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RESEARCH ARTICLE

Long-life partners or sex friends? Impact of parental pair bond on offspring personality

Océane Le Bot*, Sophie Lumineau, Emmanuel de Margerie, Florent Pittet, Marie Trabalon and Cécilia Houdelier

ABSTRACT

Previous investigations reported that some traits of parental relationships, including pair-bond duration or mate behavioural compatibility, influence subsequent offspring fitness by acting on their behaviour and growth and thus their early survival. We hypothesized that the development of a pair bond between sexual partners would have a prenatal influence. This study investigated the impact of two pairing managements on the egg characteristics and development of offspring of Japanese quail (*Coturnix c. japonica*). Thirty males and 30 females were paired either continuously (C; mates together all the time) or non-continuously (NC; pairs met only three times a week for 5 min). Separation–reunion tests evaluated parental pair bond. Egg yolk testosterone and androstenedione levels were evaluated, and the somatic and behavioural development of C and NC chicks was assessed. Our results revealed that members of C pairs were attached to their mates and, although no significant differences in androgen levels could be evidenced between egg sets, a higher proportion of C pairs' eggs were fertilized and their chicks appeared less emotive and more social. Our results revealed that the parental relationship can modulate the behavioural development of their offspring, probably via non-genetic effects, and this could play a major role in the emergence of inter-individual variability.

KEY WORDS: Attachment, Hormonal assay, Pair bond, Precocial bird, Prenatal influence

INTRODUCTION

Phenotypic variability is a fundamental trait that is determined by various mechanisms and plays a central role in the adaptation of species to their environment (Scheiner, 1993). Although genes provide the innate matrix of individuals, non-genetic environmental factors influence their construction throughout their life. Parental effects are known to influence individual development through non-genetic inheritance mechanisms (Bjorklund, 2006; Houdelier et al., 2013). Mammal and bird mothers especially can modulate their offspring's behaviour. After giving birth, mothers' behavioural and/or maternal care characteristics are known to influence their offspring's emotive and social behaviours in the long term (Bertin et al., 2008; Fairbanks, 1996; Francis et al., 1999; Pittet et al., 2014). Before birth, various maternal factors can also influence offspring phenotype. Mammal mothers' intrinsic characteristics influence their offspring's physiological and behavioural development, through physiological differences. For example, in hyenas (*Crocuta crocuta*), high levels of maternal plasmatic androgens at the end of gestation, related to maternal rank, induce higher frequencies of offspring aggressive and mounting behaviours (Dloniak et al., 2006). The maternal plasmatic androgen levels of rodents during gestation decrease with maternal age, and could be related to lighter progeny at birth, delaying their sexual development and impairing their learning abilities (Matt et al., 1986; Tarín et al., 2003; Wang and vom Saal, 2000). Researchers have reported similar influences of maternal age for birds: egg yolk hormonal modulations are related to maternal characteristics (Adkins-Regan et al., 2013; Gil, 2008; Groothuis and Schwabl, 2008; Groothuis et al., 2005). For instance, chicks of older Japanese quail (*Coturnix c. japonica* Linnaeus) parents were heavier at hatching, their sexual development occurred sooner, and their emotional reactivity was lower in the presence of novelty but higher following social separation (Guibert et al., 2012). These results could be related to the lower levels of yolk testosterone in older females' eggs (Guibert et al., 2012; Okuliarová et al., 2009).

Moreover, reports highlight the fact that the female's environment during the reproduction period has a strong prenatal influence. Thus, an aversive social environment during gestation can increase offspring emotive behaviours, impair their cognitive abilities and impair their social, sexual and maternal behaviours. These effects are linked to modulation of the expression of the hypothalamo-pituitary–adrenocortical (HPA) axis, inducing in particular modification of plasmatic concentrations of glucocorticoids and androgens in the female (Braastad, 1998; Kaiser and Sachser, 2005; Welberg and Seckl, 2001). Similarly, negative social interactions during the laying period affect chicks' physiological and behavioural phenotypes in relation to modulation of the hormonal contents of the female's eggs [social instability (Guibert et al., 2010; Mazuc et al., 2003); breeding density (Reed and Vleck, 2001; Schwabl, 1997); aggression by conspecifics (Whittingham and Schwabl, 2002)]. Avian sexual partners that develop a pair bond, even for a short time, reap benefits that include mutual preening, fewer conflictual interactions between mates, joining forces against intruders, food sharing and/or behavioural synchronization (Amat, 2000; Emery et al., 2007; Komdeur and Hatchwell, 1999; Orcutt and Orcutt, 1976). Parental relationship is known to impact investment in offspring, and so to influence their development; most studies investigate this relationship in terms of long-term survival for offspring, often of altricial species. The researchers suggest that male attractiveness influenced egg steroid levels as egg testosterone levels were higher when females were exposed to attractive males (Gil et al., 1999; Gil et al., 2004; Kingma et al., 2009), and this could influence chick development (Groothuis et al., 2005). Once a pair has formed, pair-bond duration impacts breeding success directly (Black, 1996; Hall, 1999; Nisbet and Dann, 2009). In particular, clutch size, hatching success and chick weight are directly linked to parental pair-bond duration of little penguins, *Eudyptula minor* (Nisbet and Dann, 2009). Besides this relationship duration, behavioural compatibility between mates influences offspring indirectly, as the more the traits

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of the two mates correspond, the better their activities, including nest attendance, are coordinated, and so the greater their breeding success (Coulson, 1966; Coulson, 1972; Spoon et al., 2006). In contrast, divorce between long-lasting pairs can occur in some species; although this adaptive strategy induces females to leave their male partner (Coulson, 1966; Diamond, 1987; Ens et al., 1996), their reproductive success can increase despite the immediate costs of breaking a long-lasting pair bond [e.g. fledging failure (Jacot et al., 2010)]. When biparental care occurs, the presence of both parents is crucial for rearing young successfully (Hall, 1999). Experiments show that one parent can compensate for its failing partner (i.e. experimentally removed or handicapped) by, for example, increasing nest attendance and chick feeding, but this compensation remains limited and induces growth and survival consequences for offspring (Bijleveld and Mullers, 2009; Lendvai and Chastel, 2008). However, the fact that most of these studies concern altricial species, implying postnatal parental care, makes it difficult to separate prenatal and postnatal influences of parental relationships.

Our study evaluated the impact of Japanese quail pair bond on offspring phenotype. Using two different pairing situations, i.e. a male and a female were either continuously housed in the same cage (continuously, C) or not (non-continuously, NC), we focused on the consequences of pairing situation on the partners' relationship and female behaviour, their egg characteristics and development of their offspring. Under natural conditions, male and female quail form monogamous pairs during the reproduction period at least until egg incubation starts – pair bonds last for 38 days on average (Nichols, 1991). Throughout this period, the two mates develop a special relationship, synchronizing their activities independently of those of their neighbours, staying close to each other, actively searching for their partner when separated and actively defending their partner from attacks by other mating conspecifics (Nichols, 1991; Orcutt and Orcutt, 1976; Potash, 1975). Japanese quail pair bonds are reinforced by individual recognition (Riters and Balthazart, 1998), and a preference to mate with an affiliate partner (Galef, 2008). In this context of pairing, we hypothesized that continuous pairing would facilitate the development of a pair bond between partners and modulate offspring behavioural development via non-genetic prenatal effects.

RESULTS

Adults

Activity in the home cage

Except for the interactions of C females with their male, C and NC females in their home cages expressed all the behaviours that we assessed in similar proportions, before and after the treatment, indicating no influence of pair situation on their activity budgets (Mann–Whitney *U*-tests, $P>0.05$).

Emotional reactivity

Tonic immobility (TI) duration of females did not differ significantly between the two pairing sets either before or after treatment (Mann–Whitney *U*-test, before treatment: C females 119.13±22.96 s, NC females 73.60±14.15 s, $U=78$, $P=0.16$; after treatment: C females 91.07±20.32 s, NC females 119.20±20.22 s, $U=83.5$, $P=0.24$).

Pair bond

A principal component analysis (PCA) differentiated the reactions of C and NC quail to separation and reunion with their pair mate. The projection of variables is illustrated in Fig. 1, presenting the centroids of individual projections for each set; loadings of variables on each dimension are illustrated in Fig. 2. The first dimension discriminated males according to their activity when in pairs, i.e. before separation and after reunion with their female. It separates C males expressing comfort behaviours from NC males expressing more high observations, courtship and sexual vocalizations in the presence of females, indicating that their pair bond is not well established (Mann–Whitney *U*-tests of individual scores, C females −0.40±0.22, NC females 0.40±0.25, $U=64$, $P=0.046$). The second dimension discriminated individuals according to their level of distress when separated from their mate, opposing C males that expressed active search for their female on the upper part of the axis (high observation, crowing and locomotion) and NC males that expressed comfort behaviours on the lower part of the axis (C females 0.53±0.20, NC females −0.53±0.22, $U=192$, $P<0.001$; Fig. 1).

The PCA indicated a similar pattern for females. First, an activity when in pairs dimension differentiated C females that expressed

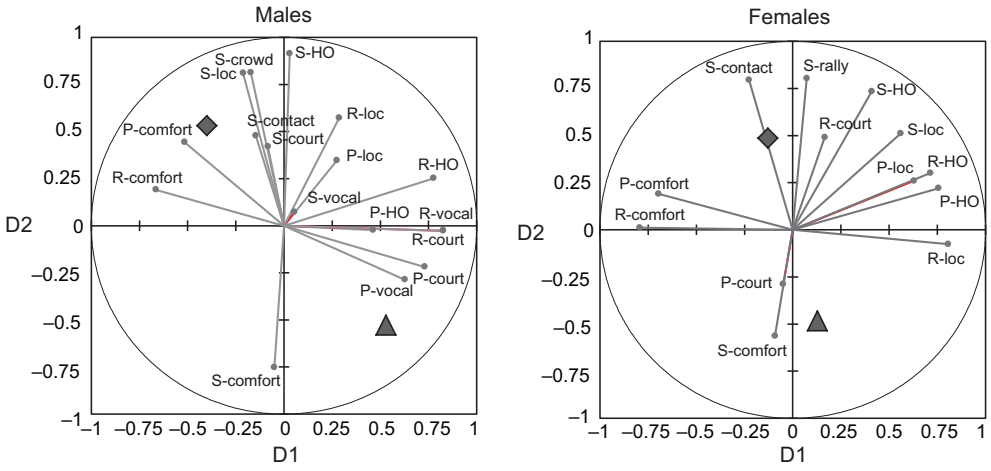


Fig. 1. Males and females of continuous pairs search for their partner when separated. The distribution in relation to the first two principal components extracted after principal component analyses (PCA) is shown. Code: P, paired; S, separated; R, reunited; loc, locomotion; HO, high observation; comfort, comfort behaviours; court, courtship; vocal, male courtship vocalizations; crowd, male crowing expressed during separation; rally, female rally calls expressed during separation; contact, contact calls emitted by both sexes during separation. The first (D1) and the second (D2) dimensions accounted, respectively, for 24.01% and 23.53% of total variation for males and 34.07% and 25.35% for females. Diamond, centroid of individual coordinates for the continuously paired (C) set; triangle, centroid of individual coordinates for the non-continuously paired (NC) set.

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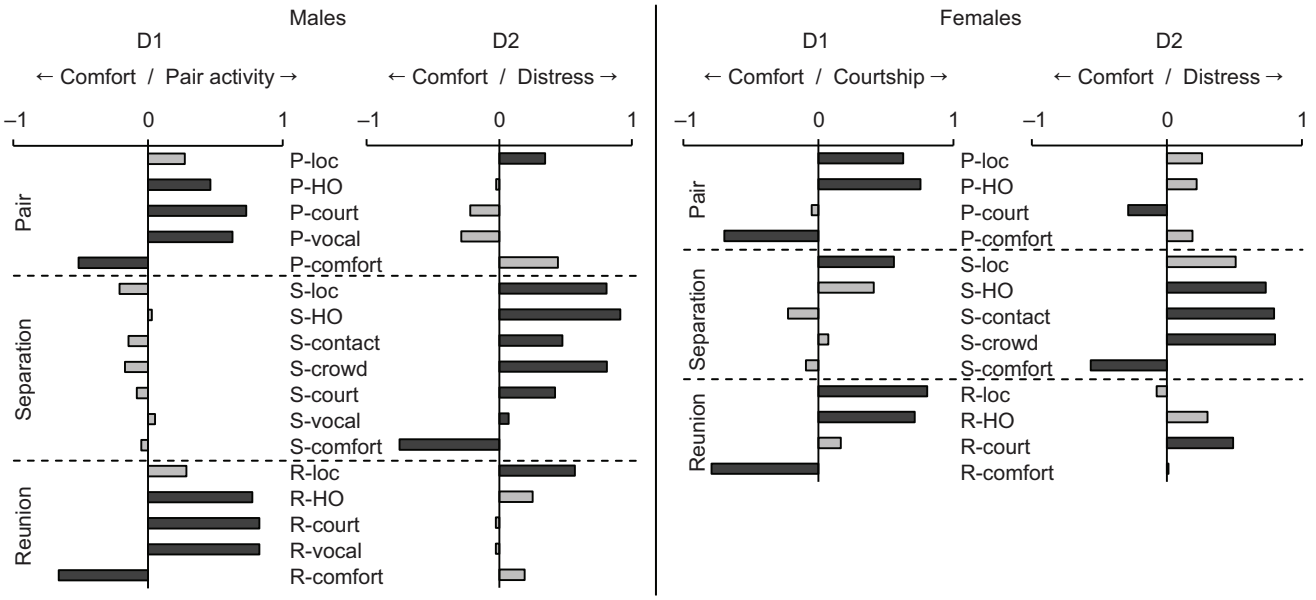


Fig. 2. Loading of variables for males and females for the two first dimensions (D1 and D2) of the PCA. Code: P, paired; S, separated; R, reunited; loc, locomotion; HO, high observation; comfort, comfort behaviours; court, courtship; vocal, male courtship vocalizations; crowd, male crowing expressed during separation; rally, female rally calls expressed during separation; contact, contact calls emitted by both sexes during separation. D1, pair behaviour; D2, reaction to separation. Black bars indicate values >±0.40.

comfort behaviours (on the left in Fig. 1) from NC females that expressed high observation and locomotion (on the right). We found no significant differences between sets for this dimension (C females –0.14±0.29, NC females 0.14±0.22, U=85, P=0.263). The second dimension concerned mainly distress when separated, contrasting C females that actively searched for their mate by expressing vocalizations and high observation (upper part of the axis in Fig. 1) with NC females that expressed comfort behaviours during separation (lower part of the axis; C females 0.54±0.25, NC females –0.54±0.18, U=192, P<0.001). Moreover, distress during separation was associated with courtship displays after reunion (Fig. 1).

Eggs

C pairs tended to produce more fertilized eggs than did NC pairs (Mann–Whitney U-test: C pairs 91.32±6.61%, NC pairs 85.35±5.32%, U=269, P=0.092). We could not find any other differences between the two sets concerning either egg traits (number laid, mass, dimensions, proportions of albumen, yolk and shell) or the hormonal concentrations of androgens (testosterone and androstenedione) (Table 1).

Offspring

Development

We found no significant differences between sets concerning sex ratio (percentage of male C chicks 46.99%, NC chicks 51.16%, χ^2=3.84, d.f.=1, P>0.05). Chicks of C pairs tended to be lighter than chicks of NC pairs at hatching (Table 2); this difference became significant after 1 week. Cloacal vent width data did not reveal any differences in sexual development between the two sets (ANOVA, P>0.05).

Emotional reactivity

TI tests did not reveal any differences between C and NC chicks concerning either TI duration (Mann–Whitney U-test: C chicks 59.65±8.44, NC chicks 71.46±7.32, U=255.5, P=0.17) or number of inductions (C chicks 1.23±0.08 s, NC chicks 1.20±0.09 s, U=368.0, P=0.61). Emergence tests revealed that C chicks emerged from the box faster than NC chicks (Fig. 3A). Furthermore, C chicks tended to walk more after their emergence (frequency for C chicks 14.0±0.80, NC chicks 11.9±0.85, U=281, P=0.073). No other differences could be found (Mann–Whitney U-test, P>0.05). C chicks expressed fewer active (pacing, wall pecking) and passive (fear postures, low and high observation) fear reactions in the open field (Fig. 3B). They also pecked their environment less than did NC chicks (ground: C chicks 1.77±0.45, NC chicks 7.96±2.05, U=169, P=0.005; walls: C chicks 0.35±0.16, NC chicks 2.17±0.63, U=178, P=0.003). Similarly, C chicks were less fearful in the presence of a novel object (neophobia), expressing fewer active fear reactions (Fig. 3B) and preening more frequently (C chicks 3.15±0.50, NC chicks 1.82±0.36, U=187, P=0.037).

Social behaviour

Although no significant differences were evident during the separation test, when C chicks were isolated from their conspecifics in an unfamiliar environment (i.e. open-field and novel object tests),

Table 1. Egg characteristics for the continuous and non-continuous sets

	C	NC	P
Number	23.67±0.83	25.13±0.46	0.23
Mass (g)	13.98±0.28	13.85±0.29	1.00
Length (mm)	35.00±0.33	34.69±0.31	0.53
Width (mm)	27.02±0.16	27.00±0.19	0.93
Shell proportion (%)	8.47±0.22	8.69±0.16	0.29
Yolk proportion (%)	31.73±0.67	32.16±0.48	0.82
Albumen proportion (%)	59.80±0.52	59.14±0.47	0.31
Testosterone (ng g^{-1})	9.69±0.89	10.60±1.07	0.72
Androstenedione (ng g^{-1})	141.07±14.58	163.50±20.42	0.78

Birds were paired either continuously (C; mates together all the time) or non-continuously (NC). Mean (±s.e.m.) number, mass, length and width of all eggs laid from the third week to the end of the protocol, i.e. 28 days, for each set (N_C=355, N_{NC}=277) are shown. Component values (%) and androgen levels (ng g^{-1}) were calculated for one egg per female (N_C=13, N_{NC}=15).

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Table 2. Mean mass of chicks of the C and NC sets

	Mass (g)		
	C	N	*P*
Day 1	9.83±0.11	10.08±0.10	0.09
Day 8	36.15±0.78	38.22±0.56	**0.03**
Day 15	105.49±1.87	108.02±1.16	0.25
Day 21	161.01±2.54	164.02±1.61	0.32
Day 28	218.50±2.98	215.74±3.16	0.53

Data (means ± s.e.m.) are given in relation to age [from hatching (day 1) to 4 weeks old (day 28); *N*=175].
Bold indicates significant difference (ANOVA, **P*<0.05).

they called sooner (in the novel object test: C chicks 144.5±20.55 s, NC chicks 225.68±22.11 s, *U*=148.5, *P*=0.004) and more frequently (Fig. 4A) than did NC chicks. In the presence of unfamiliar conspecifics, C chicks entered the runway corridor later than did NC chicks (C chicks 15.69±8.25 s, NC chicks 10.43±4.71 s, *U*=252.5, *P*=0.019), but reached zone P quicker and spent less time far from unfamiliar conspecifics (Fig. 4B). Moreover, C chicks tended to emit distress calls sooner (C chicks 274.65±10.40 s, NC chicks 289.43±6.97 s, *U*=305, *P*=0.061) and more frequently (C chicks 5.88±2.23, NC chicks 2.23±1.22, *U*=310.5, *P*=0.080) than did NC chicks.

DISCUSSION

Our results showed that pair type influences the link between sexual partners and has an effect on offspring development. We demonstrated that pair type influenced attachment between males and females: continuous pairing, but not non-continuous pairing, favoured the development of a pair bond. Whereas C males and females expressed comfort behaviours frequently when they were together, they expressed high levels of distress, such as locomotion, vigilance and vocalizations, when they were separated. These behaviours are generally associated with the search for their social partner by isolated birds and are used as an indicator that Japanese quail pair bonds are well established (Mills et al., 1997; Potash, 1975; Rodríguez-Teijeiro et al., 2003; Thomson, 1964). These search behaviours are related to increased plasma corticosterone levels, this physiological state being linked to disturbances (Remage-Healey et al., 2003; Shepherd and French, 1999). Following reunion, C females displayed courtship and this activity is known to be related to a good relationship between pair mates (Saint-Jalme, 1990). Comparatively, NC males and females expressed comfort behaviours during separation, and males expressed vigilance, locomotion, vocalizations and courtship when their female was present. The fact that NC pairs show no distress during separation and more vigilance following reunion indicates that their pair bonds are not yet established. This is reinforced by the fact that NC males increased the frequency of their courtship displays following reunion whereas in a natural situation courtship decreases after pairing (Saint-Jalme, 1990).

Our results showed that both members of a pair express attachment to their partner. Under natural conditions, quail partners synchronize their activities, remaining in close proximity but at some distance from conspecifics (Nichols, 1991; Orcutt and Orcutt, 1976; Potash, 1975). This type of pair bond can be beneficial for a male by increasing his paternity assurance and decreasing extra-pair copulations (Beecher and Beecher, 1979; Birkhead, 1979; Birkhead et al., 1987). Thus, the development of a particular attachment to their female is important for mate guarding. Although a male does not provide any offspring parental care, he could have an indirect effect on the fitness of both his partner and his offspring. The presence of male white-tailed ptarmigans (*Lagopus leucura*), a Galliform with a mating system similar to that of Japanese quail, can increase foraging by females as a result of their vigilance against predators and competitors (Artiss and Martin, 1995). Moreover, male quail frequently offer food, especially invertebrates, to their female and this can be beneficial for the female's energy state and thereby offspring survival (Pinczon Du Sel, 1994). Overall, a female's attachment to her mate could be important for her own fitness.

Our results revealed that the development of a pair bond affected offspring phenotype. C chicks were lighter than NC chicks after hatching and 1 week later, although egg mass did not differ significantly between sets. Chicks of C parents presented a lower emotional reactivity, as they entered into a novel environment more quickly, and expressed less fear there and less neophobia in the presence of a novel object. Furthermore, their social motivation was higher than that of NC offspring. Indeed, C chicks called sooner and more frequently when alone in a novel environment, reached unfamiliar conspecifics quicker and stayed near them for longer than did NC chicks. So, the existence of a pair bond in quail can modulate the growth and the behavioural phenotype of their offspring through a non-genetic prenatal process. The effects of female mammal and bird social/sexual environment on the phenotype of offspring has been analysed mainly in the context of a stressful social maternal environment. Prenatal social stress of female mammals (due to social instability, crowding and agonistic social encounters) increases their offspring's emotional reactivity and impairs their social behaviours (Braastad, 1998; Kaiser and Sachser, 2005). Social instability of laying Japanese quail increases their offspring's emotional reactivity in the presence of novelty and when separated from conspecifics (Guibert et al., 2010). In contrast to stressful social situations, our study shows that the establishment

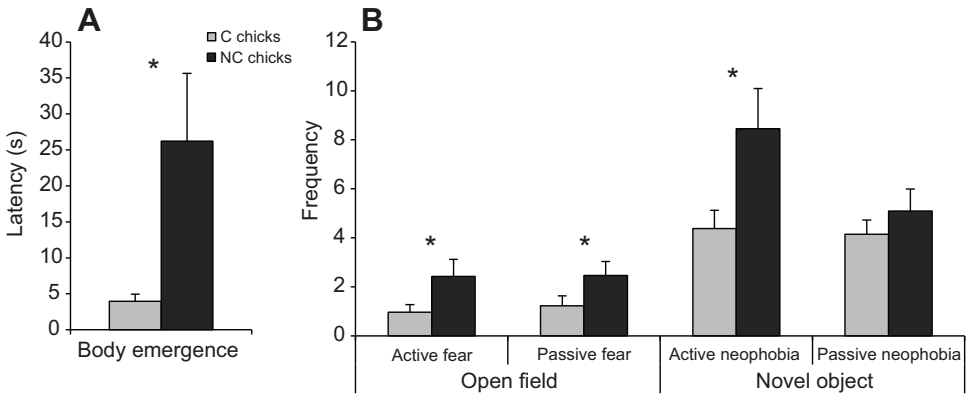


Fig. 3. Chicks of C pairs are less fearful. (A) Latency of emergence and (B) frequency of fear behaviours in the open-field and novel object tests for C and NC chicks. Active fear/neophobia: stereotypic locomotion and wall pecking in the open field, locomotor avoidance of the novel object; passive fear/neophobia: static fear postures and freezing, low and high observation postures. Means are given with s.e.m., Mann–Whitney *U*-test, **P*<0.05.

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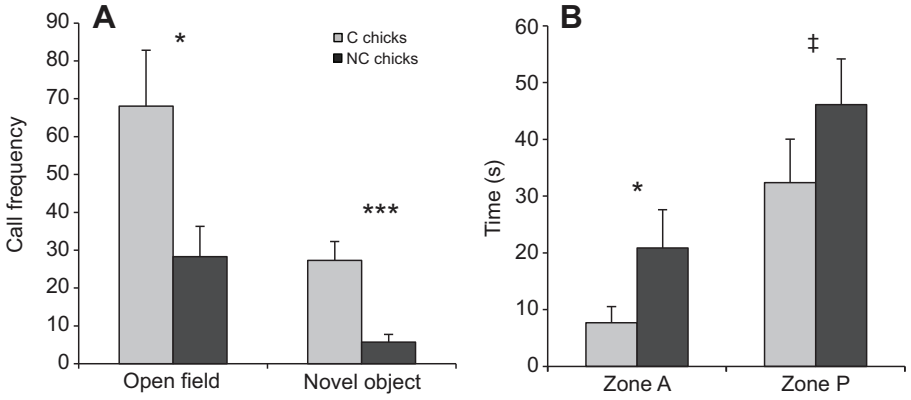


Fig. 4. Chicks of C pairs are more social. (A) Call frequency in the open-field and novel object tests and (B) time spent in zone A of the runway and latency to arrive in zone P for C and NC chicks (see Materials and methods for runway description). Means are given ±s.e.m., Mann–Whitney *U*-tests, ‡P<0.1, *P<0.05, ***P<0.001.

of a particular relationship with a partner can influence, prenatally, offspring phenotypic development. Interestingly, whereas social stress induced an increase of quail offspring's emotional reactivity (Guibert et al., 2010), the establishment of a pair bond decreased offspring emotivity. Similar effects have been observed when females are exposed to positive stimulation. For example, a physically enriched maternal environment (i.e. a cage with numerous objects and a nest) decreased female mice pups' emotional reactivity in an open field (Maruoka et al., 2009). Similarly, tactile stimulations of pregnant rat dams (i.e. gentle stroking with a baby's hairbrush) reduced their pups' anxiety in an open field (Muhammad and Kolb, 2011). We cannot tell from our study whether continuous pairing is a more favourable situation for the females than non-continuous pairing. The fact that the behaviours (emotional reactivity, activities in the home cage) and reproductive performances of females of the two sets were similar seems to exclude potential effects of stress on NC quail, and, in contrast, the effects of a positive environment on C females. However, we showed that the ability of quail to build a pair bond significantly influenced their offspring's behavioural development, in a similar way to the effects of positive stimulations.

In our study, the effects of pairing type on offspring growth and behaviour cannot be related to modulation of egg yolk androgen levels as these levels were similar in the two sets of eggs. Previous studies showed that prenatal effects appear linked to modulation of yolk hormonal content. Indeed, many factors can influence yolk composition, such as females' intrinsic characteristics [e.g. maternal age (Guibert et al., 2012)] and social environment [e.g. social instability (Guibert et al., 2010; Mazuc et al., 2003), breeding density (Reed and Vleck, 2001; Schwabl, 1997; Pilz and Smith, 2004), aggression by conspecifics (Whittingham and Schwabl, 2002)]. Moreover, males' characteristics influence androgen levels in eggs: females paired with attractive males, or paired with males that emit attractive song, lay eggs with higher levels of testosterone (Gil et al., 1999; Gil et al., 2004; Kingma et al., 2009). These yolk hormonal modulations can affect offspring growth and behaviour (Groothuis et al., 2005; Gil, 2008), but these effects differ among studies and species. Increased yolk testosterone levels can enhance, or conversely impair, chick growth (Schwabl, 1996; Eising et al., 2001; Pilz et al., 2004; Navara et al., 2006; Sockman and Schwabl, 2000; Rubolini et al., 2006, Guibert et al., 2010; Guibert et al., 2011). Similarly, increased yolk androgen levels can be either positively or negatively associated with chicks' emotional reactivity (Daisley et al., 2005; Okuliarová et al., 2007; Bertin et al., 2009; Guibert et al., 2010; Guibert et al., 2011). Thus, relationships between yolk androgen modulations and chick morphological and behavioural development are not clear and could be modulated by hormonal concentrations (with a U-shaped effect) (Groothuis and Schwabl, 2008), but also by other egg components. Avian egg yolks contain many components of maternal origin including carotenoids (Sauveur, 1988), thyroid hormones (McNabb and Wilson, 1997; Wilson and McNabb, 1997), or albumen antibodies and lysozymes (Boulinier and Staszewski, 2008) that can be modulated by the female's environment and affect offspring growth and immunity (Gil, 2008; Saino et al., 2002). Moreover, yolk oestrogens, progesterone and corticosterone can also influence offspring development (Hayward and Wingfield, 2004; Groothuis et al., 2005; Gil, 2008; Henriksen et al., 2011). In this study, we hypothesize the involvement of mesotocin and vasotocin, homologues of mammalian oxytocin and vasopressin (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012), in these prenatal effects. As the formation of bird pair bonds appears to be modulated by mesotocin and vasotocin, we reasonably suggest that our two sets of quail did not present the same levels of these nonapeptides. This raises questions concerning the influence of potential modulation of the oxytocinergic system on offspring. Vasotocin has been found in hen ovaries (Chaturvedi et al., 1994; Saito and Grossmann, 1999) and it is precisely in the ovary, during yolk formation, that maternal state can modulate egg hormonal deposition, impacting subsequent offspring development. All these considerations implicate nonapeptides in the prenatal influence of parental relationships, probably through cascading actions. So, a variety of mechanisms could be involved in the observed prenatal maternal effects.

Prenatal influence could also include epigenetic inheritance processes. Many authors have investigated the involvement of histone acetylation and DNA methylation in the epigenetic regulation of genes (Borrelli et al., 2008; Danchin et al., 2011; Franklin et al., 2010; Jensen, 2013). For example, a stressful environment can alter the hypothalamic gene expression profile in the hen, changes that are also found in their chicks, suggesting either inheritance or acquisition in the specific egg environment (Lindqvist et al., 2007). Similarly, among the multiple paths of epigenetic inheritance, the involvement of fathers must not be neglected. Our study focused on pair-bond relationships that include both the male and female as potential vectors of prenatal influence. In this context, males can have a direct impact on their offspring through their gametes, potentially impacted by their living conditions (i.e. an attachment to a female or not in our case) via epigenetic mechanisms. Several reports have investigated epigenetic paternal inheritance in mammals, in contrast to maternal effects, when maternal effects on germ cells are difficult to separate from effects of gestating environment (Anderson et al., 2006; Carone et al., 2010; Wei et al., 2014). No study to our knowledge has investigated paternally induced transgenerational inheritance in birds that could

occur either directly through germ lines or indirectly through female modulation of yolk hormones, implicating both parents in prenatal effects on offspring.

Prenatal modulation of offspring development raises the question of its adaptive consequences on offspring survival and parental fitness. Prenatal maternal influences can be adaptive by preparing offspring for particular postnatal environments. In a competitive context (for example, a high-density population), heavier offspring could be advantaged as they could express stronger competitive behaviour [i.e. aggressiveness (Groothuis et al., 2005)]. Thus, the adaptive consequences of prenatal maternal effects are related to postnatal living conditions. Although being lighter at fledging could induce greater subsequent fitness costs and poorer survival rates (Jarvis, 1974), a lower body mass at hatching could, in contrast, be an advantage in an expected restrictive environment (for example, when food is scarce) (Henriksen et al., 2011). A recent report suggests that the adaptive significance of maternal prenatal influences should be considered more globally, by investigating systematically long-term consequences for the fitness of both mothers (or parents) and offspring (Sheriff and Love, 2013). The different phenotypes of our chicks could be related to two different strategies. C chicks were lighter but less emotive, so they could compensate for their lower mass by more opportunistic behaviours (e.g. exploration of new food sources). This phenotype could be adaptive in a less-constraining or stable environment (in terms of social/sexual interactions and food availability) that could be mimicked in the context of continuous pairing (i.e. with a stable sexual relationship). By contrast, NC chicks were heavier but more fearful, and this could limit their exploratory behaviour but also increase their survival rate (by enhancing their vigilance) in a more constraining environment. Our non-continuous pairing situation can mimic this more constraining environment as sexual interactions are unstable. So, for parents, their social/sexual context could carry information about environmental conditions that could modulate offspring phenotype to increase their fitness. The characteristics of relationships between sexual partners can be an important factor influencing the phenotypic variability of populations in their evolution process.

Conclusions

We show here for the first time that continuous interaction between a male and a female Japanese quail is important for pair bonding, and that a subsequent attachment forms a prenatal parental influence that strongly impacts chick social and emotive behaviour. By addressing the consequences of a lasting male–female relationship on the behaviour of both parents and offspring, our experiment opens an innovating field of research, especially regarding the mechanisms underlying the effects of the sexual environment as a prenatal influence.

MATERIALS AND METHODS

Ethics

This study was approved by the regional ethics committee (agreement number: R-2012-SLu-01). 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).

Parental generation

Subjects

Adult Japanese quail, *Coturnix c. japonica* (6 weeks old) came from a broiler line and were provided by an industrial farm (Les cailles de Chanteloup, Ille-et-Vilaine, France). On their arrival at the laboratory 30 males and 30 females were placed individually in batteries in the same room (male cages: 35×25×21 cm; female cages: 33×50×23 cm) at 20±2°C and with a 12 h:12 h light:dark cycle; this experimental room was designed in order that all birds could have visual and auditory contact with congeners, and even some tactile contact with neighbours. Water and food were provided *ad libitum*. After 2 weeks of habituation, 30 pairs were formed randomly, and each female met her assigned male for 1 h, the male being placed in the female's cage. This meeting occurred two additional times, 3 and 6 days later. After the third meeting (defined as day 1 of the pairing protocol) and for 6 weeks, 15 males were left in their female's cage (continuously paired: set C), whereas the other 15 males were returned to their cages and were put in their female's cage three times a week for 5 min between 09:30 h and 10:30 h (non-continuously paired: set NC). This pairing time for the NC set does allow fertilization of all eggs as sperm can be stored in a female's genital tract for several days without any loss of capacity (Birkhead and Møller, 1993).

Behavioural observations

Activity in the home cage

We assessed the activity of the females in their home cage by instantaneous scan sampling on days 11 and 35 of the protocol (30 scans per activity budget with 5 min intervals), when NC males were not in their female's cages. Hidden behind a one-way mirror, each scanner recorded: maintenance behaviour (preening, dust bathing, resting and defecation); locomotion (walking, running); 'pacing' stereotypic locomotion (i.e. walking back and forth); exploration (i.e. pecking the floor and cage); jumps; observation posture based on body position: vigilance (i.e. fixed posture – a quail stands upright on her tarsi and stretches her neck), high (i.e. similar to vigilance posture but the quail is not standing), medium (i.e. body neither stretched nor crouching) and low observation posture (i.e. the quail appears frightened and glances at her environment with neck stretched parallel to the floor, lower than her body); and fear posture (standing or walking slowly with ruffled feathers, or freezing, i.e. fixed low posture).

Emotional reactivity

Tonic immobility tests evaluated the emotional reactivity of the females of the two sets. In a dark room, a subject was maintained on her back for 10 s, head facing downwards, in a U-shaped wooden device. Tonic immobility (TI) duration, an anti-predator behaviour, is positively correlated with a subject's level of fear (Mills et al., 1994). Induction is successful when the quail does not move for at least 10 s. TI duration (i.e. until the quail stands up, with a maximum of 300 s) and the number of inductions (maximum of 5) were recorded. This test was replicated once in the week before pairing of C and NC subjects, and 6 weeks after.

Pair bond

Separation–reunion tests assessed the level of affiliative attachment between the male and the female of each pair 2 weeks after pairing. First, we observed the behaviour of each member of the pair when they were together in a novel environment (a 100×44×32 cm cage with wood shavings) for 6 min. Then, the male was removed and placed in a similar cage in another room for 6 min so that the two members of the pair could neither see nor hear each other. Finally, the male was placed again with the female for 6 min. During these three phases, we noted all behaviours: locomotion, maintenance, observation posture, stereotypic pacing, vocalizations [contact calls (i.e. a short distance vocalization), female rally calls and male crowing (i.e. long distance vocalizations and male courtship vocalizations)] and sexual interactions (i.e. male and female courtship displays and copulations). To avoid an effect of NC male's arrival during the test, each NC male was placed in his female's cage for 5 min before the test started.

Eggs

Collection and incubation

As the formation of a pair bond can take several days and as the formation of a yolk takes 7 days (Sauveur and Picard, 1987), fertilized eggs from each pair were collected only after 15 days of pairing and then for 28 days. Eggs were marked and measured (mass, length and width) each morning. Broken eggs were recorded but not measured. All eggs were then stored at 16±1°C but we used only the last 10 eggs produced by each pair to produce chicks.

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These eggs (*N*=300) were artificially incubated in the laboratory (Ova-Easy Advance 380, Brinsea©, USA). During the first 14 days in the incubator, eggs were maintained at 37.7°C with a relative humidity of 45% and with a 4 deg automatic rotation of the plate twice a day. During the last 3 days, temperature was decreased to 37.2°C, humidity was raised to 60% and plate rotation was stopped. Eggs that did not hatch were opened to evaluate embryonic development.

Hormonal assays

One week before eggs were placed in the incubator, one of each pair's eggs was frozen for subsequent hormonal analyses. Our enzyme immuno-assays (EIA) were adapted from a previous method (Guibert et al., 2010). For steroid extraction, frozen yolks were separated from the shell and albumin and then weighed. As the distribution of hormones varies between egg strata (Möstl et al., 2001), the entire yolk was mixed before analysis. Each yolk was then suspended in 10 ml of water and vortexed. For testosterone assay, 1 ml of the suspension was transferred into a new vial. The suspension was then diluted with 4 ml of methanol, vortexed for 30 min and stored at −20°C overnight in order to precipitate apolar lipids. Afterwards, samples were centrifuged (−10°C, 2500 ***g***, 10 min) and 10 µl of the supernatant were transferred into a new vial, centrifuged again in order to be dried (60°C, 2500 ***g***, 10 min), and finally dissolved in 500 µl of EIA buffer. For androstenedione assay, 100 µl of the yolk and water solution was diluted in 1 ml of diethylether, vortexed and stored similarly as for testosterone. After centrifugation, 10 µl of the supernatant was centrifuged and dried, then dissolved in 100 µl of EIA buffer, 10 µl of this solution being diluted again in 990 µl of EIA buffer. The concentrations of testosterone and androstenedione were evaluated according to EIA kit procedures (Enzo Life Sciences and Oxford Biomedical Research, respectively). The concentration of hormones in yolk samples was estimated using a standard plot and expressed as ng g^{-1} of yolk. Intra-assay coefficients for testosterone and androstenedione were under 10.12% and 6.58%, respectively; inter-assay coefficients were under 15.11% and 4.13%, respectively.

Chicks

Housing and development

As hatching of eggs laid the same day can be spread out over 3 days, we kept only chicks that hatched on the 17th day of incubation (this day corresponding to the hatching peak) for this study in order to standardize age and early experience. After hatching, 86 chicks of the C set and 89 chicks of the NC set were marked with coloured rings around their tarsi and housed by set in small rooms (200×200 cm, ~45 chicks per room) with wood shavings covered by a mesh mat to facilitate chick locomotion during their first week. Two heaters (38±1°C) and a green light on continuously (i.e. 24 h) were placed in each box with a 12 h:12 h light:dark cycle, and chicks were fed *ad libitum* with starting poultry meal. After 2 weeks, the heaters and green lights were removed and the starting meal was gradually replaced by adult poultry meal. Chicks were weighed at hatching, and then each week for 5 weeks. Their sexual development was assessed 3 weeks after hatching, when males and females could be differentiated by plumage, by measuring the cloacal vent.

Behavioural tests

Behavioural tests assessed C and NC chicks' intrinsic emotional reactivity (TI), in the presence of novelty or social isolation (i.e. emergence, open-field and novel object tests), and social motivation (i.e. runway test). All observations, except the runway tests, which were screened, were recorded behind a one-way mirror. We tested two chicks of each parental pair, one male and one female, selected randomly, to counterbalance possible parental effects or chick sex effects. When it was not possible to test one chick of each sex of a pair, we tested two chicks of the same sex. Finally, as parental pairs with no or a single chick were excluded from chick analyses (two C pairs), we tested 56 chicks, 26 C (12 males and 14 females) and 30 NC (17 males and 13 females). We describe below the test protocols in relation to chick age.

TI (phd 10)

This took place on post-hatching day (phd) 10, and the protocol was similar to that used for adult quail (see 'Behavioural observations', above).

Separation test (phd 11–12)

To assess the reactivity of chicks when separated from their conspecifics, all the chicks of one room were transferred into another identical room. Then, each test subject was placed alone in the centre of its home room, and observed for 3 min. The observer recorded latencies of first step and first call, the number of steps and calls, maintenance behaviours, locomotion, pacing, exploration, jumps, observation postures and fear postures, similar to those described above for their parents.

Emergence test (phd 14–15)

The chick was placed in a little opaque box (18×18×18 cm) for 1 min, during which the latency of the first distress call and number of calls were recorded. One side of the box was then opened and the subject was allowed 3 min to leave the box and go into a large lighted cage (62×60×33 cm) with wood shavings. Emergence latency is positively correlated with fear level (Jones, 1987; Mills and Faure, 1986). This latency was recorded, as were, all behaviours of chicks in the large cage, for 3 min: latency of the first distress call and number of calls, maintenance behaviours, locomotion, exploration, jumps, observation postures and fear postures.

Runway test (phd 17–18)

The chick was placed for 1 min behind a transparent wall, at one end of a corridor (width 30 cm, length 100 cm). The social stimulus was a cage with three same-aged unfamiliar chicks placed at the opposite end of the corridor. The observer recorded latency and number of distress calls when the quail was in the start zone, then he raised the transparent wall so that the subject had access to the corridor. The observer recorded latency to emerge into the corridor; the chicks were allowed a maximum of 3 min to emerge. Once the chick was in the corridor, the observer recorded, for 5 min, time spent in the different zones of the corridor, which was divided into three, 32 cm-long zones (named A–C, beginning near the start zone) and one, 4 cm-long zone (P: close to the conspecifics' cage). The observer also recorded the latency and number of distress calls, the number of jumps, fear postures and contacts with conspecifics that could be either positive (exploration of cage) or negative (aggression of conspecifics in cage). These traits indicate the level of social motivation of a chick (Mills et al., 1995; Suarez and Gallup, 1983).

Open-field test (phd 24–26)

A chick was placed in the dark in the middle of a polygonal enclosure (nine sides, area 1 m^2) with white opaque walls (60 cm high) and wood shavings. The observer switched on the light and then recorded all behaviours for 5 min to assess the subject's fear and social motivation levels (Faure et al., 1983): latencies of first step and of first call, and number of steps, calls, maintenance behaviours, locomotion, exploration, jumps, observation postures and fear postures.

Novel-object test (phd 24–26)

Immediately after the open-field test, the light was switched off, an unfamiliar object (a yellow and black T-shape object, 20 cm high) was placed against a wall, and the subject was placed against the opposite wall before the light was switched on again. The observer recorded for 10 min all the behaviours of the chick: moving away, escape, jumps and fear postures, i.e. activities that are positively correlated with fear (Jones, 1996); approach, exploration (of the environment and novel object), feeding, maintenance behaviours and observation postures. Simultaneously, an instantaneous scan sampling recorded every 10 s where the chick was located: in the cage half opposite the novel object or close to it or in a third zone corresponding to a semicircle around the novel object with a diameter of one chick length.

Statistical analyses

A PCA was used to analyse pair-bond test data to extract pair behaviours when together and during separation. The centroid of the individual coordinates of each set was calculated to locate C and NC males and females on the variable axes. ANOVA were used to compare continuous variables (mass, cloacal vent width) of sets, after checking that data were normally distributed. All the other variables (chick and parent behaviours, PCA individual scores, yolk hormonal levels) were analysed using non-parametric tests (Mann–Whitney). Some data were pooled to categorize variables, as for instance fear behaviours (passive fear included freezing, low and high

observations, active fear included stereotypic and avoidance locomotion and wall pecking), and comfort (preening, dust bathing and resting). Kendall's non-parametric concordance was used to evaluate correlations between parent and chick behaviours. Data were analysed with Statistica 10 and XLstat 2011.3.02.

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