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Unpredictable mild stressors on laying females influence the composition of Japanese quail eggs and offspring's phenotype

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ABSTRACT

Maternal stress effects on offspring development have been studied largely in rodents and primates, and to a lesser extent in farm animals. Potential lack of knowledge concerning prenatal stress on farm animals is regrettable because they are frequently subjected to a variety of husbandry stressors. Above all, effects of maternal stress on poultry offspring have been neglected. Prenatal effects in birds are known to involve maternal steroids present in eggs. In this study, we investigated the effects of daily unpredictable stressors applied to laying Japanese quail on their offspring's morphological and behavioural development. We also investigated the effects of our procedure on female reproductive output and on egg characteristics (weight, composition, yolk sex steroid levels). Our procedure induced only a mild stress: laying and egg fertilization rates of stressed females were not impaired; they remained similar to those of control females. Nonetheless, our stress procedure had an impact on some egg characteristics: stressed females' eggs were heavier, contained more albumen and tended to have higher yolk testosterone levels than control females' eggs. Stressed females' offspring hatched earlier, were heavier at hatching and had a different growth pattern than did control females' offspring. They also appeared to have a higher emotional reactivity than control chicks when encountering a novel environment and they reacted more strongly following social separation. Our study revealed that mild stressors applied to laying Japanese quail can increase the emotional reactivity of their chicks and suggested that maternal stress effects on offspring are mediated by changes in egg composition and yolk testosterone levels. We stress the particular relevance to the poultry industry of our findings highlighting the importance of taking into account the environment of laying females, as this environment can influence their progeny's behaviour and therefore their subsequent adaptation to husbandry conditions and ultimately their welfare.

1. Introduction

Maternal stress effects on offspring development have been studied largely in rodents and primates. In these species, prenatal stress strongly affects the general development of offspring, impairing their growth, hypothalamic–pituitary–adrenal (HPA) axis activity, their fearfulness, cognitive abilities, social behaviour (play, aggression) and reproductive behaviour (sexual and maternal behaviour) (Braastad, 1998; Kaiser and Sachser, 2005; Welberg and Seckl, 2001). These effects have been shown to be mediated by modifications of maternal glucocorticoid

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and androgen plasmatic concentrations under stressful conditions (Barbazanges et al., 1996; Welberg and Seckl, 2001; Wewers et al., 2005).

Few studies concern prenatal stress effects on farm animals, although these animals are frequently subjected to a variety of physical and psychosocial stressors as, for instance, thermal stressors, sudden noise and movements, transport, handling, social mixing or isolation. In farm mammals, prenatal stress also appears to affect offspring's HPA axis activity, as these young subsequently present higher cortisol levels in standard or challenging situations (Haussmann et al., 2000; Lay et al., 1997; Roussel et al., 2004; Sloboda et al., 2002). Prenatal stress amplified piglets' immediate behavioural responses to acute pain (Rutherford et al., 2009) and sows' maternal behaviour was altered as aggression of pups increased (Jarvis et al., 2006).

In poultry, maternal stress effects on offspring have been far less studied. In chicken, offspring of hens subjected to an unpredictable feed restriction treatment during laying presented a higher fearfulness level and spent less time eating in a competitive ability test than did control offspring (Janczak et al., 2007). We demonstrated previously that the emotional reactivity of the offspring of Japanese quail females submitted to social instability during their laying period was higher in a novel environment and following social separation than that of the offspring of females maintained in a stable social environment (Guibert et al., 2010). Corticosterone (stress hormone) injections into eggs, a method mimicking maternal stress, induced similar effects on chicks' behaviour (Freire et al., 2006; Janczak et al., 2006). The prenatal stage in birds occurs in the egg, which contains all the resources essential for the embryo's development. Egg quality and composition vary according to the laying females' living conditions. For example, high environmental temperatures (heat stress) and housing conditions affect egg weight and/or egg components' (shell, albumen, yolk) weight and quality (Banga-Mboko et al., 2003; Downing and Bryden, 2008; Holt et al., 2011; Mashaly et al., 2004; Vits et al., 2005), suggesting potential effects on offspring's general development. A special attention is now paid to the effects of variations in the physical environment of laying females on their egg steroid concentrations (Janczak et al., 2009; Nätt et al., 2009). Indeed, most studies concerning prenatal maternal effects in birds focus on the effects of steroids of maternal origin present in egg yolks (Gil, 2008; Groothuis et al., 2005). Androgens, and especially testosterone, are considered to be the main mediators of maternal effects. For example, yolk testosterone levels increase under stressful social conditions (increased breeding density: Schwabl, 1997; Pilz and Smith, 2004; but see Mazuc et al., 2003; social instability: Guibert et al., 2010; aggressiveness: Whittingham and Schwabl, 2002). Moreover, experimental elevation of yolk androgens via injections into eggs revealed the effects of these hormones on subsequent offspring's short- and long-term growth, immunity and behaviour (begging, aggressiveness, emotive and social behaviour) (Gil, 2008; Groothuis et al., 2005). Even if egg corticosterone injections affected chicks' behavioural development (Freire et al., 2006; Janczak et al., 2006), the potential role of egg corticosterone in mediating maternal stress effects on offspring remains to be elucidated. Indeed, when laying females were subjected to stress no differences of corticosterone concentrations in either yolk or albumen between stressed females' eggs and control females' eggs were found although their offspring's behaviour differed (Janczak et al., 2007; Lindqvist et al., 2007). Furthermore, a recent study challenged the actual presence of corticosterone in egg yolks (Rettenbacher et al., 2009). Avian yolks contain many other steroids of maternal origin whose effects are still poorly investigated. The role of androstenedione, for example, is beginning to attract attention. This androgen is found in much higher concentrations than testosterone in the yolk of precocial birds' eggs (Hackl et al., 2003; Möstl et al., 2001) and its effects on offspring seem to differ from those of testosterone (Hegyi and Schwabl, 2010). The precursor of androgens, progesterone, is also attracting more attention. This steroid is the most abundant in avian eggs (Hackl et al., 2003; Lipar et al., 1999; Möstl et al., 2001) and its concentrations vary according to females' phenotype (Bertin et al., 2009a) and living conditions (Bertin et al., 2008).

Multiple factors in avian husbandry implying the physical environment (noise, limited space), the social environment (social disruption) or a combination of both environments (high stocking densities, large group sizes) can induce stress in laying hens (Bilčík et al., 1998; Campo et al., 2005; Fahey and Cheng, 2008; Onbaşılar and Aksoy, 2005). Here, we focus on the effects of physical environment stressors. We therefore investigated the effects of daily unpredictable stressors on laying Japanese quail (*Coturnix c. japonica*) females on the behaviour and growth of their progeny. We assayed the concentrations of yolk testosterone, androstenedione and progesterone as potential mediators of maternal stress. We hypothesised that our stress procedure would increase yolk steroid levels and, considering the results of our previous study (Guibert et al., 2010), especially those of testosterone. We also hypothesised that maternal stress would alter offspring's growth and emotional reactivity.

2. Material and methods

2.1. Birds and stress procedure

Thirty-nine 12-week-old laying Japanese quail females from a commercial line were randomly separated into two groups: one group corresponded to the stressed group ($N = 20$) and the other one to the control group ($N = 19$). Each group was placed in a different room, but under the same conditions: a temperature of $19 \pm 1\,^{\circ}C$ and a photoperiod running from 07:00 h to 21:00 h (14:10 h light: dark cycle). In these rooms, females were housed in individual cages ($22 \times 20 \times 15$ cm). The layout of these cages allowed visual and auditory contact between birds. Water and food were provided *ad libitum*.

After 11 days of habituation to their housing conditions, the birds of the stressed group were exposed to brief stressors three times a day for 24 consecutive days, whereas the control birds were left undisturbed. We applied a combination of stressful acts, which included unpredictability and sudden movements, known to induce stressor-effects on

domestic fowl (Jones, 1996). Stressors used were: (i) individual stressors such as waggling a red flag under or over the female's cage, crumpling paper under the female's cage or blowing on the female and (ii) collective stressors such as shaking a group of cages or emitting a sudden noise (compressing a plastic bottle, dropping a metal object). The time of the day (between 08:30 h and 19:00 h) and the type of stressor used were chosen randomly, including nonetheless a nocturnal stress (at 06:30 h or 21:30 h) at least every other day. During each stress session, the stressor was applied twice. During the stress procedure, the experimenter was hidden behind a cardboard screen with observation windows and observed the females' reactions. These observations showed that the females reacted strongly to our stressors (expressing flights, jumps) and that this reactivity persisted during the stress sessions.

2.2. Mating and egg collection

Every 3 days, females from both groups met individually with a male for mating (eight mating sessions in all). One group of mature males ($N = 20$) was used for the two experimental sets of females. Two males were always associated with the same four females, two from the stressed group and two from the control group, and met the two stressed females and the two control females alternately during the experiment. Both the male and the female were removed from their home cages and placed together in a "neutral" cage. This cage was located near the females' room to minimize transport of females. Pairs stayed together for a few minutes until copulation had occurred. Daily copulations are not necessary as Japanese quail females can store sperm for several days (Birkhead and Møller, 1993).

As the formation of individual yolks lasts 7 days (Sauveur and Picard, 1987), fertilized eggs from each female were kept only from the 7th day after the beginning of the stress procedure. They were collected daily and marked according to the female who laid them for 16 days. They were weighed every 3 days from the beginning of egg collection. During collection, at the middle of the experiment, one egg per female was stored at −20 °C for later steroid hormone analysis. Most of the collected eggs ($N = 242$ for the control group, i.e. 12.74 ± 0.83 egg per control female and $N = 273$ for the stressed group, i.e. 13.65 ± 0.51 egg per stressed female) were stored at 16 ± 1 °C until incubation.

2.3. Yolk steroid analysis

Steroid extraction and assays (enzyme immunoassay) followed a method similar to that described by Bertin et al. (2008) and Guibert et al. (2010). Unfortunately, four control females' eggs were broken during transport and could not be used for analysis. Therefore, only 15 control females' eggs and 20 stressed females' eggs were analysed. For steroids extraction, the frozen yolk was separated from the eggshell and the albumin, as described by Lipar et al. (1999) and Hackl et al. (2003). Yolks and dry eggshells 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. As the distribution of hormones varies between the layers of the egg (Hackl et al., 2003; Möstl et al., 2001) the entire yolk was mixed before being assayed. Each yolk was suspended in 10 ml of water and vortexed twice for 30 s. Samples were then stored overnight at 4 °C. Samples were then vortexed and 1 ml of the suspension was transferred into a new vial. The suspension was then diluted with 4 ml methanol, vortexed for 30 min and stored at −20 °C overnight to precipitate apolar lipids. Samples were then centrifuged (−10 °C, $2500 \times g$, 10 min). Ten microliters of the supernatant were transferred into a new vial, dried under a stream of nitrogen at 60 °C and then dissolved in 500 μl enzyme immunoassay (EIA) buffer. The extract was then diluted 1:5 with EIA buffer. Ten microliters of this last solution were used for testosterone and androstenedione assays. For progesterone assays, we used 10 μl of the solution with an additional 1:10 dilution. For full descriptions of antibodies and validation of enzyme immunoassays, see Palme and Möstl (1994), Hirschenhauser et al. (1999), Möstl et al. (2001). We measured yolk testosterone in seven assays, androstenedione in eight assays and progesterone in seven assays. The inter-assay variation was 14.2%, 8.7% and 10.1% respectively for the low-level pool and 5.2%, 8.7% and 7.1% for the high-level pool. The intra-assay variation was 8.5%, 4.2% and 9.2% respectively.

2.4. Chick rearing

Eggs collected for incubation were placed in an incubator and maintained at 37.7 °C with a relative humidity of 45% and an automatic rotation of 45° twice a day. Three days before hatching (day 14 of incubation), the temperature was decreased to 37.2 °C, the humidity was raised to 60% and egg rotation was stopped. Eggs were placed on a wire mesh grid with plastic dividers. All eggs from one female were placed together in one compartment of the grid so that the chicks of a given female could be identified post-hatching. Hatchings were checked three times a day (in the morning, in the early and in the late afternoon) and newly hatched chicks were removed from the incubator following each check. Chicks were identified using coloured leg-rings. The hatching date of each chick (day 17 or 18 of incubation) was recorded. No candling during incubation was done to spot non-developing eggs, so unhatched eggs were opened to determine if they were fertilized or not (i.e. presence or absence of an embryo). Egg fertilization (number of fertilized eggs per number of incubated eggs × 100) and hatchability of fertile eggs (number of hatched eggs per number of fertilized eggs × 100) were calculated for each female.

Groups of eight chicks from the same experimental set, but from different mothers, were then housed in wire mesh cages ($100 \times 70 \times 62$ cm) with opaque lateral walls. Thus, 10 groups from control females ($N = 80$ control females' chicks) and 10 groups from stressed females ($N = 80$ stressed females' chicks) were formed. A warming bulb (38 ± 1 °C) was placed in each cage to ensure chicks' thermoregulation until they were 10 days old. After that, when chicks were able to regulate their own temperature, the warming bulbs were switched off and the temperature in the room was maintained at 20 ± 1 °C. Chicks were

exposed to a 10:14 h light:dark cycle. Water and food were provided *ad libitum*. The general development of chicks was followed by weighing them weekly, from hatching until they were 5 weeks old, using electronic scales. Sex was determined via sexual dimorphic plumage when chicks were 3 weeks old. Sex-ratios did not differ significantly between the two sets of chicks (control females' chicks: 39 females and 41 males; stressed females' chicks: 37 females and 43 males; Chi-square test, $\chi^2 = 0.10$, $df = 1$, $P = 0.75$).

2.5. Behavioural tests

Classical ethological tests for poultry, based on different social and potentially fearful situations, were used to assess the general emotional reactivity of chicks (Forkman et al., 2007). Fifty chicks of control females (five per group) and fifty chicks of stressed females (five per group) were tested in the tests described below. Sex-ratios did not differ significantly between these two sets of chicks (control females' chicks: 22 females and 28 males; stressed females' chicks: 23 females and 27 males; $\chi^2 = 0.04$, $df = 1$, $P = 0.84$).

2.6. Separation test

When they were 9 days old, chicks were tested in a separation test in their familiar environment. This test followed a protocol similar to that described by Guibert et al. (2010). The test chick was isolated in its home cage by removing its seven cage mates who were then placed in a familiar box in another room. Latency of first call, latency of first step, number of calls, number of steps and number of jumps made by the chick were then recorded for 3 min. This test, which does not involve any aspect of environmental novelty, evaluates the emotional reactivity of chicks to social separation. The number of calls, the number of steps and the number of jumps are considered to be positively correlated to their reactivity (Launay, 1993). Eighty minutes at least elapsed between tests of two chicks from a same cage to ensure that they were habituated again to their home cage before a new test begun.

2.7. Tonic-immobility test

The tonic-immobility test followed the protocol described by Jones (1986). Chicks were tested when they were 10 days old. The test chick was taken to another room. The chick was then placed on its back in a U-shaped wooden cradle and restrained in this position for 10 s prior to release. Two parameters were recorded: (i) the number of inductions required to obtain tonic-immobility lasting at least 10 s, with a maximum of five induction attempts, and (ii) the duration of tonic-immobility, i.e. the time between the release of the chick and its standing up, allowing a maximum of 300 s. TI duration is positively correlated to fear level (Mills et al., 1994).

2.8. Emergence test

Chicks were tested when they were 13–14 days old. This test followed a protocol similar to that described by Mills and Faure (1986). 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 containing the chick was placed on the left side of a wooden box ($83 \times 60 \times 35$ cm). The floor of the wooden box was covered with wood shavings and the side of the wooden box facing the experimenter was a glass window. The cardboard box was kept closed for 1 min. The side of the cardboard box facing the new environment was then opened and the lights switched on. The experimenter recorded latency of emergence of the head and latency of emergence of the entire body (full emergence) into the new environment for each chick. When a chick had not left the cardboard box after 3 min, the cardboard box was closed and a maximum score of 180 s was recorded. When a chick left the cardboard box, the experimenter recorded latency of first call, number of calls and numbers of locomotor acts (walks and runs), of exploratory acts (floor and side pecking) and of high-posture observations made by the chick for 3 min. The high-posture observation is a typical posture when a chick stands upright on 'tiptoes', with its body very straight. Latency of emergence from a sheltered area into an unfamiliar open area is a good estimate of emotional reactivity: fearful or timid animals take longer to emerge (Archer, 1976; Jones, 1987; Mills and Faure, 1986).

2.9. Open-field test

Chicks were tested in the open-field when they were 17–18 days old following a protocol similar to that described by Mills and Faure (1986). The 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 (Ø 120 cm, *H* 70 cm) with a linoleum floor. Lights were then switched on and the experimenter, hidden behind a two-way mirror, recorded latency of first call, latency of first step, and numbers of calls, steps, exploratory acts (floor and wire-netting pecking) and high-posture observations made by the chick for 5 min. This test evaluates the emotional reactivity of chicks to both a novel environment and separation from conspecifics (Forkman et al., 2007).

All experiments were approved by the departmental direction of veterinary services (Ille et Vilaine, France, Permit number 005283) and were performed in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC).

2.10. Data analysis

Kolmogorov–Smirnov tests were used to determine whether data sets were normally distributed. Laying rates, fertilization rates, hatchability rates, and behavioural data were not normally distributed, so Mann–Whitney *U*-tests were applied. Yolk hormone data were log-transformed ($Y+1$) and analysed with a MANOVA and individual one-way ANOVAs. Egg composition and egg weight data were normally distributed and analysed using ANOVAs. Weights of control and stressed females eggs (six data points per female in all) were compared using a repeated measures ANOVA and subsequent post-hoc Fisher LSD tests. Chick-weight data were also analysed using a repeated measures ANOVA (maternal treatment × chicks' age) and subsequent post-hoc Fisher LSD tests. Chi-square tests were used to

compare chicks' hatching dates. Data are represented as means ± standard error of the mean (SEM). All analyses were performed using Statview Software (SAS, Cary, NC) with significance set at $P \leq 0.05$.

3. Results

3.1. Laying rates, fertilization and hatchability of fertile eggs

Laying rates did not differ significantly between control and stressed females (respectively, 0.94 ± 0.02 and 0.94 ± 0.01 eggs per day; Mann–Whitney *U*-test, $U = 173.5$, $P = 0.62$). Neither egg fertilization rates (75.53 ± 5.05% for control females and 67.54 ± 6.81% for stressed females; $U = 165.5$, $P = 0.49$) nor hatchability rates of fertile eggs (75.18 ± 6.21% for control females and 73.43 ± 7.54% for stressed females; $U = 175.5$, $P = 0.68$) differed significantly between control and stressed females.

3.2. Egg characteristics

3.2.1. Egg weight

Egg weights were significantly influenced by our stress procedure (repeated measures ANOVA, $F_{1,37} = 6.673$, $P = 0.014$), as well as by collection day ($F_{5,185} = 2.892$, $P = 0.015$), but no significant interaction between stress procedure and collection day could be evidenced ($F_{5,185} = 1.728$, $P = 0.13$). Egg weights of stressed females and control females did not differ significantly the 1st day of collection (post-hoc Fisher LSD test, $P = 0.15$; Fig. 1). Stressed females' eggs collected the 4th day tended to be heavier than control females' eggs ($P = 0.085$; Fig. 1). Then, stressed females' eggs collected the 7th, 10th, 13th and 16th days were significantly heavier than control females' eggs (respectively: $P = 0.044$; $P = 0.010$; $P = 0.0038$; $P = 0.016$; Fig. 1).

3.3. Egg components

The weights of stressed females' eggs collected for analysis tended to be higher than those of control females' eggs ($F_{1,33} = 3.785$, $P = 0.060$; Table 1). Neither shell weights ($F_{1,33} = 0.418$, $P = 0.52$; Table 1) nor yolk weights ($F_{1,33} = 0.553$, $P = 0.46$; Table 1) differed significantly between control and stressed females eggs, but albumen weights of stressed females' eggs were higher than those of control females' eggs ($F_{1,33} = 3.948$, $P = 0.055$; Table 1).

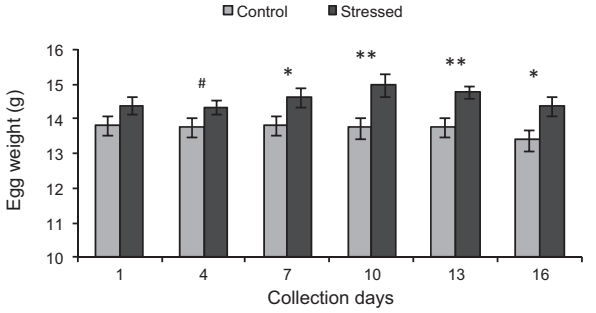

Fig. 1. Weights (g) of control ($N = 19$) and stressed ($N = 20$) females' eggs (mean ± SEM) for 6 collection days during the egg collection period. Post-hoc Fisher LSD test, $^{\#}0.1 > P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Composition of control ($N = 15$) and stressed ($N = 20$) females' eggs.

Parameters	Control females	Stressed females
Egg weight (g)	13.21 ± 0.32	14.05 ± 0.29$^{\#}$
Shell weight (g)	1.24 ± 0.09	1.30 ± 0.06
Yolk weight (g)	3.95 ± 0.16	4.08 ± 0.10
Albumen weight (g)	8.02 ± 0.21	8.66 ± 0.23$^{\#}$

Mean ± SEM; ANOVA.
$^{\#}$ $0.1 > P > 0.05$.

3.4. Yolk hormone levels

We found no overall effect on yolk hormone concentrations (MANOVA, $F_{3,31} = 1.18$, $P = 0.33$). Yolk androstenedione concentrations ($F_{1,33} = 0.099$, $P = 0.75$; Fig. 2B) and yolk progesterone concentrations ($F_{1,33} = 0.454$, $P = 0.51$; Fig. 2C) did not differ significantly between eggs of the two groups. However testosterone concentrations tended to be higher in the egg yolks of stressed females than in the egg yolks of control females ($F_{1,33} = 3.510$, $P = 0.070$; Fig. 2A).

3.5. Chicks

3.5.1. Hatching and growth

Significantly more stressed females' chicks (83.75%) than control females' chicks (63.75%) hatched on the first day (day 17 of incubation) (Chi-square test, $\chi^2 = 8.265$, $df = 1$, $P = 0.004$).

Maternal stress procedure (repeated measures ANOVA, $F_{1,158} = 5.479$, $P = 0.021$), as well as chicks' age ($F_{5,790} = 17582.765$, $P < 0.0001$) and the interaction between procedure and age ($F_{5,790} = 7.875$, $P < 0.0001$) had significant effects on chicks' body weight. Hatchlings of stressed mothers were heavier than hatchlings of control mothers (post-hoc Fisher LSD test, $P = 0.0073$; Table 2). However, weights of 1- and 2-week-old chicks did not differ significantly between the two sets ($P = 0.77$ for 1-week-old chicks; $P = 0.26$ for 2-week-old chicks; Table 2). Three-week-old stressed females' chicks tended to be heavier ($P = 0.068$; Table 2) and 4- and 5-week-old stressed

Table 2
Body weights (g) of control females' chicks ($N = 80$) and stressed females' chicks ($N = 80$) from hatching to 5 weeks old.

Age	Body weight (g)	
	Control females' chicks	Stressed females' chicks
Hatching	9.84 ± 0.10	10.23 ± 0.10**
1 week	44.87 ± 0.78	45.19 ± 0.73
2 weeks	101.39 ± 1.19	103.26 ± 1.17
3 weeks	154.44 ± 1.55	158.56 ± 1.62$^{\#}$
4 weeks	206.30 ± 1.77	212.24 ± 1.98*
5 weeks	233.25 ± 1.93	243.48 ± 2.34***

Mean ± SEM, post-hoc Fisher LSD test.
$^{\#}$ $0.1 > P > 0.05$.
* $P < 0.05$.
** $P < 0.01$
*** $P < 0.001$.

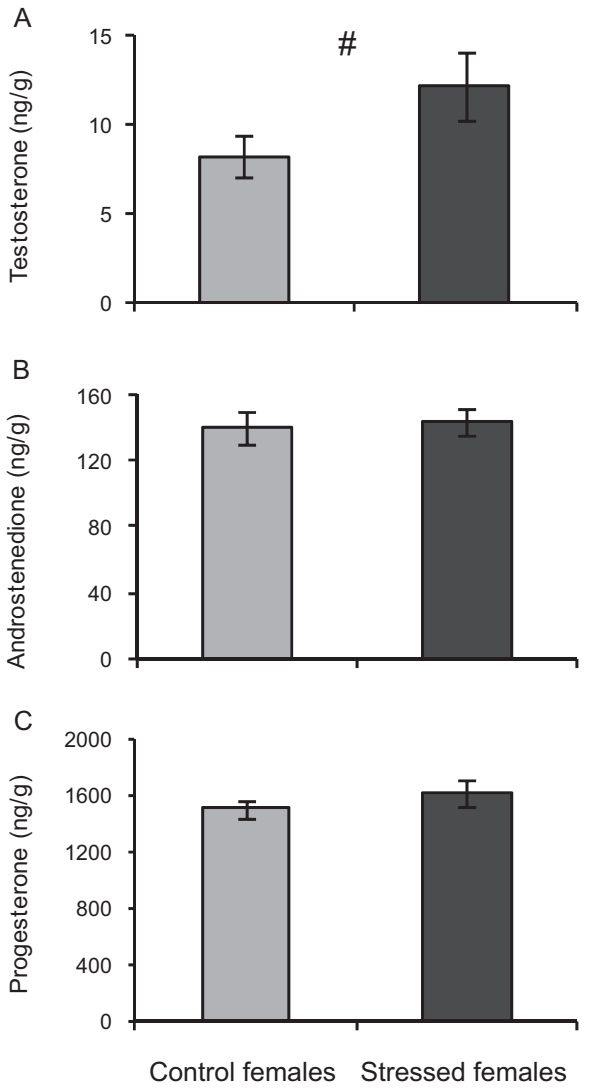

Fig. 2. Yolk testosterone (A), androstenedione (B) and immunoreactive progesterone (C) concentrations (mean ± SEM ng/g) in eggs of control ($N = 15$) and stressed ($N = 20$) females. ANOVA, $^{\#}0.1 > P > 0.05$.

females' chicks were significantly heavier than control females' chicks ($P = 0.027$ for 4-week-old chicks; $P = 0.0009$ for 5-week-old chicks; Table 2).

3.6. Behaviour

3.6.1. Separation test

Latencies of first call in the separation test did not differ significantly between the two sets of chicks (Mann–Whitney U-test, $U = 1200$, $P = 0.72$; Fig. 3A), although stressed females' chicks tended to emit more calls than did control females' chicks ($U = 980$, $P = 0.063$; Fig. 3B). Stressed females' chicks also tended to take their first step later than did control females' chicks (respectively, 8.78 ± 2.05 s and 4.80 ± 0.91 s; $U = 995.5$, $P = 0.074$) although numbers of steps did not differ between the two sets (152.64 ± 13.92 steps for control females' chicks

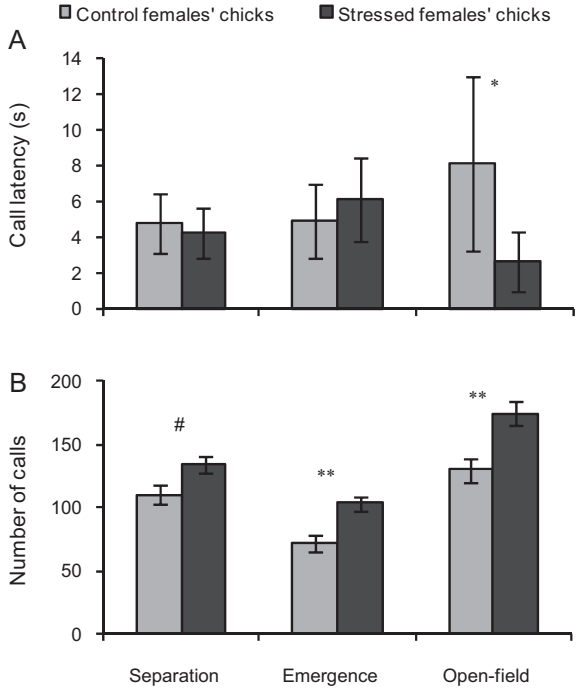

Fig. 3. Call latencies (A) and numbers of calls (B) of control females' chicks ($N = 50$) and stressed females' chicks ($N = 50$) during separation, emergence and open-field tests (means ± SEM). Mann–Whitney U-test, $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$.

and 173.24 ± 14.14 steps for stressed females' chicks; $U = 1088$, $P = 0.26$). Stressed females' chicks jumped more than did control females' chicks (respectively, 0.76 ± 0.28 and 0.22 ± 0.12 jumps; $U = 1074.5$, $P = 0.051$).

3.7. Tonic-immobility test

Neither numbers of inductions required to induce tonic-immobility (2.94 ± 0.20 for control females' chicks and 2.96 ± 0.20 for stressed females' chicks; $U = 1243.5$, $P = 0.96$), nor durations of tonic-immobility (67.78 ± 9.63 s for control females' chicks and 62.52 ± 8.80 s for stressed females' chicks; $U = 1221$, $P = 0.84$) differed significantly between the two sets of chicks.

3.8. Emergence test

Stressed females' chicks tended to put their head out of the emergence box later than did control females' chicks ($U = 992.5$, $P = 0.074$; Fig. 4), but latencies of full emergence did not differ significantly between the two sets ($U = 1047$, $P = 0.16$; Fig. 4). Eight percent of stressed females' chicks versus 0% of control females' chicks did not leave the box within the first 60 s (Chi-square test, $df = 1$, $\chi^2 = 4.167$, $P = 0.041$). All chicks of both sets left the emergence box. Latencies of first call after emergence did not differ significantly between the two sets (Mann–Whitney U-test, $U = 1136.5$, $P = 0.43$; Fig. 3A), but stressed females' chicks emitted significantly more calls than did control females' chicks ($U = 796.5$, $P = 0.0018$; Fig. 3B). Neither numbers of high-posture observations (9.72 ± 1.04 for con-

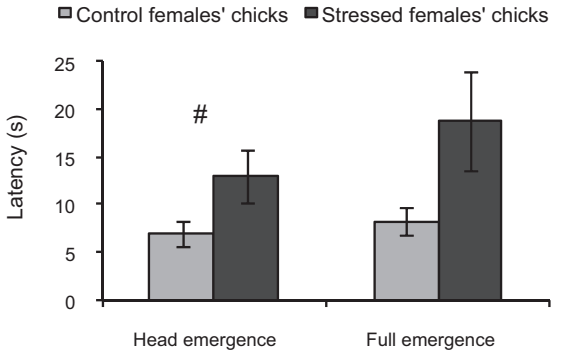

Fig. 4. Latencies (s) of head emergence and full emergence (means ± SEM) of control females' chicks ($N = 50$) and stressed females' chicks ($N = 50$) during emergence tests. Mann–Whitney U-test, $^{\#}0.05 < P < 0.1$.

trol females' chicks and 10.18 ± 0.82 for stressed females' chicks; $U = 1149.5$, $P = 0.49$), nor numbers of locomotor acts (14.26 ± 1.12 for control females' chicks and 14.96 ± 0.85 for stressed females' chicks; $U = 1116.5$, $P = 0.36$) differed significantly between the two sets of chicks. Stressed females' chicks made significantly less exploratory acts than did control females' chicks (respectively, 1.42 ± 0.37 and 2.70 ± 0.48; $U = 914.5$, $P = 0.016$).

3.9. Open-field test

Stressed females' chicks emitted their first call earlier than did control females' chicks ($U = 975$, $P = 0.039$; Fig. 3A) and emitted more calls ($U = 800.5$, $P = 0.0019$; Fig. 3B). Stressed females' chicks made also significantly more high-posture observations than did control females' chicks (respectively, 12.34 ± 0.78 and 10.14 ± 0.72; $U = 962.5$, $P = 0.047$). Stressed females' chicks took more steps during a test than did control females' chicks (respectively, 246.42 ± 24.85 and 176.60 ± 19.83; $U = 955.5$, $P = 0.042$), but latencies of first step did not differ significantly between the two sets of chicks (respectively, 12.58 ± 3.44 s and 13.76 ± 2.93 s; $U = 1117$, $P = 0.36$). Numbers of exploratory acts did not differ significantly between stressed females' chicks and control females' chicks (respectively, 6.04 ± 0.61 and 7.50 ± 0.92; $U = 1092$, $P = 0.27$).

4. Discussion

Our current study demonstrates that unpredictable stressors applied to laying Japanese quail females altered their offspring's growth and emotional responses and suggests that this maternal effect is mediated by changes in the composition of stressed females' eggs.

Stress can induce deleterious effects on poultry production such as decline of egg production and eggshell quality and/or impairment of male and female reproductive functions (Mashaly et al., 2004; Mills and Faure, 1990; Mumma et al., 2006; Shini et al., 2009). The absence of effects of our stress procedure on these parameters showed that this procedure was not perceived by our subjects as an intense stress but more as a mild stress, which can be compared to stressful events that can occur in husbandry. Our stress procedure applied to laying females had nevertheless an impact on their egg characteristics. First, stressed females' eggs appeared to be heavier that those of control females and this egg weight difference appeared related to an increase of albumen weight. While high levels of stress inhibit feeding, mild stress may in fact facilitate it. Here feeding may serve to reduce the amount of fear experienced or to adapt to the increased metabolism needed (Elsasser et al., 2000; Murphy, 1978). Interestingly, reports have shown that increasing the protein level or adding fat to the diet of laying hens leads to heavier eggs, mainly due to increase of albumen weight (Grobas et al., 1999; Keshavarz and Nakajima, 1995). Thus, our mild stress procedure may have induced females to feed more than control females and this increased feed intake may have had the same effects on egg and albumen weights as a protein or fat supplementation of their diet. However, this hypothesis remains speculative as we did not actually measure these variables in this study.

Although our stress procedure had no effect on egg androstenedione and progesterone levels, it did affect testosterone levels. Stressed females' eggs tended to contain more testosterone than did those of control females. This result is consistent with other studies showing that stressful conditions experienced by laying females of various avian species increase yolk androgen concentrations (increased breeding density: Pilz and Smith, 2004; Reed and Vleck, 2001; Schwabl, 1997; aggressive interactions: Whittingham and Schwabl, 2002) and especially with our previous report showing that Japanese quail females submitted to social instability laid eggs with higher testosterone concentrations than did females in stable social conditions (Guibert et al., 2010). This consistency across our two studies suggests a general response of quail to stress by increasing yolk testosterone levels. This finding however is in contradiction with another study showing that Japanese quail females reared under long-term restraint stress laid eggs with lower testosterone concentrations than did control females (Okuliarová et al., 2010). The type of stressor used in that study however was very different from our stress procedures, as quails were reared from 3 days old to 63 days old in hypodynamia, i.e. animals are suspended in such a way that their legs could not touch the floor (Okuliarová et al., 2010). This procedure is an extreme stressor that has profound negative consequences on birds' general condition (decreased body weight, bone growth, organ weight, sexual development) (Škrobánek et al., 2004, 2008). The profound physiological changes experienced by these quail and the fact that females' body condition is known to influence yolk testosterone levels (Verboven et al., 2003) make it difficult to compare Okuliarová et al.'s results to ours. Testosterone and other sex steroids are produced mainly by the cell layers of the follicular wall (granulosa and theca cells) that surrounds each growing oocyte (Huang and Nalbandov, 1979). The pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), regulate this follicular hormone production in response to information from the environment and the body (Groothuis and Schwabl, 2008). Our stress procedure thus seems to have stimulated the production of

testosterone at the hypothalamic–pituitary–gonadal axis level, maybe by either changing follicle receptor sensitivity or modifying LH and/or FSH production at the central hypothalamic–pituitary level (Okuliarová et al., 2010). The way steroids accumulate in the yolk still remains unknown. Groothuis and Schwabl (2008) proposed several potential mechanisms involving either passive diffusion or active regulation, but the current state of our knowledge can favour none of the proposed mechanisms.

Our maternal stress procedure had a strong impact on offspring's general development. First, chicks of stressed mothers hatched earlier, weighed more at hatching than did chicks of control mothers and grew faster post-hatching. Second, their emotional responses were more pronounced than those of controls. During emergence tests, stressed females' chicks tended to put their head out of the emergence box later than did control females' chicks and were more numerous to leave the box late, suggesting a more cautious behaviour when facing a novel environment (Archer, 1976; Jones, 1987; Mills and Faure, 1986). Furthermore, they explored the novel environment less after emergence than did control females' chicks, indicating a more pronounced emotional reactivity as fearfulness is known to inhibit exploratory behaviour in domestic fowl (Brown and Kiely, 1974; Jones, 1977). In open-field tests, stressed females' chicks expressed more locomotor activity. This enhanced locomotor activity could be interpreted as an attempt to escape (Deminière et al., 1992; Vallee et al., 1997) or as a search for conspecifics following social separation (Formanek et al., 2008; Jones and Merry, 1988). During all our tests, stressed females' chicks emitted more calls than did control females' chicks, clearly indicating active search for conspecifics (Launay, 1993). The facts that during open-field tests stressed females' chicks presented more high-posture observations and that during separation tests they jumped more indicate behavioural attempts to search for conspecifics and thus reflect their higher sensitivity to social separation (Formanek et al., 2008; Launay, 1993). Generally, chicks of stressed females showed a higher inherent fearfulness than did those of control females.

In this study, only the tonic-immobility test revealed no differences of emotional reactivity between the two sets of chicks. This result agrees with previous studies (Bertin and Richard-Yris, 2004, 2005; Richard-Yris et al., 2005) and could be linked to the high level of fear induced by this test, the strong reactivity of all animals masking behavioural differences between sets.

Phenotypic differences between chicks of stressed and of control mothers could be explained by the different prenatal environment in which they developed. Indeed, stressed mothers' chicks developed in heavier eggs containing more albumen and tending to contain more testosterone in the yolk than did control mothers' chicks. Egg albumen contains nutrients crucial for embryo development and when a proportion of the albumen was removed hatching was delayed (Ferrari et al., 2006) and hatchling body mass and size decreased (Finkler et al., 1998; Hill, 1993). The fact that stressed females' chicks developed in eggs containing more albumen could explain why they hatched earlier and weighed more at hatching than did control females' chicks. The difference in yolk testosterone contents, although non-significant in our sample, could also be involved in the phenotypic differences we observed between chicks of stressed and of control mothers. Previous reports showed strong influences of yolk androgen levels on the general development of the chicks of various avian species. Androgen injections into egg yolks showed ambiguous results: they either reduce (Eising et al., 2001; Eising and Groothuis, 2003) or increase (von Engelhardt et al., 2006; Sockman and Schwabl, 2000) incubation duration and either enhance (Eising et al., 2001; Eising and Groothuis, 2003; Schwabl, 1996) or delay (Rubolini et al., 2006; Sockman and Schwabl, 2000) chicks' growth. Similar discrepancies concern behaviour; for example, the emotional reactivity of chicks from testosterone injected eggs was either increased (Bertin et al., 2009b; Okuliarová et al., 2007) or decreased (Daisley et al., 2005). These contradictory results may reflect the complexity of prenatal influences and could be due to specific effects, dose-dependent effects, location of the injected androgen in the yolk (von Engelhardt et al., 2009; Groothuis and Schwabl, 2008), or the interaction of testosterone with other non-controlled factors such as egg size, egg component proportions or levels of other hormones (e.g. androstenedione, progesterone).

Although testosterone is an excellent candidate for implementing prenatal effects on birds, avian eggs contain many other substances of maternal origin (other sexual steroids, thyroid hormone, antioxidants, immunoglobulin) that could have an impact on their offspring's growth, immunity and behaviour (Gil, 2008; Hegyi and Schwabl, 2010). Conceivably the concentrations of these components may also vary under stressful conditions and, they alone or in interaction with elevated yolk testosterone could influence offspring development. Furthermore, despite the current debate about the effective presence of corticosterone in eggs (Rettenbacher et al., 2005, 2009), we cannot discard the possibility that egg corticosterone (either in yolk or albumen) may have been involved in the effects we observed since corticosterone injection in eggs lead to phenotypic differences in offspring (Eriksen et al., 2003; Hayward et al., 2006; Janczak et al., 2006). Finally, epigenetic mechanisms may also have influenced the present results. Indeed, recent studies in chicken showed that chronic stress in parents caused the same behavioural alterations and the same modifications of gene expression patterns in both parents and their offspring, suggesting inheritance of epigenetic modifications of the genome resulting from chronic stress from parents to offspring (Lindqvist et al., 2007; Nätt et al., 2009). Thus, a variety of mechanisms, and not just a single pathway, could be involved in the observed prenatal maternal effects.

Interestingly, the results of the current study are very similar to the results of our previous study concerning the effects of quail maternal social instability on yolk steroids and their offspring's phenotype (Guibert et al., 2010). In both studies, stressed females laid eggs with higher testosterone levels and their offspring had a different growth pattern and presented higher emotional reactivity (when encountering a novel environment and following social separation). We have therefore evidenced similar effects

of two very different types of stress (one of them acting on the social environment and the other on the physical environment) on the subsequent offspring's development, possibly mediated by an elevation of yolk testosterone levels. This probably indicates a general mechanism for the mediation of maternal stress on offspring in quail.

This current study highlights the fact that mild stressors that can be present in routine management of animals in husbandry may have great effects on the subsequent emotive behaviour of their progeny. These stressors cannot be easily detected because they do not interfere with the females' reproductive function. We stress the particular relevance to the poultry industry of our findings that highlight the importance of taking into account the environment of laying females, as this environment can influence their progeny's behaviour and therefore their subsequent adaptation to husbandry living conditions and ultimately their welfare.

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