doublecortin expression in adult males although this may be mitigated by prenatal stress (Rayen et al., 2015), but not by maternal postpartum CORT. Indeed, the increase in immature neurons due to maternal fluoxetine was only evident in the male offspring of CORT-treated dams. Although the exact mechanism of how maternal fluoxetine influences adult hippocampal neurogenesis in the offspring is not well understood, there are many possible explanations: serotonergic influences, changes in maternal care, or increased environmental enrichment via behavioral testing.

One explanation for how maternal postpartum fluoxetine could have disrupted adult offspring hippocampal neurogenesis is that fluoxetine present in the milk directly affected serotonergic regulation of hippocampal neurogenesis. Indeed, direct administration of fluoxetine to pups enhanced CA1 hippocampal dendritic spine density (Zheng et al., 2011) and hippocampal brain derived neurotrophic factor content in adult male mice (Karpova et al., 2009). This enhanced hippocampal plasticity with fluoxetine exposure is in line with findings that adult exposure to chronic fluoxetine administration stimulates hippocampal neurogenesis in adult male rats (Malberg et al., 2000; Huang and Herbert, 2006; David et al., 2009). Thus, our findings that maternal fluoxetine enhances adult hippocampal neurogenesis parallel findings from studies directly exposing pups to fluoxetine. Direct administration of fluoxetine to pups also diminished serotonin terminals in the dentate gyrus in adult male rats (Silva et al., 2010). This supports the possibility that early exposure to fluoxetine itself from the dam disrupts serotonergic regulation of hippocampal neurogenesis in the offspring.

Another alternative explanation for how maternal postpartum fluoxetine could have disrupted adult offspring hippocampal neurogenesis is that fluoxetine indirectly affected offspring hippocampal development via alterations in maternal care. In a complimentary study maternal postpartum fluoxetine reversed

CORT-induced reductions in maternal care (Workman et al., 2013b, submitted). Therefore, it is possible that the positive effect of maternal postpartum fluoxetine on maternal care resulted in enhanced neurogenesis. This is in line with findings that higher maternal care (licking and grooming) increases hippocampal plasticity in adult offspring whereas lower maternal care reduces it (Liu et al., 2000; Bredy et al., 2003; Champagne et al., 2008). Interestingly, males and females were differentially affected by maternal fluoxetine exposure. Given that there are sex differences in the amount of maternal care pups receive (Moore and Morelli, 1979), it is possible that maternal fluoxetine further skewed the amount of attention male and female pups receive and may explain the opposing effects of maternal fluoxetine on adult offspring hippocampal neurogenesis.

Another reason for maternal fluoxetine to selectively increase the density of immature neurons in males is that behavioral testing constituted exploration of a variety of different apparatuses over 10 days and may have an enriching component. Doublecortin is expressed for up to 21 days after the cell divides in rats (Brown et al., 2003), and it is possible that behavioral testing altered doublecortin expression. Environmental enrichment increases doublecortin expression in adult male and female rodents (Leal-Galicia et al., 2007; Ramirez-Rodriguez et al., 2014). Interestingly, environmental enrichment increased number of early immature neurons expressing doublecortin in adult male mice selectively in the septal (dorsal) region of the hippocampus (Tanti et al., 2013). This is consistent with our results that maternal postpartum fluoxetine increased doublecortin expression exclusively in the dorsal hippocampus. Therefore, it is possible that maternal postpartum fluoxetine in combination with the enrichment present in the battery of behavioral tests increased neurogenesis specifically in the male offspring. Alternatively, it is possible that the stress of multiple behavioral and neuroendocrine tests diminished doublecortin expression, except in the male subjects exposed to maternal postpartum fluoxetine. Generally, stress reduces hippocampal neurogenesis but is sex- and stressor-dependent (reviewed in Gobinath et al., 2014). In adult male rodents, fluoxetine can reverse the stress-induced reduction in hippocampal neurogenesis (Malberg & Duman, 2003; reviewed in Warner-Schmidt and Duman, 2006). Therefore, it is possible that maternal postpartum fluoxetine buffered against the stress of multiple behavioral tests in the adult male offspring but not in the other experimental conditions.

We also found that maternal postpartum CORT increased density of doublecortin-expressing cells in adult male offspring and diminished the density of doublecortin-expressing cells in adult female offspring. As in the case of maternal postpartum fluoxetine, it is possible that this effect of maternal postpartum CORT can be explained by increased environmental enrichment, stress resulting from behavioral testing, and/or exposure to dexamethasone and stress 3 days prior to perfusion. Developmental stress is typically associated with detrimental effects on hippocampal plasticity (reviewed in Gobinath et al., 2014). However, early life stress in the form of maternal deprivation (Oomen et al., 2010) or low maternal care (Champagne et al., 2008; Bagot et al., 2009) enhanced long term potentiation of adult-born granule cells in the dentate gyrus only in the presence of CORT. This suggests that early life adversity could promote hippocampal plasticity under mildly stressful conditions, such as multiple behavioral tests. Moreover, there are sex differences in how early life adversity affects the adult hippocampus (reviewed in Gobinath et al., 2014), which may explain the opposing effects of maternal postpartum CORT on offspring doublecortin expression in this study.

We did not find a significant effect of maternal postpartum CORT or fluoxetine on doublecortin-expression in either males or females



in the ventral hippocampus. This was surprising because we observed significant effects of these maternal treatments on anxiety-like behavior and HPA axis negative feedback regulation, and both affective behavior and HPA axis function are associated with ventral hippocampus activity (reviewed in [Fanselow and Dong, 2010](#)). However, we did observe that among the males exposed to both maternal CORT and fluoxetine, doublecortin expression selectively in the ventral hippocampus was correlated with increased anxiety-like behavior in the elevated plus maze. This suggests that developmental exposure to CORT and fluoxetine may have long-term effects on the association between immature neurons of the ventral hippocampus and neural circuits underlying anxiety-like behavior.

#### 4.5. Maternal CORT and concurrent fluoxetine during the postpartum period attenuated body mass in adult male offspring

Our results indicate that maternal postpartum CORT and concurrent fluoxetine diminished body mass only in adult male offspring, consistent with a study using prenatal dexamethasone followed by maternal postpartum fluoxetine in adult males ([Nagano et al., 2012](#)). This is consistent with findings that maternal fluoxetine diminished body mass in neonatal rats ([da-Silva et al., 1999](#)) and infants ([Chambers et al., 1999](#)). However, maternal postpartum fluoxetine alone increased body mass in adult male offspring. Perinatal exposure to SSRIs (including fluoxetine) is also associated with being overweight in boys but not girls (7 y; [Grzeskowiak et al., 2013](#)). Collectively, this suggests that maternal SSRI use can impact body mass of offspring differently depending on developmental time point and whether exposure occurred exclusively during the prenatal, postnatal, or both periods of development.

## 5. Conclusions

Our data indicate that maternal postpartum fluoxetine can have long-lasting effects on behavioral, endocrine, and neural outcomes of adult offspring in a sex-specific manner. Specifically, adult male offspring were more vulnerable to the effect of maternal postpartum fluoxetine than the female offspring with regards to anxiety-like behavior, HPA axis negative feedback regulation, and hippocampal neurogenesis. However, both adult male and female offspring exhibited more serotonin-dependent behavior in the forced swim test. Finally, maternal postpartum CORT was associated with blunted HPA activity in adult male and female offspring. Collectively, these findings bear implications for treating mothers with pharmacological antidepressants and highlight the importance of studying the consequences of maternal pharmacology on both male and female offspring.

## Conflicts of interest

The authors have nothing to declare.

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