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PRENATAL MATERNAL STRESS IS ASSOCIATED WITH BEHAVIOURAL AND EPIGENETIC CHANGES IN JAPANESE QUAIL

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Abstract

Prenatal maternal stress (PMS) influences many facets of offspring's phenotype including morphology, behaviour and cognitive abilities. Recent research suggested that PMS also induced epigenetic modifications. In the present study, we analysed, in the Japanese quail, the effects of PMS on the emotional reactivity and cognitive abilities of the F1 offspring. We also investigated in the hippocampus, the paraventricular hypothalamic nucleus and subnuclei of the arcopallium/amygdala the level of two histone post-translational modifications, H3K4me2 and H3K27me3, known to be

impacted by stress. We found that PMS does not affect F1 quail's learning abilities but increases their emotional reactivity. Moreover, we demonstrated that PMS induced an increased density of H3K27me3 positive cells, in the hippocampus, paraventricular hypothalamic nucleus and dorsal nucleus of the amygdala, but not variations of H3K4me2. As these brain regions are involved in the control of vertebrates' emotional responses, the effect of PMS on the epigenetic mark H3K27me3 could possibly be a mechanism involved in the behavioural effects we observed in F1 quail.

Keywords

Prenatal maternal stress, histone post-translational marks, emotional reactivity, learning abilities, precocial bird

1. Introduction

In many species including mammals (Weinstock, 2008), birds (Henriksen et al., 2011) as well as fish (Colson et al., 2019), lizards (Ensminger et al., 2018) and invertebrates (O'Brien et al., 2017), maternal stress during pregnancy or egg formation (i.e. prenatal maternal stress hereafter PMS), affects the development, adaptation and survival of the offspring by its long-lasting influence on their behaviour and cognitive abilities. Pioneering studies indicated that PMS can also modulate gene expression through epigenetic modifications (rat: Mychasiuk et al., 2011; chicken: Nätt et al., 2009). Epigenetic modifications correspond to the molecular processes involved in the regulation of gene expression without alteration of DNA sequence. It includes DNA methylation, noncoding RNAs as well as histone post-translational modification. Severe PMS induces a dramatic decrease of DNA methylation in rodents' hippocampus and frontal cortex and changes of hippocampal levels of histone H3 acetylation and DNA methyltransferase 1 (mice: Benoit et al., 2015; rat: Mychasiuk et al., 2011). A recent study shows that they were similar processes in birds. To mimic the effects of PMS, eggs of zebra finches were previously injected with testosterone which lead to different expression of hundreds of genes particularly in the hypothalamus (Bentz et al., 2021). Some authors suggested that influences of PMS on epigenetic marks could be implicated in offspring behavioural changes

including more hypotonia and lethargy (human: Conradt et al., 2013), modulations of locomotor activity (rat: Mychasiuk et al., 2011), greater fearfulness (human: Ostlund et al., 2016), poorer spatial learning abilities (mice: Benoit et al., 2015), more aggressive behaviour (zebra finches: Bentz et al., 2021) or different foraging strategies (chicken: Nätt et al., 2009). Birds, especially precocial, are ideal animal models to study and understand these phenomena. Precocial birds' offspring's first stages of development occur *in ovo*, out of the female, and the young can survive without a mother's presence after hatching. This makes peri- and postnatal environments easier to control in birds compared to mammals. In the current study, we thus investigated the influence of a prenatal maternal stress on Japanese quail's (*Coturnix coturnix japonica*) F1 offspring's behavioural phenotype and brain epigenetic marks. Chicks born from stressed hens usually present a stunted growth related to modulation of the yolk composition of the eggs from which they hatched (quail: Guibert et al., 2010). In order to ensure the effectiveness of our stress procedure we first analysed the egg production and hormonal content of both F0 S and NS quail and followed the development of F1 chicks. Second, we assessed F1 offspring's emotional reactivity (i.e. the predisposition of an individual to perceive and react to potentially stressful event (Boissy, 1995) and evaluated their learning capacities. Finally, we assessed, by immunohistochemistry, the impact of PMS on two histone marks, H3K27me3 and H3K4me2, in the paraventricular hypothalamic nucleus, the different subnuclei of the amygdala as well as in the hippocampus of F1 offspring. We studied these brain regions because they have important roles in birds in the control of emotional responses and learning and memory processes (quail: Lormant et al., 2020a; duck: Phillips, 1964; quail: Saint-Dizier et al., 2009), both traits we evaluated in the F1 offspring. We focused on H3K27me3 and H3K4me2 involved in silent and active chromatin, respectively (Kouzarides, 2007) because, in rodents, acute and chronic stress leads to dynamic changes in histone H3 methylation (rat: Hunter et al., 2009). We hypothesised that, in quails, PMS will influence the emotional reactivity and the learning abilities of the F1 offspring. Given the impact of PMS on epigenetic marks, we also supposed that it will modify the levels of H3K27me3 and H3K4me2 in the brain structures studied.

2. Methods

2.1. Parental generation

2.1.1. Housing and chronic stress procedure

This experiment took place in the facilities of the EthoS laboratory (Rennes, France). Thirty-two 5-month old F0 females Japanese quail (from the commercial farm, Les Cailles de Chanteloup, France) were placed individually in battery cages (35 x 24.5 x 18 cm) in two similar rooms according to their group ($18 \pm 1^\circ\text{C}$, 12h:12h light-dark cycle). These subjects were randomly allotted to one of two groups: 16 in the non-stressed group (NS) and 16 in the stressed group (S). The chronic stress procedure (CSP) applied to S F0 females has been described previously and validated for juvenile quail (Calandreau et al., 2011; Laurence et al., 2012). Briefly, S F0 females were exposed to four or five stressors a day for 24 days. Types and numbers of stressors varied randomly from day to day and could occur at anytime during the light or the dark phase. Stressors included broadcasting sudden noises or predator calls, water and air sprays, restraint, food restrictions, social instability or ambulation on a cart. See the supplementary information (SI; **Table S1**) for further details.

2.1.2. Mating and egg incubation

During the CSP, S and NS F0 quail were mated with control males ($N = 16$). These males were raised under the same conditions as the NS F0 females and were not exposed to the CSP. Two sexually mature males were always paired with the same four F0 females (two S, two NS) alternately at each mating session. Laid fertilised eggs were collected during a 16 days period, starting eight days after the beginning of the CSP to ensure that chronic stress had had an effect (e.g. yolk formation occurs in seven days in quail). Eggs ($N_{\text{NS}} = 188$, $N_{\text{S}} = 182$) were identified individually and stored at $16 \pm 1^\circ\text{C}$ until incubation (incubator: Brinsea Ova-Easy Advance 380). The first 14 days of incubation, eggs were maintained at 37.7°C , with a relative humidity of 45%, while automatically rotated 45° , every 30

minutes. From day 15 to day 17, humidity was increased to 70% and rotation was stopped to favour hatching.

2.1.3. Egg hormonal assays

Half way through the CSP, one egg was collected per female for steroid analyses (**Figure 1**). Eggs and their different component were weighted and levels of yolk testosterone, androstenedione and progesterone were evaluated at the University of Veterinary Medicine of Vienna, using the enzyme immunoassays previously described in (Möstl et al., 2001) and (Hackl et al., 2003).

2.2. Offspring

2.2.1. Housing and development

At hatching (similar fertilisation rates and hatching success for both groups, SI, **Table S2**) F1 chicks were identified individually by numbered and coloured leg rings according to their prenatal treatment. Until post-hatching day (phd) 30, F1 chicks were housed in groups of eight. Thus, 10 groups of F1 NS chicks ($N_{NS} = 80$) and 10 groups of F1 S chicks ($N_S = 80$) were formed and placed in collective cages (100 x 70 x 62 cm) equipped each with one heater ($38 \pm 1^\circ\text{C}$, removed on phd 15). On phd 30, the sex of the chicks was determined by plumage (similar sex-ratio; $N_{NS-\varnothing} = 41$, $N_{NS-\sigma} = 39$, $N_{S-\varnothing} = 32$, $N_{S-\sigma} = 48$; Chi-squared test, $X^2 = 2.040$, $p = 0.153$). Then, 32 birds per treatment and per sex were randomly selected from each group and family. They were wing banded and reallocated to collective batteries. Males and females from both groups were placed in different rooms ($18 \pm 1^\circ\text{C}$, 12h:12h light-dark cycle). From hatching to phd 35, F1 chicks were weighted individually each week. Their sexual development was assessed once a week from phd 15 to phd 35 by measuring the length of their cloacal vent (**Figure 1**).

2.2.2. Emotional reactivity

Classical ethological tests devised for poultry were used to assess several dimensions of the emotional reactivity (Mignon-Grasteau et al., 2003) of F1 chicks: an emergence test and a novel object test assessing sociality and fearfulness expressed by quail in the presence of a novel environment or a novel object (**Figure 1**). Each test took place between 08-12h and 13-18h.

Emergence test (phd 10-12; $N_{NS} = 80$, $N_S = 80$): Subjects were placed individually in a dark starting box (18 x 18 x 18 cm) which was opened after one minute. To evaluate the reactivity of F1 quail to social separation, the experimenter recorded latency of emission of their first rally call and number of rally calls they produced. The emission of rally call is considered to be positively correlated with the motivation to join conspecifics (Formanek et al., 2008). After the door had been opened, F1 quail were allowed five minutes to leave the starting box and go into a novel illuminated cage (60 x 56 x 35 cm). The experimenter, not visible for the quail, recorded latency of emergence of the quail's head which is positively correlated with its fear level (Jones, 1996).

Novel object test (phd 31; $N_{NS} = 64$, $N_S = 64$): Each individual home cage was equipped with an opaque partition (to prevent neighbours from seeing the novel object before being tested). The novel object was an unfamiliar terracotta cup (6 x 7 cm), which was placed at the entrance of the cage. During a six-minute period, the experimenter, placed 1.50 meter from the test battery, noted occurrences of fear reactions of F1 quail in the presence of this object (fear postures, alertness, jumping, avoiding, immobility, pacing).

2.2.3. Cognitive abilities

Two procedures were used to evaluate learning abilities of F1 S and NS quail: a spatial learning test and a conditioned place preference (CPP) test (**Figure 1**). These procedures involve different learning abilities and brain regions that can be affected by stress (Sandi and Pinelo-Nava, 2007). As the learning procedure is time consuming and tedious (many different sessions required for each subject), we focused on a sample of F1 females quail randomly selected in each family ($N_{NS} = 16$, $N_S = 16$). For both the spatial learning and the CPP tests, testing took place between 08-12 h and 13-20h. We controlled for a potential time effect by testing the same number of NS and S F1 quail in the same period (morning or evening) and alternately. In addition, we organised the experiment so that each F1 quail was tested both in the morning and in the evening.

Spatial learning test (phd 32-54): The spatial learning test (Lormant et al., 2020b), enabled us to assess the spatial skills and memory of F1 S and NS females quail, two traits that are functionally linked to the hippocampus (Lormant et al., 2020a). Briefly, this test took place in a decagonal arena surrounded by curtains to prevent bird's escape. Black visual cues (i.e. different geometric forms) were placed on the curtain to allow bird's orientation. Eight identical cups were placed in the arena (SI, **Figure S1**). Before the spatial learning test, birds were familiarised with the cups and mealworms (*Tenebrio molitor* used here as a reward). After the novel object test, cups were left in quail's home cage. Two mealworms were then deposited in the cups, for seven days, three times per day.

The spatial learning test included three phases. The first one was a *familiarisation* phase with all the cups baited with mealworms. A session ended once all the worms had been eaten or after 10 minutes. During each session, we recorded the first step latency and numbers of different cups visited. After three familiarisation sessions, only F1 quail that had visited at least five different cups were tested in the next phase. The second phase was a *training* phase in which only one cup, always in the same position, was baited with mealworms (reward-paired cup). F1 quail were given three training sessions per day for 12 consecutive days. Training sessions ended when quail had eaten mealworms or after five minutes. For each session, the numbers of different cups visited before the

reward cup (i.e. number of errors) were recorded. The third phase occurred on the day following the last spatial training session when F1 quail were given a *probe test*. Here, the “usual” reward cup was empty to ensure that quail did learn the location of the target cup and did not find it by smelling or seeing food. During the test, the numbers of errors made by quail before finding the reward cup were recorded. During both the training phase and the probe test a maximum score of eight was given for the number of errors if the quail did not find the reward cup.

CPP test (phd 61-68): This task allows the evaluation of a simple form of motivational memory, the ability to form an association between an environmental stimuli and a food reward (mealworms) (White and McDonald, 1993). The apparatus consisted of a wooden box (80 x 32 x 38 cm) with two compartments (40 x 32 x 38 cm) covered with different patterns (i.e. vertical vs. horizontal green and yellow bands) and separated by a removable wall. Identical cups were placed in each compartments (SI, **Figure S2**).

First, F1 quail were left to familiarise with the apparatus for 10 minutes and then trained during two sessions per day for five consecutive days. F1 quail were exposed to the two compartments during each training session. They were placed during three minutes in the first compartment and two hours later, in the other compartment. One of the compartments had its cup filled with mealworms (reward-paired compartment) while the other compartment had an empty cup (reward unpaired-compartment). The day following this training phase, F1 quail were submitted to a probe test. For three minutes, they were allowed to explore freely both compartments where both cups were present but both without mealworms. Latency to visit cups and time spent in each compartment were recorded.

2.2.4. H3K27me3 and H3K4me2 immunochemistry

To evaluate the influence of PMS on brain epigenetic marks, we assessed by immunohistochemistry the density of H3K27me3 and H3K4me2 (**Figure 1**) in key brain structures for emotional reactivity, learning and memory abilities (quail: Lormant et al., 2020a; duck: Phillips, 1964; quail: Saint-Dizier et

al., 2009). In order to minimise the number of birds collected, we focused on a sample of F1 females (phd 75; $N_{NS} = 9$, $N_S = 7$) selected within the different families. Apart from that, the selection was random and independent of the birds' behavioural results. All these females had passed the emotional reactivity tests but their learning abilities were not assessed. Indeed some authors showed that learning processes influence pattern of histone acetylation and methylation, notably for H3K4 (Dagnas and Mons, 2013; Gupta-Agarwal et al., 2012). Therefore, to avoid unwanted changes in the expression of H3K27me3 and H3K4me2, we decided not to evaluate F1 females into the spatial learning and the CPP tests.

Nine NS and seven S F1 females were lethally anaesthetised by intraperitoneal injection of pentobarbital, their brains were removed and frozen until immunohistochemistry. Once the immunochemistry procedure was done, the number of H3K27me3 or H3K4me2 positive cells was counted in the paraventricular hypothalamic nucleus (PVN), the hippocampus (Hp) and the amygdala (nucleus taeniae (TnA), as well as intermediate (IA), medial (MA), dorsal (DA), and posterior amygdaloid (PoA)). 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. See the SI for further details.

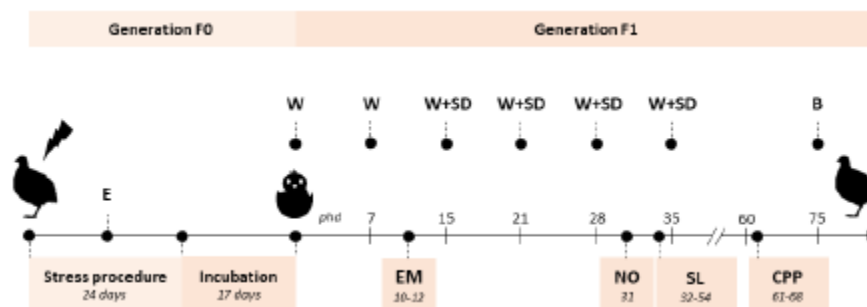


Figure 1 | Schedule of the experimentation. E = collection of one egg per F0 female for hormonal analysis; phd = post-hatching day; W = weighing; SD = measuring sexual development; EM = emergence test; NO = novel object test; SL = spatial learning test; CPP = conditioned-place preference test and B = brain collection from F1 female for H3K27me3 and H3K4me2 immunochemistry.

2.3. Statistical analyses

Statistical analyses were performed using the software R v.3.6.2 (R Core Team, 2019). Model assumptions were checked by i) visual inspection of residual and fitted value plots (package RVAideMemoire (Hervé, 2021)) and ii) evaluation of overdispersion if necessary (package AER (Kleiber and Zeileis, 2020)). When data or residuals did not respect the conditions of application of the model, non-parametric tests were used or data were transformed with the log (+1) or the square root function.

Morpho-physiological data were square root-transformed and analysed using a linear mixed model (LMM). The fixed factors included were stress effect (NS or S), sex, age and their interactions. Quail's identities were added as a random factor.

Due to a relatively small sample size, all data related to the analysis of F0 eggs and the immunohistochemistry of H3K27me3 and H3K4me2 were analysed using a Fisher-Pitman permutation test with the stress effect as factor.

For the analysis of behavioural data, latencies of head emergence were log+1-transformed and analysed using a linear model (LM). Latencies of rally call in the emergence test were processed with a generalised linear model (GLM) with gamma error structure. The numbers of rally calls in the emergence test and the numbers of fear behaviours recorded during the novel object test were analysed using a GLM with a quasi-poisson error structure. For all analyses of emotional reactivity data, stress effect, sex and their interactions were used as fixed factors.

For the spatial learning test, latencies of the first step during the familiarisation phase were first log+1 transformed and then analysed with a LMM and numbers of visits to the cups visited were analysed with a generalised linear mixed model (GLMM) with a poisson structure error and a square root link. Numbers of errors before finding the reward-paired cup during the training phase (means of the three sessions per day) were log+1 transformed and analysed using a LMM. For both the familiarisation and training phases, stress effect, day and their interactions were used as fixed factor. Quail's identity was added as a random factor. Due to the small number of individuals tested in the probe test, data were analysed using a Fisher-Pitman permutation test with treatment effect as a factor. For the familiarisation phase, two F1 S and one F1 NS quail were removed from the analysis because they never visited a cup. During the last six days of training, one S and two F1 NS quail did not reach the training threshold of at least 70% visits to the reward-paired cup (means of the three sessions per day) so they were removed from the training phase (in addition to the subjects that did not meet the threshold of five cups) and the probe test analysis. Data for one F1 S quail was also removed from the analysis for the probe test because of technical problems. The numbers of quail included for the spatial learning test analyses were: familiarisation: $N_{NS} = 15$, $N_S = 14$; training: $N_{NS} = 10$, $N_S = 10$ and probe test: $N_{NS} = 10$, $N_S = 9$. For the CPP test, the latencies to reach both cups were analysed using a GLMM with a gamma error structure. Time spent by quail in each compartment of the arena were analysed using a LMM. Stress effect, compartment and their interactions were used as fixed factors. Quail's identity was added as a random factor.

For LM(M) and GLM(M), all statistical analyses were conducted with the lme4 (Bates et al., 2015), nlme (Pinheiro et al., 2020) and car packages (Fox and Weisberg, 2019). Fisher-Pitman permutation tests were performed using the coin package (Hothorn et al., 2006). When interactions were significant, all analyses were followed by multiple comparisons (package emmeans (Lenth et al., 2020)) with a Tukey correction if necessary. Results of sex effect will be presented only if there was an interaction with stress effect.

3. Results and discussion

3.1. Validation of the PMS procedure

In order to ensure the effectiveness of PMS we analysed the egg production and hormonal content of F0 quail and followed the development of F1 chicks. We could not evidence any significant differences between our groups concerning the numbers of eggs laid or the mass of different egg components (SI, **Table S2**). However, as expected our PMS procedure induced a modulation of the yolk hormonal content. Testosterone concentrations were lower in F0 S eggs than in F0 NS eggs (mean \pm SEM, NS: 97.0 ng/yolk \pm 8.2, S: 71.0 ng/yolk \pm 7.9; Fisher-Pitman permutation, $Z = 2.139$, $p = 0.027$). F0 S eggs were also proportionally poorer in androgens (mean \pm SEM, NS: 12.1% \pm 2.4, S: 6.8% \pm 0.5; Fisher-Pitman permutation, $Z = 2.059$, $p = 0.006$) and richer in progesterone (mean \pm SEM, NS: 87.9% \pm 2.4, S: 93.2% \pm 0.5; Fisher-Pitman permutation, $Z = -2.059$, $p = 0.005$). Okuliarová and colleagues reported similar results (quail: Okuliarová et al., 2010). As previously reported in Brandt's voles (*Lasiopodomys brandtii*) and Japanese quail (Gu et al., 2020; Guibert et al., 2010), in our study PMS influenced growth of F1 chicks (LMM, effect of stress \times age: $X^2 = 21.096$, $p = 7.769e-4$; SI, **Table S3**). On phd 7, F1 S chicks' growth was stunted compared to that of F1 NS chicks ($p = 0.033$). PMS also tended to delay F1 chick's sexual development (LMM, effect of stress \times age: $X^2 = 7.749$, $p = 0.0514$; SI, **Table S2**). Altogether, these results validate the PMS procedure in quails.

3.2. Effects of PMS on the emotional reactivity and cognitive abilities of F1 quail

In the emergence test, F1 S quail's rally call latencies were higher than those of F1 NS quail (mean \pm SEM, NS: 18.6s \pm 2.9, S: 27.5s \pm 3.2; GLM gamma, effect of stress: $X^2 = 4.350$, $p = 0.037$). F1 S quail also produced fewer rally calls than F1 NS quail (mean \pm SEM, NS: 19.4 \pm 2.0, S: 13.7 \pm 1.8; GLM quasi-poisson, effect of stress: $F_{(1,156)} = 5.503$, $p = 0.020$). Both these results indicate that F1 S quail are less sensitive to social separation than F1 NS quail. F1 S quail's head emergence latencies were higher than those of F1 NS quail (mean \pm SEM, NS: 20.68s \pm 4.1, S: 34.6s \pm 7.2; LM, effect of stress: $F_{(1,156)} = 4.056$, $p = 0.046$) suggesting that F1 S quail are more fearful than F1 NS quail (Mills and

Faure, 1986). Results from the novel object test support these findings. When an unfamiliar object was placed in their home cage, F1 S quail expressed more fear behaviours than did F1 NS quail (mean \pm SEM, NS: 7.0 ± 1.0 , S: 10.5 ± 1.4 ; GLM quasi-poisson, effect of stress: $F_{(1,124)} = 4.086$, $p = 0.045$). All these results indicate that F1 S quail's emotional reactivity is greater than that of F1 NS quail. This is consistent with other studies in quail and rodent showing that PMS can increase fear and depressive or anxiety-like behaviours of the offspring (quail: Guibert et al., 2011; rodent: Weinstock, 2008).

During the familiarisation phase of the spatial learning test, first step latencies decreased over days (**Figure 2A**; LMM, effect of day: $X^2 = 39.644$, $p = 2.463e-9$; SI, **Table S4**). In addition, numbers of different cups visited increased from day to day (**Figure 2B**; GLMM poisson, effect of day: $X^2 = 15.847$, $p = 3.622e-4$; SI, **Table S4**), confirming that quail became familiarised with the task and the arena. Interestingly, irrespective of the day, F1 S females' first step latencies were higher than those of F1 NS females (**Figure 2A**; LMM, effect of stress: $X^2 = 5.720$, $p = 0.017$; SI, **Table S4**). Numbers of visits to the different cups also differed between F1 S and NS females (**Figure 2B**; GLMM poisson, effect of stress \times day: $X^2 = 9.349$, $p = 0.009$; SI, **Table S4**). On the first day of familiarisation, F1 S females visited significantly less different cups than did F1 NS females (**Figure 2B**; $p = 0.018$). These findings support the idea that F1 S females present a greater emotional reactivity, which influenced their abilities to explore and to become familiarised with the arena (quail: Guibert et al., 2011; Lormant et al., 2020b). Over the 12 consecutive days of training, the numbers of errors made both by F1 S and by F1 NS females decreased (**Figure 2C**; LMM, effect of day: $X^2 = 61.306$, $p = 5.300e-9$; SI, **Table S4**). This demonstrates that birds memorised progressively the location of the rewarded cup. Finally, we compared F1 S and NS female's performances in a probe test. As assumed from the results for training, we could not evidence any

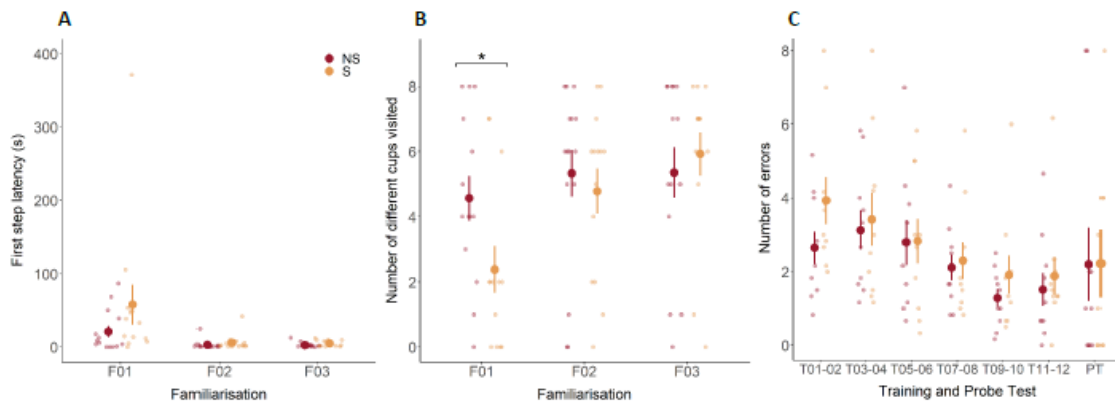


Figure 2 | Spatial learning test. (A) Latency to take first step (s) and (B) Numbers of visits to the different cups by F1 S and NS females during the familiarisation phase ($N_{NS} = 15$, $N_S = 14$). (C) Numbers of errors made by F1 S and NS females before finding the reward-paired cup, during training (data over blocks of 2 days) ($N_{NS} = 10$, $N_S = 10$) and during the probe test (PT; $N_{NS} = 10$, $N_S = 9$). Dots represent individual means for each day or group of days. Trend curves are linear regressions. * $p < 0.05$. See the SI, Table S3.

differences between F1 S and NS females concerning numbers of errors made during the probe test (**Figure 2C**; mean \pm SEM, NS: 2.5 ± 1.0 , S: 2.8 ± 1.0 ; Fisher-Pitman permutation, $Z = -0.017$, $p = 1$). The spatial learning abilities of our quail thus do not seem to have been affected by PMS. This is rather surprising because it is generally reported, notably in rodents, that PMS has negative effects on the spatial skills and the development or functioning of the hippocampus, the brain region supporting these abilities (rat: Gué et al., 2004; Modir et al., 2014). However, some authors suggest that PMS did not always affect these abilities negatively and could even have a facilitating effect (rat: Cannizzaro et al., 2006; Vallée et al., 1997). In their review mostly based on rodents, Sandi and Pinelo-Nava (Sandi and Pinelo-Nava, 2007)), based on an inverse U-shape model, suggest a non-linear relationship between stress intensity and hippocampal-dependant tasks. Moderate stress would not affect or could even promote learning and memory abilities, whereas very intense stress would be deleterious (Sandi and Pinelo-Nava, 2007).

During the probe test of the CPP test, we found that times spent by F1 females offspring in the reward-paired and -unpaired compartments were related to PMS (**Figure 3**; LMM, effect of stress x compartment: $X^2 = 4.818$, $p = 0.028$; SI, **Table S5**). Specifically, times spent by F1 S females in the two compartments were similar ($p = 0.999$) whereas F1 NS females spent significantly more time in the reward-paired compartment than in the reward-unpaired compartment ($p = 0.025$). Contrary to F1 S females, F1 NS females developed a preference for one place since they spent more time in the compartment of the arena where they received mealworms. When only times spent by the subjects in the different compartments are taken into consideration, as authors usually do (Tzschentke, 2007), this could indicate that F1 S females had poorer learning abilities than F1 NS females. However, we found that irrespective of treatment, F1 quail visited the cup in the reward-paired compartment more quickly than the cup in the reward-unpaired compartment (**Figure 4**; GLMM gamma, effect of compartment: $X^2 = 12.953$, $p = 3.194e-4$; effect of stress: $X^2 = 0.021$, $p = 0.884$; effect of stress x compartment: $X^2 = 0.085$, $p = 0.770$; SI, **Table S5**). This result underlines the fact that, regardless of time spent in either compartment of the arena, both F1 S and NS females learnt to associate an environmental cue with a food reward. However, although F1 NS females remain close to the rewarded cup, F1 S females seem to be more flexible since they spent similar times in both compartments of the arena. As previously described in rodents (Aleksandrov et al., 2001), this difference between our groups of quail may be linked to PMS-related coping strategies. If this is the case, it suggests that F1 NS females would have a more passive strategy whereas F1 S females would have a more active strategy. Although this hypothesis needs to be investigated further, our results highlight that coping styles could be fundamental in understanding differences in cognitive performances (Sih and Del Giudice, 2012).

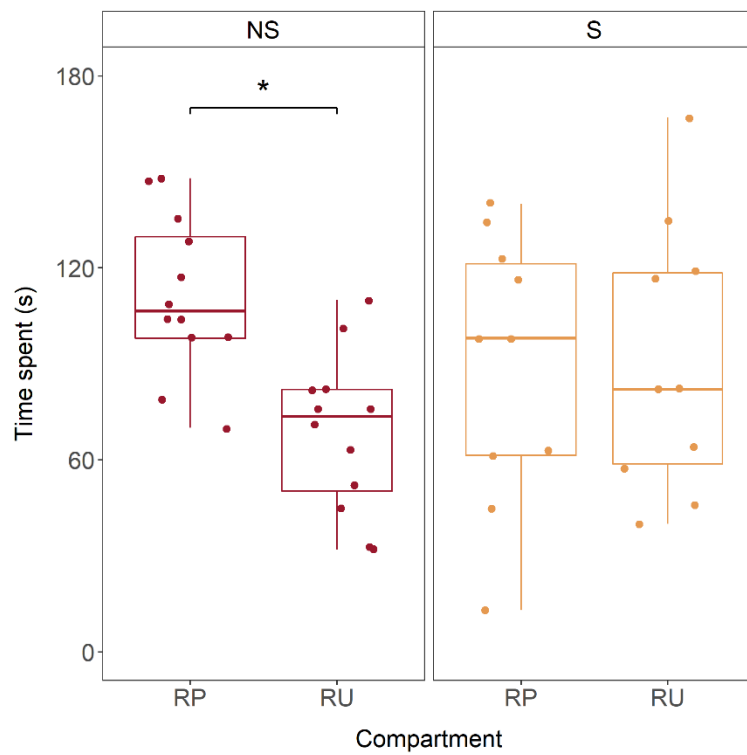


Figure 3 | Conditioned place

preference test ($N_{NS} = 12$, $N_S = 10$).

Times spent (s) in both the reward-paired (RP) and the reward-unpaired (RU) compartment. * $p < 0.05$. See the SI, Table S4.

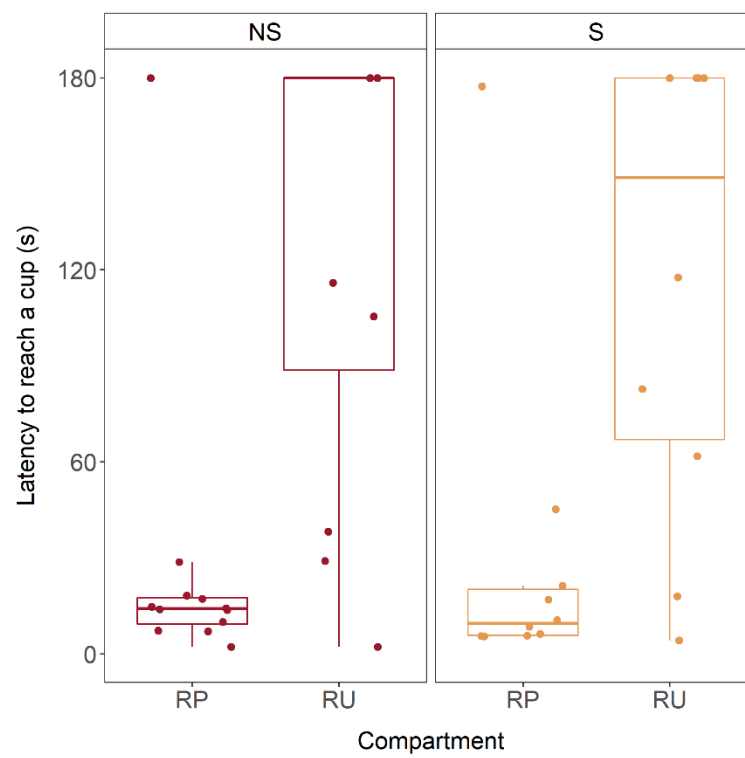


Figure 4 | Conditioned place preference test ($N_{NS} = 12$, $N_S = 10$). Latencies to visit the cup placed in both the reward-paired (RP) and the reward-unpaired (RU) compartment. See the SI, Table S4.

3.3. Effects of PMS on the histone post-translational marks in F1 quail's brain

To gain insights into the mechanisms underlying the long-term effect of PMS, we performed an immunohistochemical analysis of two epigenetic marks, H3K4me2 and H3K27me3. Epigenetic marks are known to contribute to the regulation of gene expression and their reprogramming have the potential to promote homeostatic and physiological responses either immediately, at a later stage of development and sometimes across generations (Hao et al., 2021; Mirbahai and Chipman, 2014). We investigated both H3K4me2 and H3K27me3 marks by density analysis. We could not observe any significant differences between groups for H3K4me2 in any of the brain structures we studied (**Figure 5**; Fisher-Pitman permutation, $p > 0.05$). However, the density of H3K27me3 positive cells (**Figure 5B and C**) was significantly higher in F1 S females than in F1 NS females in the hippocampus (Fisher-Pitman permutation, $Z = -2.242$, $p = 0.020$), the paraventricular hypothalamic nucleus (Fisher-Pitman permutation, $Z = -2.106$, $p = 0.025$) and the dorsal amygdala (Fisher-Pitman permutation, $Z = -2.239$, $p = 0.022$). In summary, PMS did not influence the active mark H3K4me2 but has a specific effect on the repressive mark H3K27me3 (Kouzarides, 2007). Moreover, the density of H3K27me3 positive cells was higher in key brain structures involved in the emotional response and cognition of birds (quail: Lormant et al., 2020a; duck: Phillips, 1964; quail: Saint-Dizier et al., 2009). Although causal

relationships cannot be made here, this result interestingly mirrors the behavioural differences we evidenced between F1 S and NS quail. We actually showed that F1 S quail presented a higher emotional reactivity since they were more fearful when exposed to a novel environment or a novel object. Furthermore, while we did not show any detrimental effects of PMS on the learning abilities per se of F1 S females (i.e quail learned to locate the target cup in the spatial learning test and the rewarded-paired compartment in the CPP test), our findings from both the spatial learning and the CPP tests confirm that it did affect quail's emotional reactivity. F1 S females took longer to become accustomed to the spatial arena and presented a different coping strategy in the CPP test. Therefore, by modifying their emotional reactivity PMS may have indirectly influenced the learning abilities of F1 S females. Birds' and mammals' fear reactions involve the amygdala and its related nuclei (duck: Phillips, 1964; quail: Saint-Dizier et al., 2009). The higher density of H3K27me3 positive cells in the PVN and the dorsal nucleus of the amygdala of F1 S females could be associated with their greater emotional reactivity (i.e. results from the emotional reactivity tests and the familiarisation phase of the spatial learning test). The same hypothesis could be made for the higher density of H3K27me3 positive cells in the hippocampus of F1 S females. The hippocampus is well known for its involvement in spatial memory (quail: Lormant et al., 2020a) but it also appears to play an important role in the regulation of stress responses and in the expression of fear and anxiety-related behaviours (Revest et al., 2009). In line with this, recent studies evidenced that hippocampus markers of plasticity in quail were modulated significantly by their emotional reactivity (Gualtieri et al., 2019; Lormant et al., 2020b). H3K27me3 is a mark generally implicated in silent chromatin (Kouzarides, 2007). An increased density of H3K27me3 positive cells could therefore induce the silencing of genes in F1 S quail's brain.

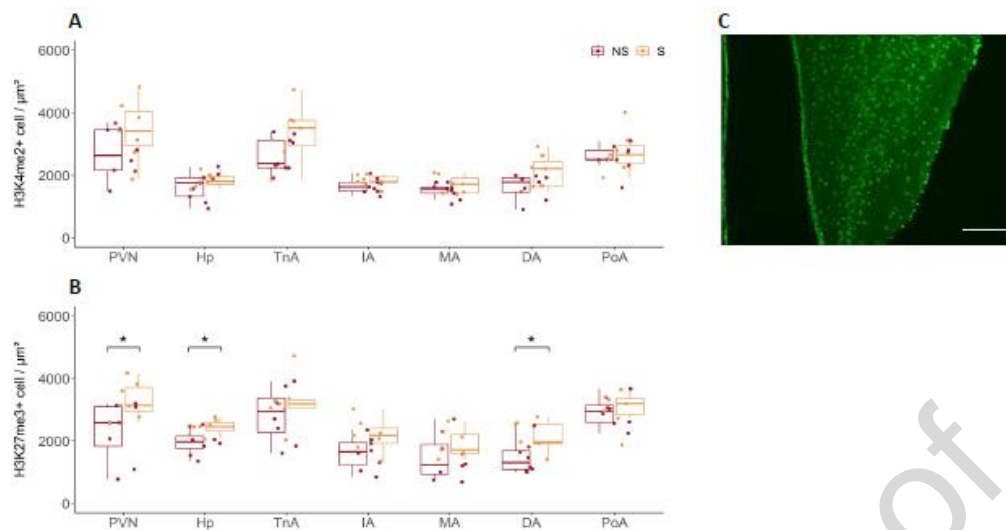


Figure 5 | Effects of PMS on H3K4me2 and H3K27me3. Density / μm^2 of (A) H3K4me2 and (B) H3K27me3 positive cells in: PVN = paraventricular hypothalamic nucleus; Hp = the hippocampus and subnuclei of the arcopallium/amygdala: TnA = nucleus taeniae; IA = intermediate; MA = medial; DA = dorsal and PoA = posterior amygdaloid of NS ($N_{\text{H3K4me2}} = 9$, $N_{\text{H3K27me3}} = 8$) and S ($N_{\text{H3K4me2}} = 7$, $N_{\text{H3K27me3}} = 7$) F1 quail. * $p < 0.05$. (C) Illustration of fluorescent H3K27me3 positive cells in the hippocampus ©PIC. Scale bar: 500 μm .

Although this hypothesis needs further investigation, we suggest that F1 S quail's behavioural changes may be associated with altered H3K27me3 signal and gene expression levels. In line with this idea, Tsankova et al. showed in male mice that a chronic social defeat induces downregulation of Bdnf transcripts (i.e. Bdnf III and Bdnf IV) in the hippocampus and increased H3K27 dimethylation at their corresponding promoters (Tsankova et al., 2006). Although our data suggest that epigenetic marks may be involved in the behavioural differences observed in F1 quail, it is also possible that these differences depend of a direct hormonal regulation (e.g. involvement of the HPA axis). The effects of PMS observed in F1 quails could be mediated by maternal androgens. Indeed, we showed that eggs from F0 S females contain less testosterone than eggs from F0 NS females. While the effects of maternal androgens on offspring's behaviour have been widely demonstrated (Groothuis et al., 2005), their effects on epigenetic marks have been demonstrated only recently. For example,

Bentz et al. showed that zebra finches from testosterone-injected eggs presented more aggressive behaviours than control males, and had several differentially expressed genes that were also differentially methylated. Their findings suggest that *in ovo* testosterone may induce epigenetic modifications that could influence aggression behaviour (Bentz et al., 2021).

In our study, we demonstrated that PMS can influence both birds offspring's emotional behaviours and specific epigenetic marks in their brain. Thus, PMS could play a fundamental role in shaping epigenome and phenotype, which could in turn influence individuals' adaptive abilities. These results raise new questions especially concerning the transmission of such effects to following generations.

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Ethical note

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the departmental direction of veterinary services (Ille-et-Vilaine, France, permit number 005283) and were realised in accordance with the European Communities Council Directive 2010/63/EU. The breeding procedure and tests were approved by the regional ethics committee (agreement number: 25552-2020052717026157_v2).

Author contributions

L.C., C.H., S.L. and M.C. designed the study; M.C. performed the behavioural experiments and analysed the data; L.C., P.C., M.G., M.M. and V.C. worked on the brain epigenome analysis; R.P., F.A.,

C.H., S.L. and M.C. worked on the physiological analysis; L.C., C.H. and M.C., wrote the manuscript; all authors discussed the manuscript.

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Highlights

- Prenatal maternal stress increases F1 offspring's emotional reactivity but has no effect on their learning abilities.
- Prenatal maternal stress influences epigenetic marks in the F1 offspring's hippocampus, paraventricular hypothalamic nucleus and dorsal nucleus of the amygdala
- Epigenetic marks changes were observed in specific brain structures involved in the behavioural differences observed in F1 quail