LONG-LIFE PARTNERS OR SEX FRIENDS? IMPACT OF PARENTAL PAIR BOND ON OFFSPRING PERSONALITY

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Summary

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, 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 five minutes). 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 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.

Keywords: attachment; hormonal assay; pair bond; precocial bird; prenatal influence.

1. 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 (Schiener, 1993). Although genes provide the innate matrix of individuals, non-genetic environmental factors

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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, high levels of hyenas’ (Crocuta crocuta) 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). Rodents’ maternal plasmatic androgen levels 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; Tarin et al., 2003; Wang and vom Saal, 2000). Authors report 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 females’ 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 the females’ plasmatic concentrations of glucocorticoids and androgens (Braastad, 1998; Kaiser and Sachser, 2005; Welberg and Seckel, 2001). Similarly, negative social interactions during the laying period affect chicks’ physiological and behavioural phenotypes in relation to modulation of females’ eggs’ hormonal contents (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. Authors 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 chicks’ 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 weights 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 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). On the contrary, 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 its failing partner (i.e. experimentally removed or handicapped) by increasing for example 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’s 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 or not, we focused on the consequences of pairing situation on partners’ relationships and females’ behaviours, 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.

2. Results

2.1. Adults

Activity in home cage. Except for C females’ interactions with their male, C and NC females
in their home cages expressed all the behavioural items assessed in similar proportions, before
and after the treatment, evidencing no influence of pair situation on their activity budgets
(Mann-Whitney U-tests, p > 0.05).

Emotional reactivity. Females’ tonic immobility durations did not differ significantly
between the two sets either before or after treatment (Mann-Whitney U-test, before treatment:
$m_C = 119.13 \pm 22.96$ s, $m_{NC} = 73.60 \pm 14.15$ s, $U = 78$, $p = 0.16$; after treatment: $m_C = 91.07 \pm$
$20.32$ s, $m_{NC} = 119.20 \pm 20.22$ s, $U = 83.5$, $p = 0.24$).

Pair-bond. A PCA analysis 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, $m_C = -0.40 \pm 0.22$, $m_{NC} = 0.40 \pm$
$0.25$, $U = 64$, $p = 0.046$). The second dimension discriminated individuals according to their
level of distress when separated from their mate opposing the C males that expressed active
search for their female on the upper part of the axis (high observation, crowing and
locomotion) to NC males that expressed comfort behaviours on the lower part of the axis ($m_C = 0.53 \pm 0.20$, $m_{NC} = -0.53 \pm 0.22$, $U = 192, p < 0.001$).

The PCA analysis indicated a similar pattern for females. First, an activity when in pairs dimension differentiated C females that expressed comfort behaviours (on the left) from NC females that expressed high observation and locomotion (on the right). We found no significant differences between sets for this dimension ($m_C = -0.14 \pm 0.29$, $m_{NC} = 0.14 \pm 0.22$, $U = 85, p = 0.263$). 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) to NC females that expressed comfort behaviours during separation (lower part of the axis) ($m_C = 0.54 \pm 0.25$, $m_{NC} = -0.54 \pm 0.18$, $U = 192, p < 0.001$). Moreover, distress during separation was associated with courtship displays after reunion (fig. 1).

2.2. Eggs

C pairs tended to produce more fertilized eggs than did NC pairs (Mann-Whitney U-test: $m_C = 91.32\% \pm 6.61\%, m_{NC} = 85.35\% \pm 5.32\%, U = 269, p = 0.092$). We could not evidence any other differences between the two sets concerning either egg traits (number laid, weight, length, proportions of albumen, yolk and shell, or the hormonal concentrations of androgens (testosterone and androstenedione) (table 2).

2.3. Offspring

2.3.1. Development

We found no significant differences between sets concerning sex ratio ($C_{males} = 46.99\%, N_{Cmales} = 51.16\%, \chi^2 = 3.84, df = 1, p > 0.05$). Chicks of C pairs tended to be lighter than chicks of NC pairs (table 2) at hatching, this difference became significant after a week. Cloacal vent width data did not reveal any differences in sexual development between the two sets (ANOVA: $p > 0.05$).

2.3.2. Emotional reactivity
Tonic immobility tests could not evidence any differences between C and NC chicks, concerning either TI durations (Mann-Whitney U-test: $m_{Cc} = 59.65 \pm 8.44$ s, $m_{NCc} = 71.46 \pm 7.32$ s, $U = 255.5$, $p = 0.17$) or numbers of inductions ($m_{Cc} = 1.23 \pm 0.08$ s, $m_{NCc} = 1.20 \pm 0.09$ 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 ($m_{Cc} = 14.0 \pm 0.80$, $m_{NCc} = 11.9 \pm 0.85$, $U = 281$, $p = 0.073$). No other differences could be evidenced (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 openfield (fig. 3b). They also pecked their environment less than did NC chicks (ground: $m_{Cc} = 1.77 \pm 0.45$, $m_{NCc} = 7.96 \pm 2.05$, $U = 169$, $p = 0.005$; walls: $m_{Cc} = 0.35 \pm 0.16$, $m_{NCc} = 2.17 \pm 0.63$, $U = 178$, $p = 0.003$). Similarly, C chicks were less fearful in the presence of the novel object, expressing fewer active fear reactions (fig. 3b) and preening more frequently ($m_{Cc} = 3.15 \pm 0.50$, $m_{NCc} = 1.82 \pm 0.36$, $U = 187$, $p = 0.037$).

### 2.3.3. Social behaviour

Although no significant differences could be evidenced during the separation test, when C chicks were isolated from their conspecifics in an unfamiliar environment (i.e. openfield and novel object tests), they called sooner (in the novel object test: $m_{Cc} = 144.5 \pm 20.55$ s, $m_{NCc} = 225.68 \pm 22.11$ 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 ($m_{Cc} = 15.69 \pm 8.25$ s, $m_{NCc} = 10.43 \pm 4.71$ s, $U = 252.5$, $p = 0.019$), but reached the zone P quicker and spent less time far from unfamiliar conspecifics (fig. 4b). Moreover, C chicks tended to emit distress calls sooner ($m_{Cc} = 274.65 \pm 10.40$ s, $m_{NCc} = 289.43 \pm 6.97$ s, $U = 305$, $p = 0.061$) and more frequently ($m_{Cc} = 5.88 \pm 2.23$, $m_{NCc} = 2.23 \pm 1.22$, $U = 310.5$, $p = 0.080$) than did NC chicks.

### 3. 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 continuously paired 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 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 associated with 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, non-continuously paired 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 partners express attachment to their partner. Under natural conditions, quail partners synchronize their activities, remain in close proximity but at some distance from conspecifics (Nichols, 1991; Orcutt and Orcutt, 1976; Potash, 1975). This type of pair bonds 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 female 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 females’ foraging by their vigilance against predators and competitors (Artis and Martin, 1995). Moreover, male quail frequently offer food, especially invertebrates, to their female and this can be beneficial for females’ 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. Chicks of continuous pairs were lighter than NC chicks after hatching and one week later, although egg weights did not differ significantly between sets. Chicks of continuously paired parents presented a lower emotional reactivity, as they entered into a novel environment more quickly, 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, chicks of continuous pairs 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 mammal and bird females’ social/sexual environment on the phenotype of their offspring have been analysed mainly in the context of a stressful social maternal environment. Prenatal social stress of mammal females (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). By contrast to stressful social situations our study shows that the establishment of a particular relationship with a partner can influence, prenatally, offspring 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 are observed when females are exposed to positive stimulations. 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 hair-brush) reduced their pups’ anxiety in an open-field (Muhammad and Kolb, 2011). We cannot know, here, whether a continuous pairing is a more favorable situation for the females than non-continuous pairing. The fact that the behaviours (emotional reactivity, activities in home-cage) and reproductive performances of females of the two sets were similar seems to exclude potential effects of stress on non-continuous paired quail, and by contrast, the effects of a positive environment on continuous paired females. However, we showed that the ability for quail to build a pair bond influenced significantly their offspring’s behavioural development, in a way similar 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 appeared linked to modulation of yolk hormonal contents. 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 having perceived attractive song emitted by males, 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 on the contrary impair, chicks’ growth (Schwabl 1996; Eising et al. 2001; Pilz et al. 2004; Navara et al. 2006; Sockman & Schwabl 2000; Rubolini et al. 2006, Guibert et al. 2010; 2011). Similarly, increased yolk androgen levels can be either positively or negatively associated with chicks’ emotional reactivity (Daisley et al. 2005; Okuliarova et al. 2007; Bertin et al. 2009; Guibert et al. 2010; 2011). Thus relationships between yolk androgen modulations and chicks’ 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 females’ environment and affect offspring growth and immunity (Gil, 2008; Saino et al., 2002). Moreover, yolk estrogens, 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 can hypothesize the implication of mesotocin and vasotocin in these prenatal effects. As the formation of birds’ pair bonds appears to be modulated by mesotocin and vasotocin, homologues of mammals’ oxytocin and vasopressin (Klatt and Goodson, 2012; Perdersen and Tomaszycki, 2012), 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 hens’ ovary (Chaturvedi et al., 1994; Saito and Grossman, 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 suggest implication of 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 change hens’ hypothalamic gene expression profile, 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 male and female as potential vectors of prenatal influence. In this context, males can have a direct impact on 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 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 females’ 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 to 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), lighter weight at hatching could be on the contrary 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. Chicks of continuous pairs were lighter but less emotive, so they could compensate their lighter weight by more opportunistic behaviours (i.e. exploration of new food sources for example). 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, chicks of non-continuous pairs 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.

Conclusion

We show here for the first time that continuous interactions between a male and a female Japanese quail is important for pair bonding, and that in turn a subsequent attachment forms a prenatal parental influence that strongly impacts chicks’ 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.

4. Material and methods

4.1. 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).
4.2. Parental generation

4.2.1. 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 x 25 x 21 cm; female cages: 33 x 50 x 23 cm) at 20 ± 2°C and with a LD 12:12 light cycle; this experimental room has been designed in order that all birds could have visual and auditory contacts with congers, and even some tactile ones 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 one hour, the male being placed in the female’s cage. This meeting occurred two other times, 3 and 6 days later. After this 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 3 times a week for 5 minutes between 09:30 and 10:30 am (non-continuously-paired: set NC). This time between two pairing of NC can ensure fecundation of all eggs as sperm can be stored in a female’s genital tract for several days without any loss of its fertilizing capacity (Birkhead and Møller, 1993).

4.2.2. Behavioural observations

Activity in 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-minutes intervals), when NC males were not in their female’s cages. Hidden behind a one-way mirror, each scan 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 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 postures (i.e. the quail appears frightened and glances at her environment with neck stretched parallel to the floor, lower than
her body), and fear postures (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 is maintained on her back for 10 seconds, 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 seconds. TI duration (i.e. until the quail stands up, with a maximum of 300 seconds) and the number of inductions (maximum of 5) are recorded. This test was replicated once the week before, and 6 weeks after, pairing C and NC subjects.

**Pair-bond.** Separation-reunion tests assessed the level of affiliative attachment between the male and the female of each pair two weeks after pairing. First, we observed the behaviour of each member of the pair when they were together in a novel environment (a 100 x 44 x 32 cm cage with wood shavings) for 6 minutes. Then the male was removed and placed in a similar cage in another room for 6 minutes 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 minutes. During these three phases, we noted all behaviours: locomotion, maintenance, observations, stereotypies, vocalizations (contact calls i.e. a short distance vocalization, female’s rally calls and male’s 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 five minutes before the test started.

### 4.2.3. 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 (weight, 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 ten eggs produced by each pair to produce chicks. 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 45°
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 incubation, one of each pair’s
eggs was frozen for subsequent hormonal analyses. Our enzyme immuno-assays (EIA) were
adapted from Guibert et al.’s method (2010). For steroid extraction, frozen yolks were
separated from 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 10ml of water and vortexed. For testosterone assay, 1ml of the
suspension was transferred into a new vial. The suspension was then diluted with 4ml of
methanol, vortexed for 30min and stored at -20°C overnight in order to precipitate apolar
lipids. Afterwards samples were centrifuged (-10°C, 2500g, 10min) and 10µl of the
supernatant were transferred into a new vial, centrifuged again in order to be dried (60°C,
2500g, 10min), and finally dissolved in 500µl of EIA buffer. For androstenedione assay,
100µL of the yolk and water solution was diluted in 1mL of diethylether, vortexed and stored
similarly as for testosterone. After centrifugation, 10µL of the supernatant were centrifuged
and dried, then dissolved in 100µL of EIA buffer, 10µL of this solution being diluted again in
990µL 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 concentrations of hormones in yolk samples were estimated using a
standard plot and expressed as ng/g 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.

4.3. Chicks

**4.3.1. Housing and development**

As hatching of eggs laid the same day can be spread out over three 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
continuous pairs (Cc set) and 89 chicks of non-continuous pairs (NCc set) were marked with
coloured rings around their tarsi and housed by set in small rooms (200 x 200 cm, approximately 45 chicks per room) with wood shavings covered by a mesh mat to facilitate chicks’ locomotion during their first week. Two heaters (38 ± 1 °C) and a green light on 24h/24h were placed in each box with a LD 12:12 light cycle, and chicks were fed ad libitum with starting poultry meal. After two weeks, 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.

4.3.2. Behavioural tests

Behavioural tests assessed C and NC chicks’ intrinsic emotional reactivity (tonic immobility), in the presence of novelty or social isolation (i.e. emergence, openfield and novel object tests), and social motivation (i.e. runway test). All observations, except the runway tests that 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 parent 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 (i.e. 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.

Tonic immobility (Post-hatching day, PHD, 10). The protocol was similar to that used for adult quail (see 4.2.2.).

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 minutes. The observer recorded latencies of first step and first call, numbers of steps and calls, maintenance behaviours, locomotion, pacing, exploration, jumps, observation postures and fear postures that are similar to those described above for parents.

Emergence test (PHD 14-15). The chick was placed in a little opaque box (18 x 18 x 18 cm) for one minute, during which latency of 1st distress call and number of calls were recorded.
One side of the box was then opened and the subject was allowed 3 minutes to leave the box and go into a large lighted cage (62 x 60 x 33 cm) with wood shavings. Emergence latency is positively correlated with fear level (Jones, 1987; Mills and Faure, 1986). This latency was recorded and, for 3 minutes, all behaviours of chicks in the large cage: latency of 1st distress call and numbers of calls, maintenance behaviours, locomotion, exploration, jumps, observation postures and fear postures.

**Runway test (PHD 17-18).** The chick was placed for 1 minute behind a transparent wall, at one end of a corridor (width = 30 cm, length = 100 cm). The social stimulus was a cage with 3 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 subjects were allowed a maximum of 3 minutes to emerge. Once the chick was in the corridor, the observer recorded, for 5 minutes, time spent in the different zones of the corridor, which was divided into three 32cm-long zones (named A to C, beginning near the start zone) and one 4cm-long zone (P: close to the conspecifics’ cage). The observer also recorded the latency and number of distress calls, the numbers 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).

**Openfield test (PHD 24 to 26).** A chick was placed in the dark in the middle of a polygonal enclosure (9 sides, area = 1 m²) with white opaque walls (60 cm high) and wood shavings. The observer switched on the light and then recorded all behaviours for 5 minutes to assess the subject’s fear and social motivation levels (Faure et al. 1983): latencies of first step and of first call, and numbers of steps, calls, maintenance behaviours, locomotion, exploration, jumps, observation postures and fear postures.

**Novel-object test (PHD 24 to 26).** Immediately after the openfield 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 light was switched on again. The observer recorded for 10 minutes 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 environment and novel-object), feeding, maintenance behaviours, and observation postures. Simultaneously an instantaneous scan sampling recorded every 10
seconds where the chick was located: half-cage opposite the novel-object or close to it or a 3\textsuperscript{rd} zone corresponding to a semicircle around the novel object with a diameter of one chick-length.

4.4. Statistical analyses

A PCA analysed 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. ANOVAs were used to compare continuous variables (weight, cloacal vent width) of sets, after checking that data were normally distributed. All the other variables (chicks’ and parents’ behaviours, ACP 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 evaluated correlations between parents’ and chicks’ behaviours. Data were analysed with Statistica 10 and XLstat 2011.3.02.

Acknowledgements

We thank Dr A. Laurence and C. Petton for their help during experimental stage, and we are grateful to Dr Ann Cloarec for the improvement of the writing of the manuscript.

References


**FIGURE & TABLE CAPTIONS**

**Table 1.** Egg characteristics for the two sets (C and NC). Mean (± SEM) number, weight, length and width of all eggs laid from the third week to the end of the protocol, i.e. 28 days, for each set ($N_C = 355$, $N_{NC} = 277$). Component values (in %) and androgen levels (ng/g) were calculated for one egg per female ($N_C = 13$, $N_{NC} = 15$).

**Table 2.** Mean (± SEM) weights of chicks of both sets (Cc and NCc) in relation to age (from hatching to 4 weeks old) ($N=175$). Bold characters: significant differences. ANOVA, *p < 0.05.

**Fig. 1.** Males (left) and females (right) of continuous pairs search for their partner when separated. Distribution in relation to the first two principal components extracted after principal component analyses (PCA). First letter: P: when in pair, S: when separated, R: after being reunited. Second letters: LOC: locomotion, HO: high observation, COMF: comfort behaviours, COUR: courtship, VOC: males’ courtship vocalizations; CROW: males’ crowing expressed during separation, RAL: females’ rally calls expressed during separation and CONT: contact calls emitted by both sexes during separation. The 1st and the 2nd 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 C set and triangle: centroid of individual coordinates for the NC set.

**Fig. 2.** Loading of variables for males (left) and females (right) for the two first dimensions (D1 and D2) of the PCA. First letter: P: when in pair, S: when separated, R: after being reunited. Second letters: LOC: locomotion, HO: high observation, COMF: comfort behaviours, COUR: courtship, VOC: males’ courtship vocalizations; CROW: males’ crowing expressed during separation, RAL: females’ rally calls expressed during separation and CONT: contact calls emitted by both sexes during separation. Black bars: values > ± 0.40.

**Fig. 3.** Chicks of C pairs are less fearful. (a) latency of emergence and (b) frequency of fear behaviours in the openfield and novel object tests for C and NC chicks. Active fear: stereotypic locomotion and wall pecking in the openfield, locomotor avoidance of the novel object; passive fear: static fear postures and freezing, low and high observation postures. Means are given with SEM, Mann-Whitney U-test: *p < 0.05.
Fig. 4. Chicks of continuous pairs are more social. (a) call frequency in the openfield and novel object tests and (b) time spent in zone A of the runway and latency to arrive in zone P for Cc and NCc sets. Means are given ± SEM, Mann-Whitney U-tests: # p < 0.1, * p < 0.05, *** p < 0.001.
### Table 1

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<td>Number</td>
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<td>Weight (g)</td>
<td>13.98 ± 0.28</td>
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<td>Testosterone (ng/g)</td>
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<td>141.07 ± 14.58</td>
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### Table 2

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<td>D15</td>
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<td>D21</td>
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<td>164.02 ± 1.61</td>
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<tr>
<td>D28</td>
<td>218.50 ± 2.98</td>
<td>215.74 ± 3.16</td>
<td>0.53</td>
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**FIGURES**

**FIG. 1**

![Diagram](image)

**FIG. 2**

**Males**

D1: pair behaviour  
D2: reaction to separation

- comfort / pair activity  
- comfort / distress

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<tr>
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<th>Reunion</th>
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<td>P.COUR</td>
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</tr>
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<td>P.VOC</td>
<td>S.VOC</td>
<td>R.VOC</td>
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**Females**

D1: pair behaviour  
D2: reaction to separation

- comfort / courtship  
- comfort / distress

<table>
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<tr>
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<th>Reunion</th>
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<td>P.HO</td>
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<td>P.VOC</td>
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**Males**

D1: pair behaviour  
D2: reaction to separation

<table>
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<td>P.HO</td>
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<td>P.VOC</td>
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**Females**

D1: pair behaviour  
D2: reaction to separation

<table>
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<tr>
<td>P.VOC</td>
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Legend:
- C: comfort
- M: distress
FIG. 3

(a) Latency (sec) for body emergence.

(b) Frequency of active and passive fear, active and passive neophobia.

FIG. 4

(a) Call frequency in openfield and novel object.

(b) Time (sec) in time zone A and latency zone P.

* denotes statistical significance.