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Trans-generational effects of prenatal stress in quail

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The prenatal environment is a source of phenotypic variability influencing the animal's characteristics. Prenatal stress affects not only the development of offspring, but also that of the following generation. Such effects have been best documented in mammals but can also be observed in birds, suggesting common processes across phylogenetic orders. We found previously that Japanese quail females stressed during laying produced offspring with higher fearfulness, probably related to modulation of testosterone levels in their eggs. Here, we evaluated long-term effects of prenatal stress by analysing reproductive traits of these F_1 offspring and, then, the development of their subsequent (F_2) offspring. The sexual behaviour of F_1 prenatally stressed (F1PS) males was impaired. F1PS females' eggs contained less yolk and more albumen, and higher yolk testosterone and progesterone levels than did F_1 prenatal control females. The fearfulness of F_2 prenatally stressed quail was greater than that of F_2 prenatal control quail. These F_2 behavioural differences paralleled those evidenced by their parents, suggesting trans-generational transmission of prenatal stress effects, probably mediated by egg compositions of F1PS females.

1. Introduction

Environment plays a fundamental role in evolutionary processes. By interacting with an organism's developmental process, it induces the emergence of phenotypic variability and thereby leads to patterns of species adaptation [1]. Maternal influences are an important environmental driver of this phenotypic variability as they intervene from the earliest development stage. Thus, maternal stress during pregnancy influences mammals' offspring morphology [2], physiology [3] and behaviours [2,4]. These prenatal effects are likely to be mediated by modifications, owing to stress, of maternal plasma glucocorticoid and androgen concentrations [4,5]. Interestingly, maternal stress effects also appear to influence the growth and behaviour of subsequent generations [6,7,8]. This trans-generational transmission of maternal effects opens new perspectives for understanding variability in animal populations and could be of fundamental importance for phenotypic evolution.

Prenatal environmental influences can also affect bird's development. These influences can intervene during embryo development (incubation) and thereby implicate both direct and indirect parental factors such as light, temperature or incubation effort [9,10]. Prenatal influences can also occur before embryonic development, during egg formation. In this case, prenatal influences involve the modulation of egg characteristics, and especially hormonal contents, and are considered prenatal maternal effects [11]. In this context, maternal stress affects the phenotypes of the offspring. The offspring of female poultry submitted to stressful events during laying exhibited greater levels of fearfulness [12–14] and lower competitive abilities than did offspring of control females [12]. These prenatal stress effects could be mediated by modifications of steroid hormone levels of maternal origin present in eggs [15]. Indeed, yolk testosterone levels increase in eggs of females living under stressful conditions during laying [13,14,16,17]. The effects of prenatal exposure to elevated yolk

testosterone levels have been studied mainly by applying yolk hormonal injections. This has been shown either to favour or to impair offspring's characteristics [18]. So, yolk testosterone injections can make Japanese quail's (*Coturnix coturnix japonica*) chicks either less [19] or more fearful [20]. Moreover, prenatal testosterone exposure appears to impair birds' reproductive characteristics, suggesting long-term effects of prenatal environment and possibly trans-generational consequences [21,22]. Although yolk testosterone levels play a key role in prenatal influences, other hormones could also be involved. Thus, androstenedione is an interesting androgen, found in much larger amounts than testosterone in precocial birds' egg yolks [23,24] and its effects on offspring differ from those of testosterone [25]. In addition, progesterone is an androgen precursor and the most abundant steroid in avian yolks surrounding avian embryos during the early developmental stages [23,24]. Moreover, yolk progesterone levels vary in relation to females' living conditions (relationship with humans) [26] and phenotypes (birds selected for a high or low level of fearfulness) [27], thus reinforcing the idea of a potential role of this steroid on offspring phenotype. Thus, a more general analysis of hormonal modulation in eggs should provide a better understanding of prenatal influences in birds.

Previously, we showed that laying Japanese quail females submitted to unpredictable stressors produced chicks exhibiting enhanced fearfulness, probably related to the modulation of testosterone levels in their eggs [14]. The present experiment investigated long-term effects of this prenatal stress on offspring and on their descendants. We hypothesized that the effects of prenatal stress on offspring phenotype would be trans-generational and transmitted to the next generation possibly related to changes in the yolk steroid levels of prenatally stressed females' eggs. First, we evaluated the consequences of prenatal stress on males' copulation behaviour and on females' laying rates and egg characteristics, including egg composition and yolk steroid levels (testosterone, androstenedione, progesterone). Second, we monitored the morphological and behavioural development of the offspring of these prenatally stressed birds.

2. Material and methods

(a) F_1 subjects origin and housing

F_1 quail were the offspring of females that had been either submitted or not to unpredictable stressors during their laying phase (details in the electronic supplementary material, section S1). Thirty-five prenatally stressed quail (F1PS: 21 females, 14 males) and 41 prenatal control quail (F1PC: 28 females, 13 males) from this previous study were raised until they were adult for the present study. F1PS and F1PS males were housed in the same room. To thwart possible effects of social interactions between F1PC and F1PS females on their eggs' hormone levels [13], females of the two groups were housed in two different rooms. In both rooms, quail were housed in individual $22 \times 20 \times 15$ cm cages with food and water provided ad libitum in common troughs placed in the front and behind cages. Housing conditions were standardized: same room dimensions ($2.25 \times 1.85 \times 2.80$ m), same spatial arrangement of cages, similar temperature ($19 \pm 1^{\circ}$C) and light : dark cycle conditions (14 L : 10 D) and same caretaker.

(b) F_1 males' sexual behaviour

Before the breeding period, F_1 males' copulation behaviour was evaluated in encounters with females. Copulation in quail includes several phases: first, the male grabs the back of the female's head or neck in his beak (grab response). The male then climbs onto the female's back with both feet (mount response) and brings his cloaca in contact with the female's cloaca, making a series of cloacal thrusts (cloacal contact response). This response sequence can easily be interrupted, and the male may have to make several grab and mount attempts before making cloacal contact [28]. When they were between 30 and 32 weeks old, each sexually mature male was tested five times in a female encounter test. Twelve sexually mature females from a commercial farm were used for these encounters. Each male was presented a different unfamiliar female and at a different time in the morning (between 07.30 and 11.30) for each encounter. During encounter, one male and one female were placed in a wooden cage ($83 \times 60 \times 35$ cm) with wood shavings on the floor. The side of the box facing the experimenter was a glass window. The experimenter noted for 5 min all copulation attempts (response sequences interrupted after the grab response and those interrupted after the mount response) and effective copulations (complete response sequences, from the grab response to the cloacal contact response).

(c) Breeding and egg collection

The breeding period began when the F_1 birds were 38 weeks old. F1PC females were mated with F1PC males, and F1PS females were mated with F1PS males. Females were presented a male for mating three times a week (six sessions in all). A female met a different male, and a male met approximately two females (from one to three according sessions) during each mating session. Two F1PS males were not used for breeding, as they did not copulate with females. Pairs stayed together in a small cage for a few minutes. Eggs were collected daily for 13 days, identified according to the female that had laid it and weighed. One egg per female was collected, on the same day for all females (28 F1PC eggs, 21 F1PS eggs), and stored at -20°C for hormone analyses. Most of the eggs (252 F1PC eggs, 9.0 ± 0.4 egg per female; 200 F1PS eggs, 9.5 ± 0.5 egg per female) were stored at $16 \pm 1^{\circ}$C until incubation.

(d) Yolk steroid analysis

Levels of yolk steroids were measured, using the enzyme immunoassays methods described in Möstl *et al*. [23] and Hackl *et al*. [24] (details in the electronic supplementary material, section S2). We measured yolk testosterone, androstenedione and progesterone in two assays. The intra-assay variations were 8.5 per cent, 4.2 per cent and 9.2 per cent, respectively.

Yolk and eggshell were weighed, and albumin weight was obtained by subtracting the weight of the eggshell plus the weight of the yolk from the total weight of the egg.

(e) F_2 chick rearing

Eggs were placed in an incubator for 18 days. During the first 14 days, eggs were maintained at 37.7°C, 45 per cent of relative humidity and with an automatic rotation of 45° twice a day. For the last days, the temperature was decreased to 37.2°C, the humidity was raised to 60 per cent, and egg rotation was stopped to facilitate hatching. At hatching, leg rings identified chicks according to their mother. Hatching date of chick (day 17 or 18 of incubation) was recorded. Unhatched eggs were opened to determine whether they were fertilized or not (presence/absence of an embryo). Egg fertilization rates (n fertilized eggs per n incubated eggs $\times$ 100) and hatchability rates of fertile eggs (n hatched eggs per n fertilized eggs $\times$ 100) were calculated for each F_1 female.

F_2 chicks were housed in groups of four from the same experimental group, but from different mothers. Thus, 10 F_2 prenatally stressed (F2PS) groups ($n = 39$ F2PS chicks; one group included three chicks) and 10 F_2 prenatal control (F2PC) groups

($n = 40$ F2PC chicks) were formed. Each group was housed in a $100 \times 70 \times 62$ cm cage with solid walls that did not allow chicks to make visual contact with others groups. All groups were maintained in a single experimental room. A warming bulb (38 ± 1°C) was placed in each cage to ensure chicks' thermoregulation until they were 10 days old. After this, warming bulbs were switched off, and the temperature in the room was maintained at 20 ± 1°C. Chicks were exposed to a 10 L : 14 D cycle. Water and food were provided ad libitum. The general development of chicks was monitored by weighing them weekly, from hatching until they were three weeks old, using electronic scales. Their sex was determined by their sexual dimorphic plumage when they were three weeks old. Sex-ratios did not differ between the two groups (F2PC chicks: 15 females, 25 males; F2PS chicks: 19 females, 20 males; chi-squared test, $\chi^2 = 1.014$, d.f. $= 1$, $p = 0.31$).

(f) Behavioural tests

Classical ethological tests devised for poultry were used to assess the emotional reactivity of F_2 chicks [29]. These tests are the same as those previously used to evaluate the fearfulness of the F_1 generation [14].

(i) Emergence test

F_2 chicks were tested when they were from 14 to 15 days old. The test chick was taken from its home cage to a dark room in a cardboard box ($18 \times 18 \times 18$ cm). The cardboard box was placed on the left side of a wooden box ($83 \times 60 \times 35$ cm). After 1 min, the cardboard box was opened, and lights were switched on. The experimenter recorded latency of chick's emergence into this novel environment. When a chick did not leave the box within 3 min, a maximum score of 180 s was recorded. When a chick left the box, the experimenter noted, for 3 min, latency of its first call, number of calls, locomotor acts (walks, runs), exploratory acts (pecking floor per sides) and high-posture behaviour (chick stands upright on 'tiptoes', holding its body very straight). Latency of emergence is a good estimate of emotional reactivity: fearful animals take longer to emerge [30].

(ii) Open-field test

F_2 chicks were tested when they were from 16 to 17 days old. A test chick was taken from its home cage to a dark room in a cardboard box. The chick was then put in the centre of a wire netting cylinder (diameter 120 cm, height 70 cm) with a linoleum floor. Lights were switched on, and the experimenter (hidden behind a two-way mirror) noted latency of first call, latency of first step, and number of calls, steps, exploratory acts and high-posture behaviour for 5 min. This test evaluates the fearfulness of chicks to both a novel environment and separation from conspecifics [29].

Previous studies reported significant correlations between poultry emotional reactivity data revealed by open-field and emergence tests, although these correlations were not unequivocal [30]. Indeed, emotional reactivity (fearfulness) is a complex trait, composed of different aspects of fear and is influenced by the nature of the stressful event and by the individual's characteristics [29]. Thus, fear reactions can differ among contexts, and a combination of tests is usually needed to assess the general emotional reactivity [29].

All experiments were approved by the departmental direction of veterinary services (permit no. 005283) and performed in accordance with the European Communities Council directive (86/609/EEC).

(g) Data analyses

Kolmogorov–Smirnov tests were used to determine whether datasets were normally distributed. Laying rates, fertilization rates, hatchability rates were not normally distributed, so Mann–Whitney U-tests were used. Data for evaluating F_1 males' sexual behaviour were mean numbers of copulation attempts and copulations during the five encounters. A MANOVA and individual one-way ANOVAs analysed log-transformed ($Y + 1$) yolk–hormone data. ANOVAs analysed arcsin-square-root-transformed proportions of egg components. Data for incubated egg weight comparisons were mean egg weight per F_1 female and were analysed using ANOVAs. F_2 chick-weight data were analysed using one-way repeated measures ANOVAs (maternal prenatal experience × chicks' age). F_2 chicks' behaviours (not normally distributed) were analysed using a general linear model on ranking values. A two-way ANOVA evaluated cage and prenatal effects, with cage effect as a random factor. Data are represented as means ± standard error of the mean (s.e.m.). Analyses were performed using MINITAB Statistical Software with significance level set at $p \leq 0.05$.

3. Results

(a) F_1 males' sexual behaviour

During sexual encounters, F1PS males made significantly fewer copulation attempts (2.44 ± 0.60 versus 4.23 ± 0.53; Mann–Whitney U-test: $U = 45$, $p = 0.025$) and fewer copulations (1.21 ± 0.37 versus 2.19 ± 0.31) than did F1PC males ($U = 44$, $p = 0.022$).

(b) F_1 females' laying rate and egg characteristics

Laying rates of F1PS and F1PC females did not differ significantly (respectively, 0.88 ± 0.03 and 0.90 ± 0.02 egg per day; Mann–Whitney U-test: $U = 286$, $p = 0.87$).

Mean weights of eggs collected for incubation did not differ between F1PS and F1PC females' eggs (respectively: 14.02 ± 0.18 g and 14.14 ± 0.23 g; ANOVA: $F_{1,47} = 0.148$, $p = 0.70$), neither did the weights of eggs collected for hormonal analyses (13.81 ± 0.17 g and 13.83 ± 0.23 g; $F_{1,47} = 0.004$, $p = 0.95$). Proportions of egg shell to egg weight did not differ between F1PS and F1PC females' eggs (12.61 ± 0.32% and 12.53 ± 0.19%; $F_{1,47} = 0.029$, $p = 0.87$), but F1PS females' eggs contained proportionally more albumen (59.92 ± 0.73% versus 58.13 ± 0.36%; $F_{1,47} = 5.711$, $p = 0.021$) and less yolk (27.48 ± 0.70% versus 29.34 ± 0.39%; $F_{1,47} = 6.176$, $p = 0.017$) than did F1PC females' eggs.

No significant overall effect on yolk hormone concentrations was evidenced (MANOVA: $F_{3,45} = 1.793$, $p = 0.16$). However, when data for each hormone were analysed separately, we found that testosterone concentrations tended to be higher in F1PS females' egg yolks than in F1PC females' eggs, but this trend was not significant (ANOVA: $F_{1,47} = 3.224$, $p = 0.079$; figure 1*a*). Yolk androstenedione concentrations did not differ between the two groups of females ($F_{1,47} = 0.548$, $p = 0.46$; figure 1*b*). However, F1PS females' egg yolks had higher progesterone concentrations than did F1PC females' eggs ($F_{1,47} = 4.975$, $p = 0.031$; figure 1*c*).

(c) Fertilization and hatchability of eggs

Fertilization rates of F1PS females' eggs were significantly lower than those of F1PC females' eggs (respectively: 46.26 ± 6.16% and 66.23 ± 5.27%; Mann–Whitney U-test: $U = 180$, $p = 0.021$). Hatchability rates of fertile eggs did not differ between F1PS and F1PC females' eggs (respectively: 46.75 ± 10.62% and 50.87 ± 6.63%; $U = 217.5$, $p = 0.77$).

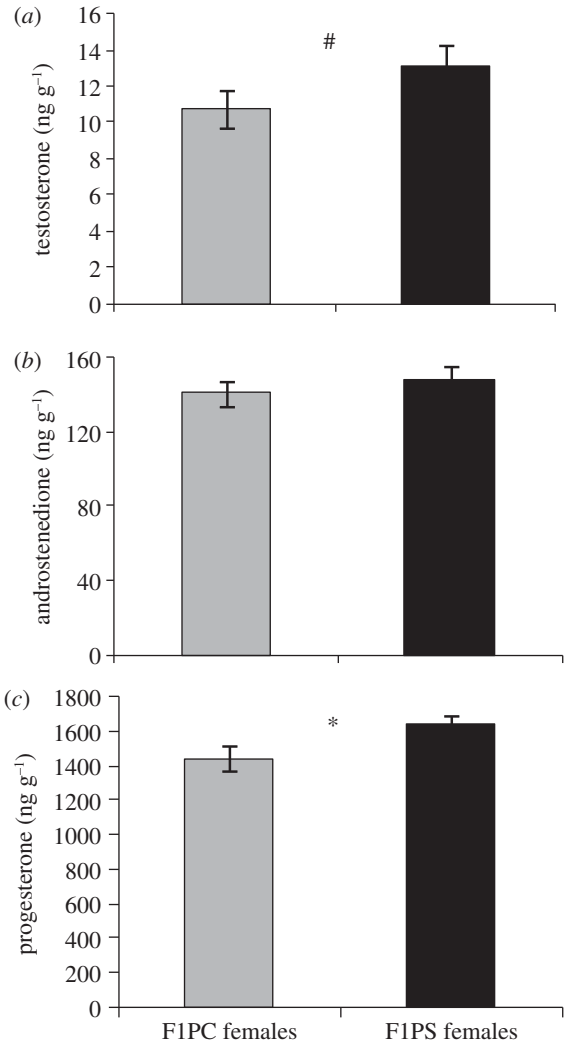

Figure 1. (*a*) Yolk testosterone, (*b*) androstenedione and (*c*) progesterone concentrations (mean ± s.e.m. ng g^{-1}) in F1PC ($n = 28$) and F1PS ($n = 21$) females' eggs as measured by ELISA. ANOVA: $^{\#}0.1 > p > 0.05$, $^{*}p \leq 0.05$.

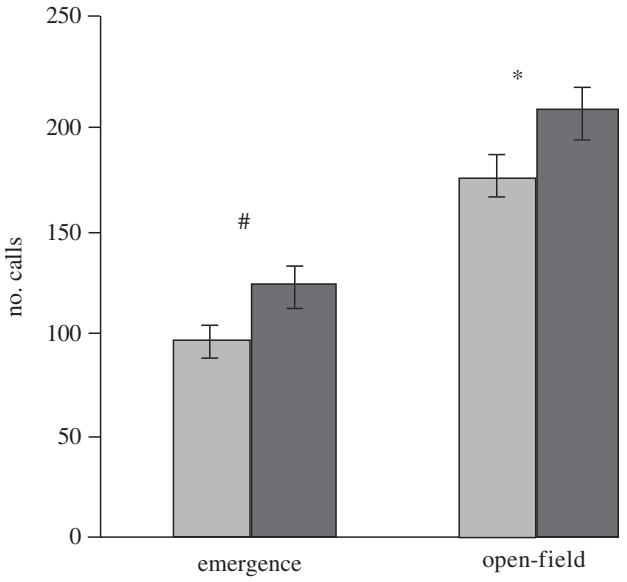

Figure 2. Numbers of calls (mean ± s.e.m.) emitted by F2PC ($n = 40$) and F2PS ($n = 39$) chicks in emergence and open-field tests. ANOVA: $^{\#}0.1 > p > 0.05$, $^{*}p \leq 0.05$. Light grey bars, F2PC chicks; dark grey bars, F2PS chicks.

(d) F_2 chick characteristics

Mother's treatment did not influence hatching dates and body weight of chicks (detailed data in the electronic supplementary material, section S3).

In the emergence test, F2PS chicks emerged in the novel environment significantly later than did F2PC chicks (respectively, 34.1 ± 7.9 s and 19.9 ± 6.8 s; ANOVA: $F_{1,78} = 5.63$, $p = 0.029$). Numbers of chicks that left the box did not differ between the two groups (38 F2PC chicks and 36 F2PS chicks; chi-squared test: $\chi^2_2 = 0.241$, $p = 0.62$). After emergence, F2PS chicks emitted their first call sooner than did F2PC chicks (respectively, 6.11 ± 2.29 s and 8.21 ± 2.95 s; ANOVA: $F_{1,73} = 5.21$, $p = 0.035$) and they tended to emit more calls ($F_{1,73} = 3.10$, $p = 0.09$; figure 2). Numbers of high-posture behaviours (F2PC chicks: 9.29 ± 1.31; F2PS chicks: 12.22 ± 1.60; $F_{1,73} = 1.42$, $p = 0.25$) of locomotor acts (F2PC chicks: 11.90 ± 1.33; F2PS chicks: 14.72 ± 1.71; $F_{1,73} = 0.82$, $p = 0.38$) and of exploratory acts (F2PC chicks: 4.13 ± 0.66; F2PS chicks: 3.72 ± 0.57; $F_{1,73} = 0.01$, $p = 0.93$) did not differ between the two groups of chicks. No cage effects were evident for these parameters (ANOVA, all $p > 0.10$).

In the open-field test, neither latency to first step (F2PC chicks: 4.98 ± 0.78 s; F2PS chicks: 10.92 ± 4.00 s; ANOVA: $F_{1,78} = 0.41$, $p = 0.53$), nor numbers of steps (F2PC chicks: 301.55 ± 26.59; F2PS chicks: 339.23 ± 30.17; $F_{1,78} = 0.86$, $p = 0.37$), nor numbers of high-posture behaviours (F2PC chicks: 9.73 ± 0.82; F2PS chicks: 11.64 ± 0.98; $F_{1,78} = 1.91$, $p = 0.18$) differed between F2PC and F2PS chicks. Latency to first call did not differ between the two groups (F2PC chicks: 8.32 ± 5.65s; F2PS chicks: 2.13 ± 0.61s; $F_{1,78} = 0.41$, $p = 0.53$), but F2PS chicks emitted more calls than did F2PC chicks ($F_{1,78} = 6.93$, $p = 0.0017$; figure 2). Moreover, F2PS chicks made fewer exploratory acts than did F2PC chicks (respectively, 4.44 ± 0.70 and 7.30 ± 1.07; $F_{1,78} = 4.51$, $p = 0.048$). No cage effects were evident for these parameters (ANOVA: all $p > 0.10$), except for latency to first call ($F_{18,78} = 1.91$, $p = 0.033$).

4. Discussion

This study revealed long-term effects of prenatal stress on reproductive traits of birds of the first generation (F_1) and, a trans-generational effect on the behaviour of second generation (F_2) offspring.

First, the sexual behaviour of prenatally stressed (F1PS) males was impaired: they made fewer copulation attempts and performed fewer copulations during sexual encounters than did prenatal control (F1PC) males. Similarly, prenatally stressed male rats initiated copulation less often and failed to ejaculate during copulation more frequently than did control rats [31]. Moreover, F1PS females laid eggs that contained relatively less yolk and more albumen than did F1PC females. Furthermore, yolks of F1PS females' eggs tended to have higher testosterone concentrations and had significantly higher progesterone concentrations. Prenatal stress is known to induce abnormal regulation of the hypothalamic–pituitary–adrenal (HPA) axis in offspring, resulting in higher basal levels of corticosteroids and differences in the activation of the HPA axis between prenatally stressed and control offspring after exposure to stress [32]. Our results suggest that prenatal stress also affects the regulation of the hypothalamic–pituitary–gonadal axis. Prenatally stressed female rats had higher basal progesterone levels when adult than did control females, suggesting that gonadal steroid metabolic routes could be affected by prenatal

stress [33]. Finally, prenatal stress affected fertility given that egg fertilization rates of F1PS females were lower than those of F1PC quail. This effect could be of male origin, related to their impaired sexual behaviour, and/or of female origin in relation to potential effects of prenatal stress on the physiology of fertilization, as in mammals [34]. These prenatal stress effects on quail's reproductive functions could be mediated by the hormonal characteristics of their embryonic milieu, as F1PS quail developed in eggs that tended to contain more testosterone [14]. Exposure to androgens during embryonic development can impair female and male reproduction. For instance, the egg production and fertility of female pheasants (*Phasianus colchicus*) from testosterone-injected eggs were lower [22]. The male Chinese quail (*Coturnix chinensis*) from testosterone-injected eggs had smaller testes than did control males, stressing the detrimental effects of high testosterone levels during embryonic development on males' reproduction system [21].

We showed that prenatal stress had a trans-generational effect on F_2 offspring. Indeed, behaviour of F2PS and F2PC chicks differed. F2PS chicks took longer to leave the emergence box than did F2PC chicks, suggesting a more cautious behaviour phenotype [30]. Furthermore, they explored less in a novel environment, indicating a more pronounced emotional reactivity, as fearfulness is known to inhibit exploration in domestic fowl [35,36]. In tests, F2PS chicks called earlier and more frequently than did F2PC chicks, indicating active search for conspecifics [29] and reflecting their higher sensitivity to social separation. Generally, F2PS chicks showed greater fearfulness than did F2PC chicks.

Interestingly, the behavioural differences between F2PS and F2PC chicks are similar to those between their parents evaluated in the same way [14]. Indeed, both F2PS and F1PS chicks showed longer latencies to emerge, less explorations in a novel environment and more calls during the tests than chicks from control parents. This finding suggests a trans-generational transmission of prenatal stress effects. A similar result has been reported only for rats: handling female rats during infancy reduced the activity of their offspring and grand-offspring [7], and avoidance-conditioning of female rats either before mating or during gestation increased their grand-offspring's exploratory behaviour [8].

Two pathways could explain this non-genomic transmission of phenotypes across generations [37]. One is that prenatal stress experienced by F_1 offspring could have altered the epigenetic regulation of genes in both the germ-line and in somatic tissues and, consequently, could induce a germ-line transmission of these environmentally generated epigenetic modifications to the F_2 generation [38]. Another potential mechanism is that prenatal stress altered F_1 offsprings' neural and/or hormonal regulation, thus offering a different prenatal environment to their own offspring. This modified prenatal environment could then lead to a different embryonic development and thus to phenotypic differences between the groups of F_2 offspring. Our data seem to support this second hypothesis as eggs' characteristics (yolk and albumen proportions, testosterone and progesterone levels) clearly differed between F1PS and F1PC females' eggs. The potential influence of yolk steroid hormones on chicks' behaviour is well known [18] and revealed especially, strong effects of yolk testosterone on chicks' fearfulness [19,20]. Our study, however, suggests also that progesterone could play an important role in shaping offspring behaviour. Our work shows, for the first time to our knowledge in a bird species, a trans-generational influence of maternal stress on the behaviour of F_2 offspring. This study stresses the importance of considering living conditions of one generation for understanding phenotypic variability in subsequent generations.

All experiments were approved by the departmental direction of Veterinary Services (permit no. 005283) and performed in accordance with the European Communities Council directive (86/609/EEC).

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