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Yolk testosterone levels and offspring phenotype correlate with parental age in a precocial bird

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ABSTRACT

Parents, and particularly mothers, can influence their offspring's development in non-genetic ways. Maternal effects can occur during the mothering phase as well as during the embryonic phase. Prenatal maternal effects in birds can be mediated by yolk steroid hormones that influence subsequent offspring development. Studies have focused mainly on the influence of laying females' living conditions on yolk hormonal contents, and rarely on the effects of individual characteristics. Here, we investigated prenatal influence of parent age on yolk steroid levels and on offspring phenotype. We compared Japanese quail at two different ages: at the beginning of their reproductive cycle (11 weeks old: *age 1*) and six months later, after egg production peak (37 weeks old: *age 2*). Egg composition, reproductive outcomes, and offspring growth, sexual development and behaviour were studied at both ages. We found that laying rate, fertility and chick survival rates declined between *age 1* and *age 2*. *Age 2* eggs had relatively lighter shells and higher yolk plus albumen contents; they also had lower testosterone contents. *Age 2* offspring weighed more at hatching than did *age 1* offspring; subsequently their growth patterns differed and their sexual development was more precocious. *Age 2* offspring were less emotional than *age 1* offspring when encountering a novel environment, and they appeared more sensitive to social separation. Our study shows, for the first time in a bird species, a strong impact of parental age on offspring phenotype, and especially on behaviour, an impact that is possibly mediated via modulation of yolk testosterone content.

1. Introduction

Parents influence the phenotype of their offspring through genetic inheritance, but also through non-genetic mechanisms. Mothers have been the centre of interest concerning non-genetic inheritance processes and, in many species, they play a fundamental role in influencing their offspring's general development, both during the mothering phase [1–3] and during embryogenesis [4–7]. Prenatal maternal influences in birds have aroused much interest since the discovery that avian egg yolks contain variable levels of steroid hormones of maternal origin that have a strong influence on offspring development [6–8]. Most researchers so far have investigated the effects of yolk androgens on offspring phenotype by injections into eggs and they report strong effects on offspring short and long-term growth, immunity (cellular and humoral immunocompetence) and behaviour including begging, aggressiveness, emotional and social behaviours [6,7,9].

Avian yolk steroid levels appear to be influenced by social factors experienced by laying females, such as breeding density [10–12], frequency of social intrusion [13], social instability [14], social status [15], and male attractiveness [16]. In these social contexts, modulation of yolk androgen levels could have an important adaptive value for the next generation, preparing chicks for the social environments they will encounter either during the rearing phase, after fledging during the juvenile stage or as adults [6].

Effects of the laying females' characteristics on their yolk steroid levels have been investigated to a lesser extent. The genetic origin of a female has been shown to influence the hormonal content of her eggs. Japanese quail (*Coturnix coturnix japonica*) females from a genetic line selected for long tonic immobility duration (corresponding to high inherent fearfulness) laid eggs with lower progesterone and androgen levels than did females from a line selected for short duration of tonic immobility (corresponding to a low level of emotional reactivity) [17]. Lesser black-backed gull (*Larus fuscus*) females in enhanced body condition laid eggs with reduced yolk androgen contents [18]. Finally,

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females' age appears to modulate yolk androgen levels, although effects seem to differ amongst species. Androgen levels increase with age in European starlings (*Sturnus vulgaris*) [19], whereas they decrease with age in Japanese quail [20].

Parental age has been shown to affect several aspects of bird reproduction, younger and first-time breeders are generally less efficient. In many species, egg weight and clutch size increase with female age [21,22]. Moreover, chicks of younger parents appear to have a lower survival rate than chicks of older parents; this could be linked either to their lower hatching weight (correlated to lower egg weight) that influences their postnatal survival [23] and/or to the reduced offspring-rearing abilities of younger parents compared to older parents [24,25]. Although this last aspect has generally been well analysed in bird species, parental age effects on egg laying and especially egg quality, and their consequences on the general development of young have been neglected [26].

This study investigated the effects of parental age on egg characteristics, especially yolk steroid levels and on the subsequent morphological and behavioural development of chicks. Japanese quail have a relatively short life span. They reach puberty at 6–8 weeks and attain full sexual maturity 2–3 weeks later [27]. We chose to study Japanese quail eggs and chicks at two different reproductive stages: first, at the beginning of the reproductive cycle (11 weeks old) and second, after the egg production peak (37 weeks old). We assayed the three main steroids present in quail yolk: progesterone, androstenedione and testosterone [28]. We hypothesised that yolk steroid levels would vary with parental age and that offspring morphological and behavioural development would differ according to parental age. We expected that yolk testosterone levels would decrease with parental age, as observed in a previous study on quail [20]. Furthermore, as several quail studies have shown that chicks from eggs with lower testosterone levels were less emotive than those from eggs with higher testosterone levels [14,29,30], we expected that chicks of older parents developing in eggs with lower androgen levels would present lower emotional reactivity than chicks of younger parents.

2. Materials and methods

2.1. Ethics

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).

2.2. Housing of birds and mating

Twenty-two female and 16 male Japanese quail (*Coturnix c. japonica*), obtained from a commercial farm when they were 8 weeks old, were housed in individual cages (22×20×15 cm). Males and females were housed in different windowless rooms, but under the same conditions: an artificial 14:10 h light:dark cycle and a temperature maintained at 19±1 °C. Water and food were provided *ad libitum*. Three weeks later (when 11 weeks old) when all females had begun to lay, we started the first mating period (*age 1*: February/March 2008). Females met a male three times a week. Pairs stayed together for a few minutes in a small cage until copulation had occurred. Daily copulations are not necessary, as Japanese quail females can store sperm for several days [31]. During the 10 mating sessions realised, female met a different male at each mating session. So a 10-male combination was elaborated for each female. The 10-male combinations implicated that all males ($N=16$) were mated with at least half of the females (13.75±0.3 females per male) and a similar 10-male combination was used at the most for two females. Eggs were collected daily for 17 days. Each egg was identified according to the female that had laid it and weighed. Six months later (when they were 37 weeks old), after the egg production peak [32], the same individuals (both males and females) were used for a second mating period (*age 2*: August/September 2008). The process was the same as for *age 1* and the same 10-male combinations for each female were used. Eggs were collected daily for 20 days. During the second mating period, 20 of the 22 females still laid regularly (one had stopped laying and one laid only two eggs during the collection period, thus her egg data were not taken into consideration). At the middle of each egg collecting period, one egg per female, collected the same day for all females, was frozen at −20 °C for subsequent hormonal assays. The other eggs collected were stored at 16±1 °C until incubation.

The same experienced individual performed all pairings and egg collection during both periods. During the whole experiment (also between the two periods), we maintained the birds under the same standard conditions (room, photoperiod, temperature, diet) that were controlled with particular attention. Moreover, the same caretaker took care of the birds during the whole experiment.

2.3. Chick rearing

First, 227 *age 1* eggs (*i.e.* 10.32±0.55 eggs/female) and then 236 *age 2* eggs (*i.e.* 11.80±0.72 eggs/female) were collected for incubation. For both periods, the eggs collected were placed in an incubator for 17 days. During the first 14 days, eggs were maintained at 37.7 °C with a relative humidity of 45% and with an automatic rotation of 45° twice a day. During the last three days, temperature was decreased to 37.2 °C, humidity was raised to 60% and egg rotation was stopped. At hatching, leg rings identified chicks according to their mother. At *age 1*, 148 chicks from eggs laid by the 22 females hatched (6.73±0.62 chicks/female). At *age 2*, 117 chicks from only 18 females hatched (6.50±0.72 chicks/female). Unhatched eggs were opened to determine whether they were fertilised or not (*i.e.* presence or absence of an embryo). Fertility (number of fertilised eggs per number of incubated eggs×100) and hatchability of fertile eggs (number of hatched eggs per number of fertilised eggs×100) were calculated for each female. Chick mortality during their first week of life was recorded and chick survival rate was then calculated for each female (number of chicks still alive after 1 week per number of hatched chicks×100).

Seventy-six *age 1* chicks (3.46±0.14 chicks/female) and 75 *age 2* chicks (4.17±0.23 chicks/female) were kept for experimentation. Chicks from different mothers were housed in groups of four in a 100×70×62 cm cage (for each *age*, one group included only three chicks because of the sudden death of one of the four chicks). Nineteen groups were formed and housed in the same room. A heat lamp (38±1 °C) was placed in each cage to ensure chicks' thermoregulation until they were 10 days old. After this, when chicks were able to regulate their own temperature, the heat lamps were switched off and the temperature in the room maintained at 20±1 °C. Chicks were exposed to a 10:14 h light:dark cycle. Water and food were provided *ad libitum*. To assess weight gain, chicks were weighed on electronic scales once a week, from hatching to 4 weeks old. When they were 3 and 4 weeks old, the length of their cloacal vent was measured with an electronic calliper rule to assess sexual maturation. This measure is an indicator of sexual development in quail (for both males and females) [33–35]. Sex was determined via sexual dimorphic plumage when chicks were 3 weeks old. The chicks' sex ratios did not differ significantly between *age 1* (38 females and 37 males) and *age 2* (30 females and 45 males) (Chi-square test, $\chi^2=1.722$, $P=0.19$).

The same experienced individual was responsible for all chick care and measurements for both *ages*.

2.4. Behavioural tests

Classical ethological tests devised for poultry, based on different social and potentially frightening situations, were used to assess the general emotional reactivity of chicks [36]. *Age 1* chicks ($N=76$) and

age 2 chicks ($N=75$) were given each test at the same age. All tests were performed by the same experienced experimenter.

2.4.1. Separation test

Nine-day-old chicks were observed in a separation test in their familiar environment. This test followed a protocol similar to that described by Guibert et al. [14]. The test chick was isolated in its home cage by removing its three cage mates. 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 during 3 min. This test, which does not involve any aspect of environmental novelty, evaluates the emotional reactivity of chicks to social separation. Numbers of calls, numbers of steps and numbers of jumps are considered to be positively correlated to their reactivity [37].

2.4.2. Tonic-immobility test

The tonic-immobility test followed the protocol described by Jones [38]. 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) number of inductions required to obtain tonic-immobility lasting at least 10 s, with a maximum of 5 induction trials, and (ii) duration of tonic-immobility, *i.e.* the time between the release of the chick and its standing up, with a maximum of 300 s. TI duration is positively correlated to fear level [39].

2.4.3. Emergence test

Chicks were tested when they were 14 to 15 days old. This test followed a protocol similar to that described by Mills and Faure [40]. The test chick was taken from its home cage to a dark room in a cardboard box ($18\times18\times18$ cm). The cardboard box containing the chick was placed on the left side of a wooden box ($83\times60\times35$ cm). The floor of the wooden box was covered with wood shavings and the side of the wooden box facing the experimenter was an observational glass window. The cardboard box was kept closed for 1 min. The cardboard box was then opened and the lights were switched on. The experimenter noted latency of emergence of head and latency of emergence of entire body (full emergence) into the novel environment for each chick. When a chick did not leave the cardboard box within 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 noted latency of first call, number of calls and numbers of locomotor acts (walks and runs), exploratory acts (pecking floor or side) and high-posture observations made by the chick during 3 min. The high-posture observation corresponds to a typical posture when a chick stands upright on 'tiptoes', with its body very straight. Latency of emergence from a sheltered area into an open unfamiliar area is a good estimate of emotional reactivity: fearful or timid animals take longer to emerge [40–42].

2.4.4. Open-field test

Chicks were tested when they were 16 to 17 days old following a protocol similar to that described by Mills and Faure [40]. The test chick was taken from its home cage to a dark room in a cardboard box. The chick was placed in the centre of a wire-netting cylinder (Ø120 cm, H 70 cm) on a linoleum floor. Lights were then switched on and the experimenter, hidden behind a two-way mirror, noted 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 during 5 min.

2.5. Egg analyses

Two eggs per female, one collected at *age 1* and the other at *age 2*, were analysed during the same assay (only eggs of females who had had chicks during both periods were analysed, thus $N=2\times18$). Steroid extraction and assays (enzyme immunoassay) followed a method similar to that described by Bertin et al. [43] and Guibert et al. [14]. For steroid extraction, we first separated the frozen yolk from the eggshell and the albumin. Eggshells and yolks 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 egg layers [28,44] 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 stored overnight at 4 °C. Samples were then vortexed and 1 ml of the suspension was transferred into a new vial. The suspension was diluted in 4 ml methanol, vortexed for 30 min and stored at −20 °C overnight to precipitate apolar lipids. Samples were then centrifuged (−10 °C, 2500 *g*, 10 min). 10 µl of the supernatant was transferred into a new vial, dried under a stream of nitrogen at 60 °C and then dissolved in 500 µl EIA buffer. The extract was diluted 1:5 with EIA buffer. 10 µl of this solution was used for testosterone and androstenedione assays. 10 µl of the solution after an additional 1:10 dilution was used for progesterone assays. For full descriptions of antibodies and validation of enzyme immunoassays, see Palme and Möstl [45], Hirschenhauser et al. [46], and Möstl et al. [44]. We measured yolk testosterone, androstenedione and progesterone in two assays. The intra-assay coefficients of variation were 8.5%, 4.2% and 9.2% respectively.

2.6. Data analyses

Kolmogorov–Smirnov tests were used to determine whether data sets were normally distributed. Laying rates, fertilisation, hatchability and chick survival rates were not normally distributed, so Wilcoxon signed-rank tests were applied. Yolk hormone data were log-transformed ($Y+1$) and analysed by paired Student's *t*-tests. Proportions of egg components were arc-sin-square-root transformed and compared by paired Student's *t*-tests. Data used for comparisons of incubated egg weights were mean egg weight per female at *age 1* and at *age 2* and were compared by a paired Student's *t*-test. Only data for the 18 females that had had chicks during both periods were compared (except laying rate data that also included data for the 22 females present during both periods). In order to compare only chicks from females that had had chicks at both *ages* (18 females), we had to discard data for 12 *age 1* chicks as their mothers did not have any chicks at *age 2*. For all comparisons, we had data for 64 *age 1* chicks (3.56 ± 0.15 chicks/female) and for 75 *age 2* chicks (4.17 ± 0.23 chicks/female). This did not bias the sex ratio (*age 1*: 31 females and 33 males, *age 2*: 30 females and 45 males; Chi-square test, $\chi^2=0.998$, $P=0.32$). Chicks' weights and cloacal vent lengths were analysed using repeated measures ANOVAs (parental age × chicks' age × chicks' sex); subsequent post-hoc tests (paired Student's *t*-tests) were used when required. Given that we had several offspring from a same female for both ages, we used average chick weights and cloacal vent lengths for all chicks of a female (female and male offspring separately) for these analyses. As one female had no female offspring at *age 2*, its data could not be included in the analyses. Chicks' behavioural data were not normally distributed, so we used a non-parametric test for two related samples: the two-sample matched sets test [47] that can take into account all (*age 1* + *age 2*) chicks of a given female. Data are represented as means ± standard error of the mean (SEM). All analyses were performed using Statview Software (SAS, Cary, NC) with significance level set at $P\leq0.05$.

3. Results

3.1. Laying rates

The laying rates of the 22 females present at both *ages* were significantly lower at *age 2* than at *age 1*. Mean production was

0.93 ± 0.02 egg per day at *age 1* and 0.75 ± 0.06 egg per day at *age 2* (Wilcoxon test, $Z = -3.541$, $P = 0.0004$). The laying rates of the 18 females that had chicks at both *ages* were also lower at *age 2* than at *age 1* (respectively, 0.84 ± 0.03 egg per day and 0.96 ± 0.01 egg per day; $Z = -3.001$, $P = 0.0013$).

3.2. Egg characteristics

The mean weights of eggs collected for incubation did not differ significantly between *age 1* and *age 2* (respectively, 13.88 ± 0.23 and 13.80 ± 0.237, $N = 18$; paired Student's *t*-test, $t = 0.538$, $P = 0.60$). The weight of eggs collected for analyses did not differ significantly between *age 1* and *age 2* ($t = -0.666$, $P = 0.51$; Table 1). The ratio egg shell/egg weight was lower at *age 2* than at *age 1* ($t = 2.093$, $P = 0.052$; Table 1) and, consequently, the proportion of yolk plus albumen was higher at *age 2* than at *age 1* ($t = -2.093$, $P = 0.052$; Table 1). Neither yolk proportions ($t = -0.402$, $P = 0.69$; Table 1) nor albumen proportions ($t = -0.849$, $P = 0.41$; Table 1) differed significantly between *age 1* eggs and *age 2* eggs.

Yolk testosterone concentrations were significantly lower in *age 2* eggs than in *age 1* eggs ($t = 3.320$, $P = 0.0041$; Fig. 1A). No significant differences between sets emerged for androstenedione ($t = 0.693$, $P = 0.50$; Fig. 1B) or progesterone concentrations ($t = -0.354$, $P = 0.73$; Fig. 1C). Comparisons of total amounts of hormones per yolk revealed similar results: *age 2* egg yolks contained less testosterone than did *age 1* egg yolks (*age 1*: 48.05 ± 6.97 ng/yolk and *age 2*: 31.64 ± 3.39 ng/yolk; $t = 3.005$, $P = 0.0080$), and neither androstenedione (*age 1*: 525.87 ± 31.70 ng/yolk and *age 2*: 524.78 ± 34.97 ng/yolk; $t = 0.142$, $P = 0.89$) nor progesterone (*age 1*: 5086.39 ± 203.39 ng/yolk and *age 2*: 5411.80 ± 243.39 ng/yolk; $t = -1.102$, $P = 0.29$) total amounts differed significantly between sets.

3.3. Fertilisation rate, hatchability of fertile eggs and chick survival rate

Fertilisation rate of *age 2* eggs was significantly lower (63.66 ± 5.00%) than that of *age 1* eggs (81.59 ± 4.37%) ($N = 18$, Wilcoxon test, $Z = -3.136$, $P = 0.0017$). Hatchability of fertile eggs did not differ significantly between *age 1* and *age 2* (respectively, 81.74 ± 3.60% and 83.57 ± 3.87%; $Z = -0.355$, $P = 0.72$). Chick survival rate was lower at *age 2* (95.68 ± 2.05%) than at *age 1* (100%) ($Z = -2.032$, $P = 0.042$).

3.4. Chicks' body weight and sexual development

Parental age, chicks' age and sex had significant effects on chicks' body weight (Table 2). Interactions between chicks' age and parental age and chicks' age and sex also had significant effects. Other interactions were not significant (Table 2). Post-hoc comparisons (paired Student's *t*-tests) indicated that both female and male *age 2* chicks were heavier than *age 1* chicks at hatching (females: $t = -2.949$, $P = 0.0094$; males: $t = -2.361$, $P = 0.031$; Table 3) and at 1 week (females: $t = -5.729$, $P = 0.000031$; males: $t = -4.822$, $P = 0.00019$; Table 3). However, male and female *age 2* chicks did not differ from *age 1* chicks at 2 weeks (females: $t = -1.329$, $P = 0.20$; males: $t = -0.311$, $P = 0.76$; Table 3). Differences between *age 2* and *age 1* chicks became significant again at 3 weeks for females ($t = -2.455$, $P = 0.026$) but not for males ($t = -1.250$, $P = 0.23$) (Table 3). At 4 weeks, *age 2* females tended to be heavier than *age 1* females ($t = -1.752$, $P = 0.099$), and again males did not differ ($t = -0.500$, $P = 0.62$) (Table 3).

Parental age, chicks' age and sex had significant effects on chicks' cloacal vent length (Table 2). None of the interactions was significant (Table 2). Both male and female *age 2* chicks had larger cloacal vents than did *age 1* chicks at 3 weeks (respectively, in females: 5.59 ± 0.17 mm and 5.04 ± 0.08 mm; respectively, in males: 5.10 ± 0.12 mm and 4.72 ± 0.11 mm) and at 4 weeks (respectively, in females: 6.20 ±

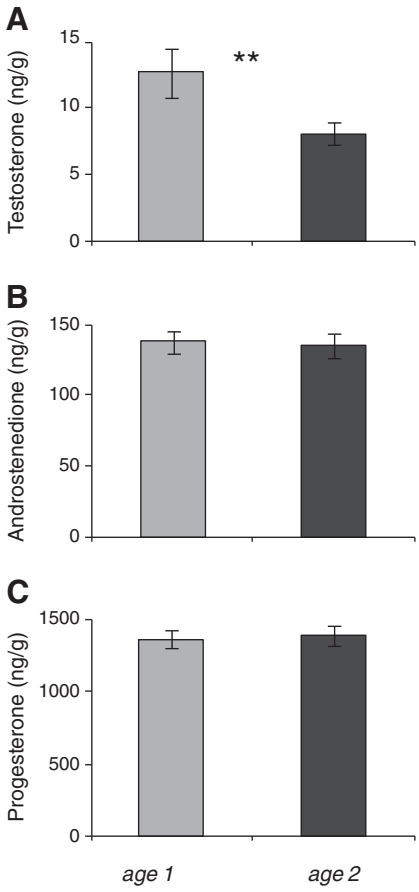

Fig. 1. Yolk testosterone (A), androstenedione (B) and immunoreactive progesterone (C) concentrations (mean ± SEM ng/g) in *age 1* and *age 2* eggs. Paired Student's *t*-test, $^{**}P \leq 0.01$.

Table 1
Composition of *age 1* and *age 2* eggs.

Parameters	*Age 1*	*Age 2*
Egg weight (g)	13.64 ± 0.32	13.88 ± 0.33
Shell/egg weight ratio, in %	12.01 ± 0.28	11.19 ± 0.29*
(Yolk + albumen)/egg weight ratio, in %	87.99 ± 0.28	88.82 ± 0.29*
Yolk/egg weight ratio, in %	28.08 ± 0.50	28.33 ± 0.64
Albumen/egg weight ratio, in %	59.91 ± 0.62	60.49 ± 0.60

Mean ± SEM; $N = 18$; paired Student's *t*-test.
* $P \leq 0.05$.

Table 2
Repeated measure ANOVAs for chicks' body weight and cloacal vent length in relation to parental age, chicks' age and chicks' sex.

Variable	Factors	d.f.	*F*	*P*
Body weight	Parental age	1,16	8.93	0.0087
	Chicks' age	4,64	5263.20	<0.001
	Chicks' sex	1,16	11.95	0.0032
	Parental age × chicks' age	4,64	3.43	0.013
	Chicks' age × chicks' sex	4,64	12.62	<0.001
	Parental age × chicks' sex	1,16	0.90	0.36
	Parental age × chicks' age × chicks' sex	4,64	0.75	0.56
Cloacal vent length	Parental age	1,16	30.03	<0.001
	Chicks' age	1,16	65.37	<0.001
	Chicks' sex	1,16	17.37	<0.001
	Parental age × chicks' age	1,16	0.074	0.79
	Chicks' age × chicks' sex	1,16	0.59	0.45
	Parental age × chicks' sex	1,16	1.08	0.31
	Parental age × chicks' age × chicks' sex	1,16	0.057	0.81

Table 3
Body weight of *age 1* and *age 2* chicks from hatching to 4 weeks old.

Age	Chicks' body weight (g)			
	Females		Males	
	Age 1	*Age 2*	*Age 1*	*Age 2*
Hatching	9.26 ± 0.16	9.91 ± 0.25**	9.39 ± 0.18	9.77 ± 0.18*
1 week	34.87 ± 1.23	41.47 ± 0.74***	33.83 ± 0.86	39.23 ± 1.19***
2 weeks	90.49 ± 1.87	93.28 ± 1.89	87.41 ± 1.61	88.14 ± 2.42
3 weeks	148.21 ± 2.57	157.38 ± 3.67*	143.13 ± 2.07	147.03 ± 3.21
4 weeks	195.92 ± 3.34	204.45 ± 4.28#	187.48 ± 2.56	189.68 ± 4.22

Mean ± SEM; paired Student's *t*-test.
0.1 > *P* > 0.05,
* *P* < 0.05,
** *P* < 0.01,
*** *P* < 0.001.

0.19 mm and 5.57 ± 0.13 mm; respectively, in males: 5.60 ± 0.15 mm and 5.20 ± 0.08 mm).

3.5. Chicks' behaviour

3.5.1. Separation test

Age 2 chicks took their first step significantly sooner than did *age 1* chicks (respectively, 7.87 ± 2.00 s and 12.31 ± 3.31 s; two-sample matched sets test, $H = 6.802$, $P < 0.01$) and took more steps (163.27 ± 14.46 steps for *age 1* chicks and 199.88 ± 10.94 steps for *age 2* chicks; $H = 4.036$, $P < 0.05$). Neither latencies of first call (5.38 ± 0.71 s for *age 1* chicks and 8.95 ± 2.56 s for *age 2* chicks; $H = 0.601$, $P > 0.05$) nor numbers of calls (108.64 ± 6.28 for *age 1* chicks and 117.21 ± 5.55 for *age 2* chicks, $H = 0.77$, $P > 0.05$) differed significantly between the two sets. More *age 2* chicks than *age 1* chicks tended to jump during a test (respectively, 21.5 ± 5.1% and 14.8 ± 7.3% chicks per female jumped; sign test, $n = 12$, $x = 3$, $P = 0.073$).

3.5.2. Tonic-immobility test

Age 2 chicks tended to need more inductions to induce tonic-immobility than did *age 1* chicks (respectively, 3.31 ± 0.17 and 2.92 ± 0.18 inductions; two-sample matched sets test, $H = 2.93$, $0.1 > P > 0.05$), but tonic-immobility durations did not differ significantly between sets (*age 1* chicks: 65.14 ± 7.63 s; *age 2* chicks: 55.96 ± 6.37 s; $H = 0.448$, $P > 0.05$).

3.5.3. Emergence and open-field tests

Age 2 chicks stuck their head out of the emergence box and emerged entirely sooner than did *age 1* chicks (respectively: $H = 7.75$, $P < 0.01$; $H = 4.324$, $P < 0.05$; Fig. 2).

After emergence, once in this novel environment, *age 2* chicks ($N = 72$) made more locomotor acts than did *age 1* chicks ($N = 59$) (respectively, 14.58 ± 1.05 and 11.27 ± 0.97; $H = 4.22$, $P < 0.05$). Similarly, in the open-field test, *age 2* chicks took significantly more steps than did *age 1* chicks (respectively, 411.64 ± 23.80 and 213.20 ± 18.82 steps; $H = 28.324$, $P < 0.001$), although latencies to take first step did not differ significantly between the two sets (*age 1* chicks: 18.69 ± 4.71 s; *age 2* chicks: 11.39 ± 2.06 s; $H = 0.0198$, $P > 0.05$). In both tests, *age 2* chicks emitted their first call sooner (emergence test: $H = 7.73$, $P < 0.01$; open-field test: $H = 17.29$, $P < 0.001$; Fig. 3A) and emitted more calls (emergence test: $H = 3.91$, $P < 0.05$; open-field test: $H = 4.622$, $P < 0.05$; Fig. 3B) than did *age 1* chicks. *Age 2* chicks made significantly more high-posture observations in both tests (emergence test: *age 1* chicks: 6.73 ± 0.76, *age 2* chicks: 10.69 ± 1.00, $H = 6.69$, $P < 0.01$; open-field test: *age 1* chicks: 7.84 ± 0.67, *age 2* chicks: 11.59 ± 0.58, $H = 19.409$, $P < 0.001$). Numbers of exploratory acts did not differ significantly between the two sets of chicks in either of these two tests (emergence test: *age 1* chicks: 8.05 ± 0.93, *age 2* chicks: 7.17 ± 0.79, $H = 0.013$, $P > 0.05$; open-field test: *age 1* chicks: 6.97 ± 0.72, *age 2* chicks: 6.84 ± 0.59, $H = 0.359$, $P > 0.05$).

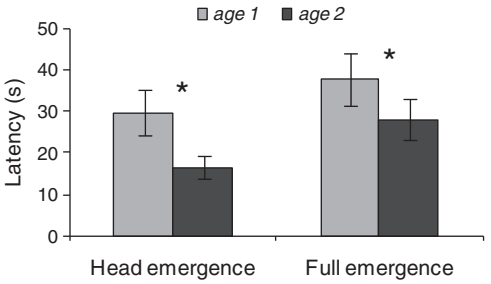

Fig. 2. Latencies (s) of head emergence and of full emergence (mean ± SEM) of *age 1* and *age 2* chicks in emergence tests. Two-sample matched sets test, $^{*}P \leq 0.05$.

4. Discussion

Here, we demonstrated that parental age affects offspring morphological and behavioural development in Japanese quail and our data suggest that these effects could be linked to variations of yolk testosterone levels due to age.

The facts that laying rates and egg shell proportions decreased when females' age increased indicate that the reproductive state of females differed between *age 1* and *age 2*. Indeed, decline of egg production and eggshell quality occurs typically at the end of the egg-laying period in domestic fowl and in Japanese quail [48,49]. Our results support previous reports that laying by Japanese quail decreases after 26 weeks [50]. The decline of egg fertilisation rates between *age 1* and *age 2*, which could be of female and/or male origin, confirms that parents were in different reproductive states at *age 1* and at *age 2*. However, compared to the 30 to 50% decline in fertility observed in 70 week-old Japanese quail [51], the reproductive function of our *age 2* parents appeared to still be at a relatively high level. Furthermore, hatchability levels were similar between *age 1* and *age 2*.

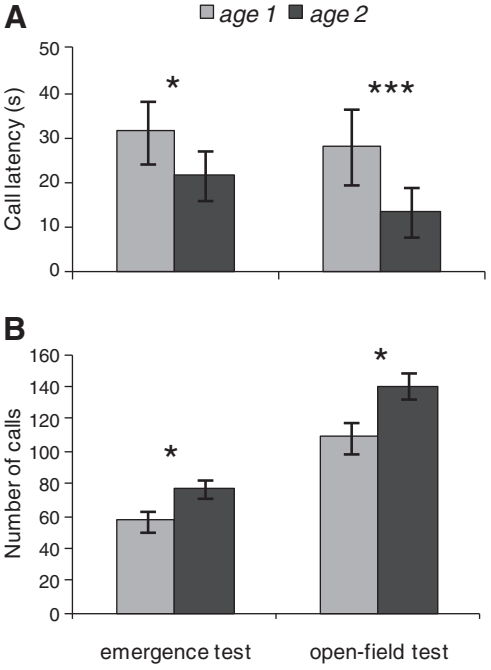

Fig. 3. Mean (±SEM) call latencies (A) and numbers of calls (B) of *age 1* and *age 2* chicks in emergence and open-field tests. Two-sample matched sets test, $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$.

Age also affected egg hormonal levels. Although yolk progesterone and androstenedione levels were not influenced by age, yolk testosterone concentrations decreased between *age 1* and *age 2*. This result is consistent with another study showing that yolk testosterone concentrations in the eggs of Japanese quail females living in groups decreased between 15 and 51 weeks [20]. This decrease of yolk testosterone content can be linked to the decline of the reproductive function of our females. Yolk testosterone is produced by cell layers of the follicular wall that surrounds the growing oocyte [52]. The pituitary hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH) regulate follicular hormone production and follicular growth [52]. Sensitivity of follicular cells to LH [53] and that of the central nervous system to LH-positive feedback stimulation [54] decrease in ageing laying hens. These mechanisms could account for the decrease of yolk testosterone content with age [20]. However, another factor could explain yolk testosterone decrease. Indeed, male characteristics have been shown to affect yolk testosterone levels. The eggs of female zebra finches paired with highly attractive males contained more androgens than did those of females paired with less attractive partners [16]. Yolk testosterone levels in collared flycatchers' eggs were not affected by male attractiveness but by male age. Females paired with old males (over one year old) produced eggs containing less testosterone than did females paired with 1-year-old males [55]. So, in our study, a decrease of male attractiveness with age or just their age could have influenced the decrease of yolk testosterone levels. In these two studies, however, pairs stayed together during the whole laying period, so a male effect on yolk testosterone levels may require the male and the female staying together for a long period and creating special bonds. Our females were force-mated with different males and stayed only a few minutes with them, three times a week. So, in this context, we suppose that a male effect on yolk testosterone level was very low or even negligible.

Age of parents influenced the general development of chicks. First, chicks of older parents were heavier at hatching than were chicks of younger parents, their subsequent growth patterns differed and their sexual development was more precocious. This is consistent with other studies showing that the weights of one-day old Japanese quail chicks increase with parental age [56,57]. Similarly, turkeys of older parents weighed more when 42 days old than did offspring of younger parents [58]. Second, the behavioural development of chicks differed in relation to their parents' age. Chicks of older parents tended to need more inductions to induce tonic-immobility, suggesting a lower anti-predator response and thus a lower underlying fearfulness [39,59]. *Age 2* chicks stuck their heads out of the emergence box and left it faster than did *age 1* chicks, indicating a less cautious behaviour when encountering a novel environment and thus lower emotional reactivity of *age 2* chicks [40–42]. During separation tests, *age 2* chicks moved sooner, took more steps and more of them tended to jump than did *age 1* chicks. This clearly reflects attempts to re-establish contact with conspecifics and, so, higher sensitivity of these chicks to social separation [37]. The same pattern concerning locomotor activity was found during emergence and open-field tests. In addition, during these tests, *age 2* chicks called sooner, emitted more calls and made more high posture observations than did *age 1* chicks, these behaviours also reflecting active search for conspecifics [37,60,61]. Thus, *age 2* chicks appeared less emotional than *age 1* chicks when encountering a novel environment, and more sensitive to social separation.

These phenotypic differences between chicks related to parental age may be linked to differences in embryos' prenatal environments. First, changes in the relative parts of egg components between *age 1* and *age 2* could influence embryos' development. Indeed, *age 2* eggs contained relatively less shell and more nutrients (yolk and albumen) than did *age 1* eggs. Differences in eggshell thickness could affect gas exchanges during incubation, and this in turn could affect embryo development [62]. The higher proportion of nutrients in *age 2* eggs could obviously be involved in the higher hatching weight of *age 2* chicks [63]. Second, chicks of older parents were exposed prenatally to less maternal testosterone than were chicks of younger parents. Previous reports indicated strong influences of yolk hormonal levels on chicks' general development. Testosterone injected into the egg yolks of various bird species affected chicks' morphological development, sometimes in different ways: either it decreased body mass at hatching or during postnatal development [29,64,65], or it enhanced growth [66–68]. Moreover, yolk testosterone levels are known to affect the emotional reactivity of young. Quail chicks from eggs with lower levels of yolk testosterone appeared less fearful than chicks subjected to higher androgen levels [14,29,30,43]. Daisley et al. [9] showed that chicks from eggs with lower yolk androgen levels were more socially dependent, but, contrary to previous studies, they were also more fearful. These discrepancies in effects of yolk testosterone reflect the complexity of prenatal hormonal influence on subsequent development and could be linked to the yolk testosterone supplementation procedure. First, differences in testosterone effects on subsequent development may be linked to hormonal dose. Indeed, hormone dose–response curves are often non-monotonic, presenting an inverted U-shape, with intermediate dosages having greater effects than either higher or lower dosages [52]. However, Okuliarova et al. [29] did not find evidence for a U-shape effect, but a simple dosage dependency between testosterone levels and behavioural parameters in tonic-immobility test. But, such a dose-dependency was not observed in relation to behavioural parameters recorded in the openfield test. Second, the mode of testosterone supplementation (injection *vs* maternal deposition) may result in these opposite effects. Originally, steroids are not uniformly distributed in the yolk of freshly laid eggs, but vary within yolk layers, with high oestradiol concentrations in the centre, high androstenedione and testosterone concentrations in the middle and high progesterone concentrations in the peripheral layer of the yolk sphere [28,44,69]. As yolk layers seem to persist, at least during the beginning of the incubation period, embryos may thus be exposed to temporal variations of the availability of these hormones during embryonic development [69]. Recently, von Engelhardt et al. [70] showed that a bolus injection of steroid hormone (dissolved in sesame oil) into the yolk concentrates near the area where the embryo develops and that, after 6 days of incubation, the hormone is still not evenly distributed in the yolk. Thus following an injection, the embryo, on the one hand, may not be exposed to the hormone at the appropriate time during its development and, on the other hand, may be exposed to a super-physiological dose of the hormone. So, the effects observed on offspring following a yolk hormone injection may not reflect the observed effects on offspring following natural exposition to hormones of maternal origin.

In the literature, a recurring theme is the adaptive value of yolk androgen level modulation. Most of the existing work on the subject is based on data from artificial prenatal exposure and on observations of egg natural hormonal variations [6,7]. Many hypotheses have been developed concerning altricial or semi-precocial species in which modulation of chicks' behaviour, in relation to androgen levels, may have a strong impact on their growth and survival. So, in some species, the high frequency of begging display in chicks from eggs with high level of testosterone could compensate the disadvantage of hatching asynchrony for the later-hatched chicks [8,71] or be advantageous for inter-brood food competition [6]. Parental age has a strong effect on offspring survival. Indeed, in many species, the reproductive success of young breeders is lower than that of older breeders. They lay smaller clutches, their eggs are smaller and the survival rates of their chicks are lower [21,22,72,73]. These facts could be linked to the poorer condition of young birds [74,75], to their inexperience in foraging or their lack of familiarity with their habitat [76,77], all aspects that can affect egg production and offspring rearing. So, higher

levels of yolk androgens in young females' eggs could compensate for the "lower" quality of their eggs and rearing behaviour. Contrary to altricial and semi-precocial chicks for which begging is fundamental for their survival, precocial chicks do not need to beg as they find food by themselves. However, they appear very dependent on their mother for temperature regulation, finding good feeding areas and predator protection. The lower reproductive success of young precocial lapwing (*Vanellus vanellus*) could be linked to their lower foraging ability and anti-predator behaviour [78]. So, in quail, a species submitted to high predation pressure, high levels of yolk testosterone in young females' eggs could compensate for their lower anti-predator behaviour by producing emotive chicks that are more attentive and/or reactive to their environment. More research, of course, is required to validate this hypothesis. However, the adaptive value of prenatal maternal effects needs to be analysed differently in precocial and altricial species in relation to different biological constraints.

Although yolk steroid levels seem to play a fundamental role in prenatal maternal effects on offspring development, an embryo's environment is complex, containing various substances. The composition of the principal nutritional components of egg yolk, lipids, appears to change with the laying female's age. Indeed, yolk fatty acid profiles vary according to broiler breeders' age [79,80] and these changes have been found to be correlated with late embryonic mortality and hatchability [81]. Egg yolks also contain antioxidants, antibodies and other hormones such as thyroid hormones, all of maternal origin, which can have an impact on offspring growth and immunity [7]. These substances may also vary with females' age and may influence offspring phenotype directly or through their interaction with testosterone. For instance, old laying hens have been shown to have over twofold lower levels of immunoglobulin G-containing cells in their ovaries than do young laying hens [82] resulting in a lower transfer of this antibody to their egg yolks [83]. Thus, in our study, yolk components other than testosterone may have been involved in inducing phenotypic differences between offspring of young and of older parents. Beyond yolk content differences related to age, differences in gamete quality (both male and female) could be involved in eliciting the phenotypic differences we observed between *age 1* and *age 2* chicks. Indeed, the decrease of fertility and of chick survival rates between *age 1* and *age 2* suggests this possibility. With ageing, accumulation of mutations in the nuclear and/or mitochondrial DNA of gametes or epigenetic changes in gene expression could occur and have consequences on offspring phenotype [84,85]. However, these gamete quality changes may occur only in very old parents. As we mentioned previously, our *age 2* quail breeders cannot be considered to be very old as their life span in the laboratory varies from 3 to 5 years and reproductive senescence of males is observed only after 3 years and that of females after 2 years [27]. Moreover, the good hatchability level of *age 2* eggs suggests that the observed effects on offspring development are not due to differences in gamete quality.

Parallel prenatal effects on offspring general development are observed between birds and mammals. In these two orders, offspring morphological and behavioural development can be influenced prenatally by maternal stress or females' living conditions [4,14,43,86–88]. These maternal influences seem to result from modulation of embryos' hormonal environment, either *in utero* [5,89,90], or in the egg [7,52]. Interestingly, testosterone concentrations in the serum of pregnant female rats and mice decreased with advancing age at gestation [91,92] thus providing the developing embryos with less testosterone in their prenatal environment. Body weight of older mother mice's offspring was lower than that of younger mothers' offspring, their puberty was delayed and their learning capacities were lower [84,92]. Our study shows that, as in mammals, bird mothers' age can affect offspring development, possibly via prenatal hormonal environment.

The mechanisms of prenatal maternal influences appear complex, involving many different factors simultaneously. The complexity of this fact is reflected by discrepancies between studies on the same or on different species (see above). Contrary to our results, Pilz et al. [19] showed that older free-living female European starlings (over one-year old) laid eggs with higher androgen levels than did one-year old females. The mechanisms modulating egg hormonal levels may differ between the two species. Whereas androgen levels in Japanese quail egg yolks appear similar across the laying sequence [20], they increase with laying order in starlings' eggs [19]. However, data for free-living birds may be the result of a combination of factors, as laying females may differ not only in age but also in living conditions or intrinsic characteristics. So, domestic species living in a controlled environment could become useful models to understand mechanisms of maternal prenatal effects on offspring development in birds.

In this study, we demonstrated how age of parents can influence their offspring's characteristics (especially their behaviour) and that this is probably linked to differences in the prenatal environment they provide their offspring. Precocial birds appear to be good models to investigate parental age effects on offspring as prenatal and postnatal influences of these effects can be separated. Our study highlights the fact that parental age *per se*, without taking into account its potential effects on postnatal care of offspring, is a strong source of individual behavioural variability.

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