

Prenatal maternal stress is associated with behavioural and epigenetic changes in Japanese quail

Supporting information

Chronic Stress Procedure

S F0 quails were repeatedly exposed to aversive events (**Table S1**) four to five times a day for 24 days. The stressors could occur during the light or the dark phase and were randomly applied to reduce the possibility of familiarisation. Four stressors were used individually: a metallic stick banged on the cage rods (thrice in 30 minutes), air or water jet (twice in 30 minutes), a physical restraint (30 minutes). The seven other stressors were used on the entire battery: transparent devices placed on the feeders to delay food access (2 hours), air flows with ventilators (30 minutes), a flag agitated around the battery simultaneously with the diffusion of a hawk's cry (thrice in 30 minutes), unexpected sounds (several times in 30 minutes), introduction of a quail from the same battery in the home cage of another quail (30 minutes) or an overcrowding (by placing the 16 quails in the same cage (60 x 42.5 x 12 cm)) sometimes with a trolley transport. When stressors were used, the experimenter (MC) was dressed with a green combination, white gloves, a hairnet and a mask, otherwise she was dressed with a white gown. When an aversive event was applied to S F0 quails, the experimenter then had a positive contact with NS F0 quails (addition of food or woodchips in the home cage).

Table S1 | Chronic stress procedure applied to F0 females. Summary table of the different stressors used during the chronic stress protocol.

	DESCRIPTION	DURATION
ENVIRONMENTAL DISTURBANCE		
Unexpected sounds	Speakers on the ground, facing the battery. 80 dB diffusion of various noises (fire alarm, horn, metallic noises, alarm clock)	30 minutes Broadcasting of several sequences of noises of variable duration interspersed with silences.
Air flow	Medium speed ventilators installed in front of the battery	30 minutes
Water or air jet	Via a sprayer or a straw	3 times over 30 minutes
Metallic stick	Stick passed laterally on the bars of the cages	3 times over 30 minutes
Restraint	Quail restrained by a wooden wall in a corner of its cage.	30 minutes
Predator sounds	Flag waved above and below the cages while a hawk call is broadcast at 80 dB through a loudspeaker placed in front of the battery	3 times over 30 minutes
FOOD DISTURBANCE		
Food frustration	Transparent board placed on the feeders before the breeding room is lit. The quails, during their morning peak of activity, see the food but cannot consume it	2 hours
SOCIAL DISTURBANCE		
Overcrowding	Quails placed in the same transport cage (78 x 43 x 13 cm). Overcrowding can be simultaneous with transport on a cart	30 minutes
Intrusion	Quail of the same battery but of a different level introduced in the cage of another female. Each quail is once resident and once intruder	30 minutes

H3K27me3 and H3K4me2 immunochemistry of F1 females quails

To evaluate the influence of PMS on brain epigenetic marks, we assessed by immunohistochemistry the density of H3K27me3 and H3K4me2 in key brain structures for emotional reactivity, learning and memory abilities (Lormant et al., 2020a; Phillips, 1964; Saint-Dizier et al., 2009). In order to minimise the number of birds collected, we focused on a sample of F1 females selected within the different families. All these females had passed the emotional reactivity tests but their learning abilities were not assessed. Subjects were lethally anaesthetised by intraperitoneal injection of pentobarbital, perfused transcardially via the left ventricle with 80 mL 1% sodium nitrite in phosphate-buffered saline, followed by 300mL of ice-cold 4% paraformaldehyde solution in 0.1M phosphate buffer containing 15% (v/v) saturated picric acid. After perfusion, brains were removed from the skull, post-fixed overnight in the same fixative and cryoprotected in a 20% sucrose-0.1% sodium azide solution in 0.1M phosphate buffer. Then they were frozen 30 seconds in a bath of -50°C isopentane. Frontal free-floating sections were cut on a cryostat (NX70, MM France, Brignais, France) at a thickness of 30 µm and stored in 0.025M Tris Buffer Saline (TBS)-0.1% sodium azide, pH 7.4 (TBSA). Sections were rinsed in TBS for five minutes and treated in 0.1% sodium borohydride in TBS under gentle agitation (30 minutes at room temperature). After four baths in TBS, sections were pre-incubated (one hour at room temperature) in TBSA-T (0.3% Triton)-1% Bovine Serum Albumin (BSA) to increase the permeability of plasma membrane. Sections were then incubated (48 hour at 4°C) in 1:500 rabbit anti-H3K27me3 primary antibody (Millipore, Molsheim, France) or in 1:1000 rabbit anti-H3K4me2 primary antibody (Abcam, Cambridge, UK) in TBSTA-1% BSA. Sections were rinsed twice in TBS-0.3% Triton (TBS-T) for five minutes and twice in TBS for five minutes. Then, sections were incubated (two hours at room temperature) in 1:2000 secondary antibody (Donkey anti-rabbit-488; Jackson ImmunoResearch, Peterborough, UK) in 0.3% TBS-saponin-0.1% BSA. After four rinses in TBS, sections were immersed in Hoechst (nuclear staining, Hoechst 33258, 2µg/mL in distilled water, Invitrogen, Cergy Pontoise, France) for two minutes and rinsed in TBS four times. Finally, sections were mounted on gelatin-coated glass slides, allowed to dry and covered-slipped using

Fluoromount-G (Southern Biotech, Birmingham, USA). The slides were stored at 4°C in the dark until analysis. The H3K27me3 or H3K4me2 positive cells were counted using a fluorescence microscope (Axioskope 2; Zeiss, Germany) with a magnification of 20X associated with a cell-count analysis software (computerized image analysis; Mercator; Explora Nova, La Rochelle, France) through a camera. H3K27me3 and H3K4me2 positive cells were counted in the hippocampus, the paraventricular nucleus of the hypothalamus and in the following subnuclei of the arcopallium/amygdala: intermediate, medial, dorsal, posterior amygdaloid and nucleus taeniae. The brain regions were identified by referring to the stereotaxic atlas of chick brain (Kuenzel and Masson, 1988), its revised nomenclature (Reiner et al., 2004) and the quail brain atlas (Baylé et al., 1974). Densities of H3K27me3 or H3K4me2 positive cells were averaged (total number of positive cells divided by the total area of the counted region) to obtain a single value per brain region.

References

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Table S2 | Characteristics of F0 NS and S females' eggs. Number (mean \pm SEM) of eggs laid per female in each group for the 16 days they were collected ($N_{NS} = 16$, $N_S = 16$). Mass, component values and hormonal levels were calculated for one egg per female ($N_{NS} = 15$, $N_S = 16$). All data in the table are expressed as mean values (\pm SEM). Significant results are in bold (* $p \leq 0.05$, *** $p \leq 0.001$, # $p \leq 0.10$).

	NS	S	p-value
Number	13.1 \pm 1.1	13.7 \pm 0.4	0.714
Fertilisation rate (%)	88.0 \pm 5.5	84.2 \pm 4.8	0.628
Hatching rate (%)	68.7 \pm 6.5	78.5 \pm 5.0	0.245
Mass (g)	13.3 \pm 0.3	13.3 \pm 0.3	0.094
Shell mass (g)	1.2 \pm 0.04	1.1 \pm 0.03	0.325
Yolk mass (g)	4.4 \pm 0.1	4.3 \pm 0.1	0.727
Albumen mass (g)	7.3 \pm 0.2	7.4 \pm 0.2	0.761
Androstenedione (ng/yolk)	383.7 \pm 38.2	294.8 \pm 25.7	0.060 #
Testosterone (ng/yolk)	97.0 \pm 8.2	71.0 \pm 7.9	0.027 *
Progesterone (ng/yolk)	4388.0 \pm 14.8	5255.2 \pm 386.9	0.139
Androgene proportion (%)	12.1 \pm 2.4	6.8 \pm 0.5	0.006 ***
Progesterone proportion (%)	87.9 \pm 2.4	93.2 \pm 0.5	0.005 ***

Table S3 | Physio-morphological characteristics of F1 chicks. Mass (g) of F1 NS and S chicks from hatching (day 1) to 5 weeks-old (day 35). From day 1 to day 21: N_{NS} = 80, N_S = 80; day 28: N_{NS} = 79, N_S = 80; day 35: N_{NS} = 77, N_S = 80. Cloacal vent length (mm) of F1 NS and S chicks from 2 weeks-old (day 15) to 5 weeks-old (day 35). From day 15 to day 21: N_{NS} = 80, N_S = 80; from day 28 to day 35: N_{NS} = 79, N_S = 80. phd = post hatching day. All data in the table are expressed as mean values (\pm SEM). Significant results are in bold (* $p \leq 0.05$).

	NS	S	p-value
Mass (g)			
phd 1	10.0 \pm 0.1	10.0 \pm 0.1	0.913
phd 7	47.2 \pm 0.6	44.6 \pm 0.6	0.033 *
phd 15	126.3 \pm 1.1	123.5 \pm 1.2	0.179
phd 21	182.3 \pm 1.5	179.6 \pm 1.7	0.316
phd 28	241.7 \pm 2.0	239.4 \pm 2.3	0.430
phd 35	268.3 \pm 2.3	272.0 \pm 2.5	0.128
Cloacal vent (mm)			
phd 15	5.3 \pm 0.08	5.3 \pm 0.08	0.564
phd 21	5.5 \pm 0.06	5.2 \pm 0.07	0.014 *
phd 28	6.7 \pm 0.08	6.6 \pm 0.07	0.261
phd 35	7.2 \pm 0.08	7.2 \pm 0.08	0.918

Table S4 | Spatial learning test. Results of statistical analyses (generalised linear or linear mixed models). Significant results are in bold (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

	χ^2	df	p-value
Familiarisation			
<i>First step latency (s)</i>			
Stress	5.720	1	0.017 *
Days	39.644	2	2.46e-09 ***
Stress * Days	1.552	2	0.460
<i>Number of different cups visited</i>			
Stress	0.628	1	0.428
Days	15.847	2	3.622e-04 ***
Stress * Days	9.349	2	0.009 **
Training			
<i>Number of errors</i>			
Stress	0.604	1	0.437
Days	61.306	11	5.30e-09 ***
Stress * Days	10.976	11	0.445

Table S5 | Conditioned place preference test. Results of **statistical analyses** (generalised linear or linear mixed models). Significant results are in bold (* $p \leq 0.05$, *** $p \leq 0.001$).

	χ^2	df	p-value
Time spent			
Groups	0.000	1	1.000
Compartment	4.958	1	0.026 *
Groups * Compartment	4.818	1	0.028 *
Latency to reach cup			
Groups	0.021	1	0.884
Compartment	12.953	1	3.194e-04 ***
Groups * Compartment	0.085	1	0.771

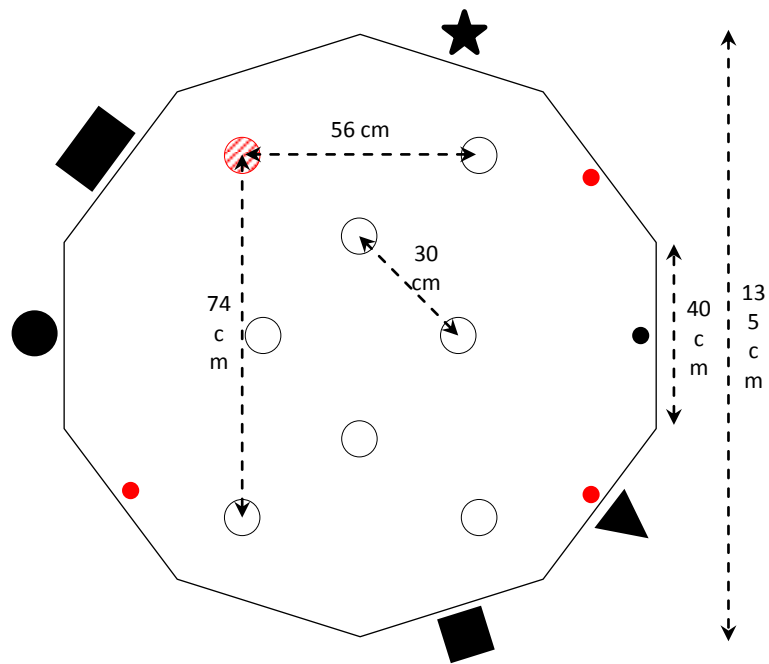


Figure S1 | Schematic representation of the device used in the spatial learning test. The 8 circles represent the 8 cups, each containing 1 mealworm during the familiarisation phase. The red circle represents the only cup (= rewarded cup) containing mealworms during the training phase. The red dots correspond to the 3 possible entrances for the training phase and the black dot correspond to the entrance for the probe test.

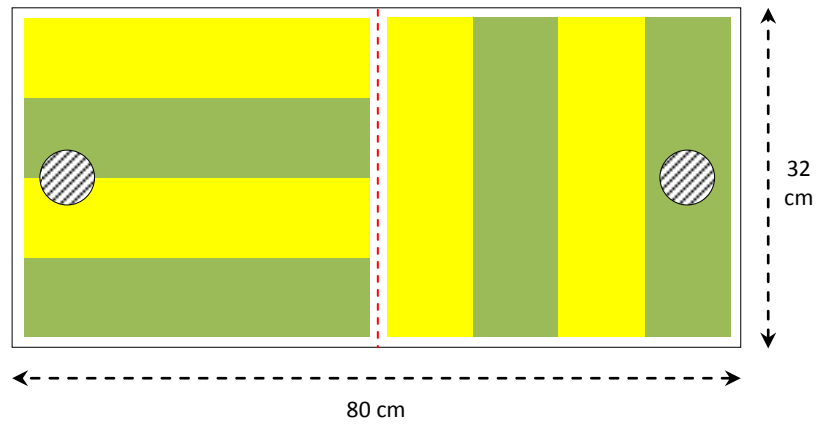


Figure S2 | Schematic representation of the device used in the conditioned place preference test.

The apparatus could be divided into 2 chambers by means of a removable wall represented by the red dotted line. Walls of the first side were covered by horizontal green and yellow stripes while walls of the other side were covered by vertical green and yellow stripes. The 2 circles represent the cups which could contain mealworms.