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Abstract

Regular visual presence of humans is known to reduce chickens' human-generated stress responses. Here we questioned whether, more than mere visual presence, human behaviour affects laying hen behaviour and subsequently their offspring's behaviour. We hypothesized that human behaviour triggers maternal effects via variations in yolk hormone levels. For five consecutive weeks, two groups of hens were exposed to the same durations of human presence (30 min twice a day, five days a week) but the behaviour of the human differed between groups. The first group (H+) was exposed to predictable arrival of the experimenter, slow movements combined with static presence, stroking during handling and human voice. Whereas the second group of hens (H-) was exposed to unpredictable arrival of the experimenter which remained silent, in motion, and did not provide stroking during handling. At the end of the treatment, we evaluated egg quality and offspring behaviour. We found that avoidance of the experimenter by H+ hens but not by H- hens decreased significantly. Fertility rates and concentrations of yolk progesterone and estradiol in H+ hens' eggs were higher than in H- hens' eggs. Fear of humans, neophobia or the capacity to solve a detour task did not differ significantly between H+ and H- chicks. Social discrimination tests showed that H+ chicks but not H- chicks typically preferred a familiar conspecific to a stranger. These results show that, with the same duration in the presence of the birds, humans through their behaviour engender variations in fertility rates, yolk hormone levels and transgenerational effects on social skills. Rarely explored, our data suggest that maternal effects influence filial imprinting. These data have broad implications for laboratory, commercial systems and conservatory programs where the inevitable presence of humans could trigger maternal effects on offspring phenotype.

Keywords: maternal effects, yolk hormones, behaviour, filial imprinting

1. Introduction

Variations in environmental conditions experienced by laying female birds engender variations in yolk steroid concentrations. These variations in yolk hormonal content subsequently engender maternal effects on embryonic development as well as on offspring morphology and behaviour [1]. These nongenomic effects of parental environment drive behavioural plasticity in a way that may constrain or allow offspring to cope better with the conditions experienced by their parents [2, 3]. Yolk hormone levels of wild avian species show a strong context-dependency within species and factors such as social conditions (density, aggressive interactions, mate quality) [4-6], parasitic infection [7], predation risk [8], or food availability can influence maternal hormone production. Although the domestic chicken is the most abundant bird species on the earth, nongenomic effects of maternal environment remain rarely explored so far [9]. As any impairment of offspring's capacities to adapt to their environment (e.g. exacerbated fearfulness) can impair domestic chicks' welfare drastically, the specific environmental cues triggering maternal effects must be elucidated further.

Maternal stress during egg formation is one of the factors that could predispose chicken to less favourable behavioural phenotypes. As recently pointed out in an on-farm study, parental stress physiology correlates with offspring's fear-related behaviours and expression of damaging behaviour [10]. Variations in yolk hormone levels are thought to play a key part in these maternal effects on offspring. One possible mechanism is thought to involve maternal plasma corticosterone levels. Indeed, experimental increase of plasma corticosterone levels -mimicking a maternal stress- during egg formation decreased the

synthesis of steroid hormones which accumulate in the yolk [11]. However, moderate environmental challenges such as moderate heat also trigger variations in yolk hormone levels and engender maternal effects on offspring independently of maternal plasma corticosterone levels [12]. These results show that the physiological mechanisms at the origin of variation in yolk hormone levels remain unclear. In addition, the environmental factors triggering maternal effects are barely explored in domestic chicken. So far, housing conditions [13], unpredictable access to food [14, 15], maternal social status [16], thermal environment [12] or maternal diet [17] are all factors identified as potential sources of maternal effects mediated by yolk hormone variations in domestic chickens. These environmental factors caused modifications in growth, feeding behaviour, or emotional reactivity of the progeny [18] [15] [12] [17]. As these behaviours are implicated in chicks' capacity to adapt to their environement, deepening our understanding of maternal effects is of importance to contribute to a better management of layer breeders.

Research has aimed to understand effects of human presence on hens' fearfulness and productivity but the potential transgenerational effects engendered by human presence during egg formation have never been investigated. In most poultry production systems, due to the large size of flocks, the birds generally have very limited physical contact with the stockperson. And, fear of humans can be detrimental for welfare and productivity [19] [20, 21]. Regular visual contact of domestic chicken with humans and gentle tactile contact are largely known to effectively reduce the expression of fear related behaviours in layers or broilers [reviewed in 22]. For example, regular visual contact with a human's slow movements or static presence reduced subsequently the avoidance of the experimenter in adult layers [19] or broilers [23]. In layers, gentle daily handling and stroking facilitate chicks' habituation to human beings [24]. In broilers, the presence of a static experimenter twice a day for 10 min reduced significantly chicks subsequent fear of humans [25]. On the

contrary, when the presence of a stockperson was associated with rapid movements, avoidance behaviours increased and first-week mortality was greater [26]. These results indicate that, more than mere visual presence, humans' behaviour is an important factor in the environment of farm birds.

In the present study, we evaluated experimentally the effects of human behaviour during egg formation on variations of hens' hypothalamic–pituitary–adrenal axis activation, yolk steroid levels and offspring phenotypes. For five consecutive weeks, we exposed two groups of hens to the same duration of human presence but the humans' behaviour differed between the two groups. The first group (H+) was exposed to predictable arrival of the experimenter (i.e. announced arrival), slow movements combined with static presence, stroking during handling and human voice. Whereas the second group of hens (H-) was exposed to unpredictability (i.e. unannounced arrival of the experimenter), more rapid movements of the experimenter which remained silent, in motion, and did not provide stroking during handling. We hypothesized that the first group would habituate to human presence and their avoidance behaviours would decrease whereas the second group would not habituate and would avoid the experimenter throughout the treatment period. We expected maternal experience with a human to be a source of variation in qualities of eggs (mass and yolk hormone levels) and in their progeny subsequent behavioural phenotypes. As maternal stress is known to be associated with increase of offspring's anxiety, we expected H- hens' chicks to be more fearful. We also investigated behaviours that are rarely considered in the literature despite being key components for adaptation to the environment or social life in gregarious animals: chicks' capacities to solve a locomotor detour problem, their social motivation and social discrimination.

2. Materials and methods

2.1 Adult hens housing conditions and treatment

Thirty-six one-year old White Leghorn hens (*Gallus gallus domesticus*) from the PEAT experimental unit (INRA, Nouzilly) were split into two groups. The groups were balanced for mass of the hens. Both groups were housed in two similar 60-m² thermo-regulated rooms. For the needs of the experiment (egg identity and individual behaviour), subjects were placed in individual wire home-pens (100 cm × 100 cm × 50 cm) with wood shavings on the floor, a nest, a perch, a drinker and a trough. Cages were adjacent to one another so that all birds had tactile, visual, and vocal contacts with one another. All the birds were maintained at a temperature of 21 ±1°C for the duration of the experiment. Water and food were available *ad libitum* during a 14-h light/10-h dark cycle.

After two weeks of habituation to the room, all the hens were submitted to the same duration of human presence for 5 consecutive weeks. The experimenter spent 30 min in each room twice a day (once in the morning and once in the afternoon), 5 days per week. The same experimenter applied the treatment in both groups, recorded the behavioural observations and made the tests.

Our aim was to reduce, in the presence of the first group (H+) (N = 19), human behaviours that are known to induce fear in poultry or other farm animals like rapid movements, arm movements or unpredictability (i.e. unannounced arrival of a human) [23-26] . Each session started by the experimenter knocking at the door before entering the room. Then, during the session, the experimenter spent one minute in front of each cage and placed one hand on a wall of the cage. He also walked slowly (2 minutes to cross the room by slaloming between cages) with minimum arm movements. The experimenter was allowed to talk freely (with a normal voice) to the animals during the whole session. When present, eggs were collected gently in the cage with as little disturbance as possible. The experimenter handled each hen only once a week for weighing. They were carried under the arm, stroked for 30 s and gently placed on a scale. Our hypothesis was that this treatment would favour

habituation of hens to humans (i.e. decrease the expression of fear-related behaviours with time).

A session with the second group (H-) (N = 19), started with the experimenter entering the room without knocking at the door. Then, the experimenter spent the whole session walking fast (1 min to cross the room by slaloming between cages) moving her arms. Although our experimental conditions are not comparable to conditions in farm systems, the absence of a static human presence is common. When eggs were present, the experimenter put one leg in the cage to collect them. The experimenter remained silent during the whole session. As H+ hens, each H- hen was handled only once a week for weighing. They were carried head down and put head down in a plastic cone placed on the scale. Our expectation was that fear of the experimenter would not decrease throughout the 5 weeks of treatment. Two hens were maintained in the room but were discarded from the experiment due to irregular laying and soft eggshells (N=17 H- hens).

2.3 Hens' behaviour

To evaluate the effects of our treatment on hens' fear of the experimenter, we conducted behavioural observations the week before the treatment and at the end of the treatment (fifth week). Observations were conducted for 1 hour in each room (30 min in the morning and 30 min in the afternoon) using scan sampling. Every 2 min, the experimenter passed in front of each cage and recorded the distance of the hen to the experimenter and its activity. When a hen was in the half of the cage near the experimenter, it was recorded as "close" to the experimenter. It was recorded as "far" when it was in the other half of the cage. Behaviour was recorded using the following repertoire: maintenance (preening, dustbathing), feeding (drinking, eating), locomotion, exploring (scratching, pecking), resting (lying) and observing (standing still with head movements).

178

179 **2.4 Hens' morpho-physiological measurements**

180 Each hen was weighed 6 times: once the week before treatment started, and once a
181 week during the 5 weeks of treatment. Eggs were collected throughout the treatment and
182 laying rates were calculated as the total mean number of eggs laid per hen per day.

183 In order to evaluate chickens' HPA activity, faecal corticosterone metabolite (FCM)
184 concentrations were measured [27] [28]. At the end of the treatment, one fresh faecal
185 dropping per hen was collected between 9:00 and 11:00 a.m. from each home cage. Each
186 sample was homogenized and stored at -20°C. From each sample an aliquot (0.5 g) was
187 extracted with 60% methanol [29] and analysed by using a cortisone enzyme immunoassay
188 (EIA) validated for chickens and previously described in detail [27]. Intra- and interassay
189 coefficients of variation were below 10% and 15%, respectively.

190 **2.5 Yolk hormones and egg components**

191 Chickens' vitellogenesis lasts 8 days on average [30]. One egg per female was
192 collected at the end of the fifth week to assay yolk hormones of maternal origin. The eggs
193 were weighed and stored at -20°C for hormonal assay. Eggshells were separated, dried for
194 24h and weighed. Frozen yolks were separated from the albumen and weighed. The weight of
195 albumen was calculated by subtracting the weights of the eggshell plus yolk from that of the
196 whole egg. We then determined the ratio of each component relative to egg mass (yolk mass /
197 egg mass; albumen mass / egg mass; shell mass / egg mass) for each female. The
198 concentrations of immunoreactive progesterone, testosterone, androstenedione and œstradiol
199 were analysed by EIAs. Details of the extraction protocol are found in [31]. For a full
200 description of the assays including specific antibodies, see [32-34]. Intra- and inter-assay
201 coefficients of variation were less than 10% and 15%, respectively.

202 **2.6 Egg collection and chicks' housing conditions**

To obtain offspring, we applied artificial inseminations on weeks 4 and 5. Eggs were collected on weeks 5 and 6 for 10 consecutive days. We collected 141 eggs from the H+ group (mean = 7.4 eggs per female) and 111 eggs from the H- group (mean = 6.5 eggs per female). 222 of the 252 eggs collected were fertile and maintained in the incubator (n = 136 H+ eggs and n = 86 H- eggs). Eggs from both groups were placed in alternative rows on each shelf of the incubator. They were maintained at 37.8°C and 56% relative humidity and turned automatically and continuously. Three days before hatching, the rotation was stopped, and the temperature was decreased to 37.6°C. Eggs were then placed in a grid constructed of a wire mesh and cardboard dividers so that chicks from both the sets could be identified by treatment and mother.

We kept 98 chicks (50 H+ and 48 H-), all hatched on the 21st day of incubation. The chicks were placed in pairs (from the same treatment but different mothers) in wire-covered plastic cages (50 cm × 40 cm × 30 cm; length × width × height) with wood shavings on the floor. Cages were placed in two rooms and balanced for treatment. They were maintained under an 11h light/13h dark cycle, with water and food available *ad libitum*. All the chicks were weighed on post-hatch days 1, 11, 18, 25 and 32. Within each pair of chicks, a focal chick was chosen randomly when they were 2 days old and was tagged with a blue-coloured mark on its head. The sex of each chick was determined by comb size at 4 weeks. The H+ group included 28 females and 22 males in total, 12 females and 12 males as tagged chicks. The H- group included 28 females and 20 males in total, 16 females and 9 males as tagged chicks.

2.7 Offspring's fear of humans

To evaluate fear of humans, each pair of chicks (n = 25 pairs of H+ chicks and n = 24 pairs of H- chicks) was transported in a transport box to a test room and placed in an experimental cage that had the same features as the home cage. The experimenter placed one

hand on an internal wall of the cage for three minutes and recorded the position of the tagged chick in the cage by scan sampling every 10s. To determine the position within the cage, the cage was divided virtually into two zones of equal surface: close zone (i.e. close to the hand) and distant zone. Fear of humans was evaluated on post-hatch day 3.

2.8 Food and object neopobia

Fear of novel food and of novel objects were assessed following protocols previously described [35] [36]. Each test was performed at the same age for all chicks (8 and 9 days old respectively). Each test was run for 180 seconds. Because chicks become distressed when they are socially isolated, we tested cage mates together (n = 25 pairs of H+ chicks and n = 24 pairs of H- chicks). Tests were performed in a test room but in an experimental cage that had the same features as their home cage. Testing started 90 minutes after the feeder had been removed from the home cage. Pairs were deposited in an opaque enclosure within the test cage, opposite to the feeding trough. After 30 seconds, the enclosure was removed, and an unseen observer, blind to the treatment, recorded the behaviour of the marked focal chick of each pair. Latency to eat (the moment swallowing was observed) and time spent eating were recorded. On post-hatch day 7, chicks were familiarized with the test cage and handling procedure. Their home cage feeding trough was placed in the test cage, filled with their usual food. This familiarization procedure was also used to control for food motivation. Food neophobia was tested on post-hatch day 8 with their feeding trough filled with millet seeds. Object neophobia was evaluated on post-hatch day 9, the novel object was an unfamiliar coloured feeder (yellow and green plastic instead of grey metal) containing their familiar food.

2.9 Open-field test

The chicks were individually (n = 50 H+ chicks and n = 48 H- chicks) tested in a novel open environment (open-field). This test is commonly used to assess fear of novel environments and reactions to separation from conspecifics [37]. Each chick was placed in the middle of an open arena (120 cm diameter) for 5 minutes. To assess their locomotor activity, two perpendicular lines were drawn in the arena, dividing the space into four equal parts. Latency of first step, number of times a subject crossed a line, latency of first distress call and number of distress calls were recorded by an unseen experimenter, blind to the treatment. This test was conducted on post-hatch days 15 and 16.

2.10 Detour task

This test was performed on all tagged chicks (n = 25 H+ chicks and n = 24 H- chicks) in a rectangular arena measuring (80 cm x 60 cm x 31 cm). For each pair, the cage mate was placed in a wire mesh goal cage (27 cm x 20 cm x 31 cm), placed at the opposite side of a starting point. The test chick was placed 30 cm away in a U-shape barrier with a wire-mesh front wall and two opaque, vertical sidewalls. To solve the problem, the chick had to move away from its cage mate, lose sight of it and go round one end of the barrier. An unseen experimenter, blind to the treatment, recorded latency to make the detour (the chick crosses the barrier with the whole body) from the start location. This test was carried out on post-hatch day 10 with a maximum duration of 600s.

2.11 Social discrimination

We evaluated the capacity of all tagged chicks of each pair (n = 25 H+ chicks and n = 24 H- chicks) to discriminate between two conspecifics. We used a simultaneous two-choice test paradigm following the protocol previously described [38] [36]. This test was performed in a rectangular arena measuring (80 cm x 60 cm x 31 cm : length x width x height). Two stimulus birds were each placed in a 27 cm x 20 cm x 31 cm compartment with a wire mesh top and

front at the opposite sides of a starting point. One of these compartments contained its familiar cage-mate and the other compartment contained an unfamiliar chick subjected to the same treatment (same age as the test chick). A "close zone" was delineated in front of each cage (14 x 27 cm). Sides were counterbalanced between trials and treatments were alternated between trials. After 30 seconds, the test bird was released, and time spent in each close zone was recorded during a five-minute period. This test was carried out on post-hatch day 19.

2. 12 Social motivation

To assess social motivation, runway tests were conducted on all tagged chicks (n = 25 H+ chicks and n = 24 H- chicks). The apparatus was a straight 145 cm-long wire-mesh tunnel with a goal cage at the end of the tunnel where the subject's cage mate was placed. The tunnel was divided into three zones of equal size: 'non-social' (far from the conspecific), 'middle' and 'social' (close to the conspecific) zones. Each pair of chicks was transferred to the test room. Then, the cage mate was placed in the goal cage, and the test chick was placed in the middle zone. The side with the social stimulus was counterbalanced between trials. An unseen experimenter, blind to the treatment, recorded time spent in each zone during 5-minute (beginning after the subject had taken its first step). This test was carried out on post-hatch day 20.

3. Statistics

The masses of adult hens were compared between treatments by using a one way repeated-measures ANOVA. Even after transformation, the behavioural data were not normally distributed (Shapiro-Wilk test) and did not have the homogeneity of variances (Levene tests) required to apply parametric statistics. Wilcoxon tests with Monte-Carlo simulations were used, within groups, to compare frequencies of behaviours between before and after the treatment. Mann-Whitney *U*-tests with Monte-Carlo simulations were used for intergroup

comparisons on laying rates, corticosterone metabolite concentrations, and parameters recorded on eggs (masses, yolk hormone concentrations). We compared fertility rates and numbers of chicks hatched from fertile eggs by using Chi-square tests. The masses of chicks were compared by using a two-way repeated-measures ANOVA with treatment and sex as factors. Analyses were performed with XLSTATS 2016.2 (Addinsoft) with significance accepted at $P \leq 0.05$. Even after transformation, chicks' behavioural data were not normally distributed and did not have the homogeneity of variances required to apply parametric statistics. We used the function aovp of the lmPerm package in R 3.4.2 to run permutation tests with treatment, sex, and the interaction treatment*sex as fixed factors with significance accepted at $P \leq 0.05$. Analyses of variance were conducted for intergroup comparisons on all behavioural parameters recorded during neophobia tests (food, object, environment), during the detour task and runway tests. In the runway tests, we compared the proportions of time spent in the social zone (time spent in the social zone / 300 seconds). For the discrimination tests, we compared the total time spent in the social zone (time spent close to the familiar + time spent close to the unfamiliar conspecific). And, within each group we compared times spent close to the familiar conspecific to times spent close to the unfamiliar conspecific using Wilcoxon tests with Monte-Carlo simulations.

4. Ethics statement

All birds were maintained at the Experimental Unit PEAT of INRA (Nouzilly, France, license number B-37-175-1). All the experiment was approved by the Ethics Committee for Animal Experimentation of Val de Loire, CEEA Vdl (reference number 02153.02) and was performed in accordance with the European Communities Council Directive 2010/63/UE. All animals were sold for rehoming at the end of the experiment.

5. Results

5. 1 Behaviour of hens

H+ hens' behaviour differed significantly between before and after the treatment. The numbers of scans with hens observed close to the experimenter increased significantly between before and after the treatment (15.95 ± 2.93 vs. 23.22 ± 2.19 scans, z = -2.58, P < 0.01) and they were observed significantly more frequently feeding, exploring and resting and less in observation after than before the treatment (Table 1). Neither the numbers of scans with H- hens close to the experimenter (18.13 ± 2.86 vs. 14.87 ± 2.78 scans, z = -1.44, P = 0.15) nor their behaviour differed significantly between before and after the treatment (Table 1).

[table 1 approximately here]

5. 2 Morpho-physiological measurements on hens

No significant effects of the treatment on the mass of hens were found throughout the treatment (treatment effect, $F_{1,31} = 1.38$, $P = 0.25$). The masses of hens of both groups, increased significantly throughout the treatment (time effect $F_{1,5} = 8.03$, $P < 0.01$; treatment x time effect, $F_{5,155} = 0.58$, $P = 0.71$) (Table 2).

[table 2 approximately here]

Mean laying rates did not differ significantly between H+ and H- hens (0.69 ± 0.03 egg per day vs. 0.73 ± 0.04 egg per day, U = 104.50, P = 0.27).

At the end of the treatment, faecal corticosterone metabolite levels did not differ significantly between H+ and H- hens (197 ± 32 ng/g *vs.* 225 ± 29 ng/g, U = 124, P = 0.34).

5. 3 Egg characteristics

The masses of eggs and egg constituents did not differ significantly between H+ and H- hens (Table 3).

346 [table 3 approximately here]

347 We found an effect of the treatment on yolk hormone levels. Significantly, higher
348 concentrations of yolk progesterone and œstradiol were found in H+ hens' eggs than in H-
349 hens eggs (Fig. 1). Concentrations of yolk testosterone and androstenedione did not differ
350 significantly between the two groups (Fig. 1).

351 [Fig.1 approximately here]

352 **5.4 Hatching success and growth of chicks**

353 After insemination, the number of H+ hens fertile eggs was significantly higher (136 out of
354 141 eggs) than that of H- hens (86 out of 111 eggs) (Chi-square = 16.57, $P < 0.001$). The
355 numbers of hatched chicks did not differ significantly between H+ (120 out of 136 eggs) and
356 H- eggs (71 out of 86 eggs) (Chi-square = 0.10, $P = 0.74$).

357 Whatever their age, masses did not differ significantly between H+ and H- chicks (treatment
358 effect: $F_{1,45} = 0.56$, $P = 0.46$) (Table 4). There was an effect of sex, with the mass of males
359 being higher than the mass of females (sex effect: $F_{1,45} = 7.09$, $P = 0.01$) and, no interaction
360 between treatment and sex (treatment*sex effect: $F_{1,45} = 0.38$, $P = 0.54$).

361 [table 4 approximately here]

362 **5. 6 Offspring fear of humans.**

363 The reactivity to humans test data showed that the numbers of scans when chicks were close
364 to the experimenter's hand did not differ significantly between H+ and H- chicks (6.20 ± 1.09
365 scans and 7.00 ± 1.09 scans respectively; treatment effect: Mean Square(MS) = 4.96, $P =$
366 0.84; sex effect: 5.71 ± 0.99 scans for females *vs.*7.76 ± 1.19 scans for males, MS = 44.92, P
367 = 0.23; treatment*sex effect: MS = 9.01, $P = 0.71$).

368 **5. 7 Offspring neophobia and open-field tests.**

Similarly, no significant differences were found between H+ and H- chicks for the neophobia (food and object) or novel environment tests (Table 5: Food neophobia: latency to eat, treatment effect: MS = 2234.81, $P = 0.19$; sex effect: 22.86 ± 6.41 s for females *vs.* 17.90 ± 8.22 s for males, MS = 104.57, $P = 0.98$; treatment*sex effect: MS = 74.49, $P = 0.71$; time spent eating, treatment effect: MS = 289, $P = 1$; sex effect: 65.43 ± 7.97 s for females *vs.* 65.76 ± 7.97 s for males, MS = 1.65, $P = 0.98$; treatment*sex effect: MS = 1127.04, $P = 0.36$; Object neophobia: latency to eat, treatment effect: MS = 1622.9, $P = 0.53$; sex effect: 125.14 ± 13.13 s for females *vs.* 106.71 ± 14.24 s for males, MS = 3298, $P = 0.29$; treatment*sex effect: MS = 2063.8, $P = 0.30$; time spent eating, treatment effect: MS = 1234.2, $P = 0.37$; sex effect: 25.07 ± 6.64 s for females *vs.* 38.47 ± 8.65 s for males, MS = 1676.1, $P = 0.28$; treatment*sex effect: MS = 1092.3, $P = 0.25$; Novel environment: latency of first step, treatment effect: MS = 1570.08, $P = 0.10$; sex effect: 34.98 ± 4.54 s for females *vs.* 37.59 ± 5.16 s for males, MS = 141.44, $P = 0.52$; treatment*sex effect: MS = 3.30, $P = 0.98$; number of lines crossed, treatment effect: MS = 2.56, $P = 0.82$; sex effect: 5.61 ± 0.72 for females *vs.* 5.12 ± 0.91 for males, MS = 5.36, $P = 0.47$; treatment*sex effect: MS = 22.03, $P = 0.27$; latency to distress call, treatment effect: MS = 12.66, $P = 0.44$; sex effect: 18.37 ± 2.11 s for females *vs.* 15.36 ± 1.03 s for males, MS = 213.44, $P = 0.42$; treatment*sex effect: MS = 11.45, $P = 0.96$; number of calls, treatment effect: MS = 2874.6, $P = 0.62$; sex effect: 212.39 ± 11.01 for females *vs.* 241.36 ± 10.90 for males, MS = 19928, $P = 0.11$; treatment*sex effect: MS = 6451.3 $P = 0.40$).

[table 5 approximately here]

5.8 Detour task

Latencies to go round the barrier did not differ significantly between H+ and H- chicks (294.40 ± 51.43 s and 274.87 ± 47.31 s respectively, treatment effect, MS = 30036, $P = 0.49$) Latencies were significantly longer for males (383.76 ± 52.75 s) than for females (210.64 ±

41.45 s) (sex effect: MS = 382307, $P = 0.03$) and there was no significant interaction between treatment and sex (treatment*sex effect: MS = 20009, $P = 0.62$).

5. 9 Social discrimination and social motivation

Total times spent close to conspecifics (time spent close to the familiar + time spent close to the unfamiliar conspecific) in the social discrimination test did not differ significantly between H+ and H- chicks (137.60 ± 11.81 s and 123.91 ± 9.24 s respectively, treatment effect: MS = 1329.64, $P = 0.31$; sex effect: 134.53 ± 10.42 s for females *vs.* 126.05 ± 10.94 s for males,MS = 570.87, $P = 0.72$; treatment*sex effect: MS = 2575.56, $P = 0.62$).

H+ chicks spent significantly more time close to their familiar conspecific than to the unfamiliar one (Fig. 2). The times H- chicks spent close to the familiar or unfamiliar conspecific did not differ significantly.

[Fig.2 approximately here]

Latencies to reach their cage mate in the runway test did not differ significantly between H+ and H- chicks (24.00 ± 7.51 s and 9.00 ± 2.66 s respectively, treatment effect: MS = 3404.2, $P = 0.09$; sex effect: 14.64 ± 6 s for females *vs.* 19.33 ± 5.55 s for males, MS = 596.2, $P = 0.33$; treatment*sex effect: MS = 624.4, $P = 0.36$). The proportions of time spent in the social zone did not differ significantly between H+ and H- chicks (0.83 ± 0.03 and 0.89 ± 0.03 respectively, treatment effect: MS = 0.05, $P = 0.21$; sex effect: 0.84 ± 0.04 for females *vs.* 0.88 ± 0.03 for males, MS = 0.01, $P = 0.88$; treatment*sex effect: MS = 0.07, $P = 0.11$).

6. Discussion

In this study we show that the quality of human presence during egg formation induced variations in yolk hormonal levels and that the capacity to discriminate in the offspring was plastic. H- hens laid eggs with significantly lower yolk progesterone and œstradiol levels

417 compared to H+ hens. And, contrary to H+ chicks, H- chicks did not discriminate between a
418 familiar and an unfamiliar conspecific.

419 Visual contact with humans can elicit behavioural withdrawal and violent escape reactions in
420 poultry, often with associated injury as well as negative impacts on egg production [39] [40]
421 [41]. As some degree of contact between poultry and humans is inevitable, many studies have
422 focused on ways to decrease the expression of fear-related behaviours. Although the treatment
423 we applied (experimenter standing still in front of each hen) would not be applicable in
424 commercial systems, our study adds support to previous findings showing that regular
425 presence associated with static moments and gentle handling is effective in reducing domestic
426 chickens' fear of humans [23] [19] [25] [42]. Indeed, after five weeks of treatment, H+ hens
427 expressed less avoidance of the experimenter than before the treatment. In addition, they were
428 observed more frequently feeding, exploring, observing or resting in the presence of the
429 experimenter after the treatment than before. As fearfulness inhibits exploration, feeding
430 behaviour or resting [43], our data show that the hens were less fearful of the experimenter at
431 the end of the treatment than before. This means that the hens' behaviour is still plastic in
432 adulthood and that visual human presence associated with predictable approach, human voice,
433 static moments, slow movements and gentle handling for weighing were effective in inducing
434 habituation to humans. As expected, we found no differences in H- hens' distance to the
435 experimenter and behaviour between before and after the treatment, showing that they had not
436 habituated to the presence of the experimenter. With our experimental setting the factors
437 contributing to hens' habituation to humans could not be disentangled. Additional studies are
438 required to determine whether a specific human behaviour (e.g. motion) would be as effective
439 as a combination of behaviours (e.g. static presence and gentle handling) in regulating fear of
440 humans. Using non-human artificial stimuli (by robots) may help deciphering the importance
441 of specific sensory stimulation (e.g. visual movements).

442 We observed no effects of the treatment on basal corticosterone levels, egg laying
443 rates, egg quality (mass of eggs and of the different components) or hatchlings' mass and
444 growth of chicks. Environmental stress can induce HPA axis activation in birds, including
445 chickens, causing a decrease in egg and offspring weights [44] [45] [46]. Elevated
446 corticosterone levels due to a corticosterone implant also reduce hens' egg mass, yolk mass
447 and hatchlings' weights [45, 47]. The absence of effects of our treatment on these parameters
448 suggests that the presence of the experimenter twice a day for 30 min may not have been
449 sufficiently stressful to induce modification of the regulation of H- hens' HPA axis and
450 subsequently engender deleterious effects on eggs. Similarly, 30-min sessions of visual
451 contact with humans three days/week was found to reduce broiler chickens' avoidance of
452 humans without affecting production parameters [23]. Our hens were probably already
453 habituated to human presence since they were exposed to humans before entering our
454 experiment. Albeit H- hens still avoided the experimenter, it is possible that we obtained a
455 difference in habituation level between the two groups with H+ hens that were more
456 habituated than H- hens. However, we found a clear effect of our treatment on fertility rates.
457 H- hens' fertility rate was lower (77%) than that of H+ hens (96%). The insemination
458 procedure was very rapid for all the hens (few seconds), but required some handling. This
459 procedure could possibly have been more stressful for H- hens than for H+ hens. Not recorded
460 in our study, the presence of stress-induced defecations after handling may have reduced the
461 success of insemination of H- hens.

462 We observed significant differences of yolk hormone levels between H+ and H- hens.
463 H+ hens' eggs had significantly higher yolk progesterone and œstradiol concentrations than
464 H- hens' eggs. Previously we observed that exposure to a moderate heat challenge
465 significantly increased hens' yolk progesterone, testosterone, and œstradiol levels [12]. The
466 quality of polyunsaturated acids in hens' diet modulated concentrations of yolk progesterone,

androstenedione, and œstradiol [17]. Natt et al. [30] reported an increase in yolk œstradiol in the eggs of hens exposed to unpredictable access to food (unpredictable diurnal light rhythm). In addition, yolk androstenedione and œstradiol levels were found to be higher in floor-housed hens than in to caged hens [13]. All these results show that yolk hormone levels are affected differently depending on maternal environment. Not always assayed in the aforementioned studies, progesterone is produced in the granulosa cells of the pre-ovary follicles and is the precursor of androgens and œstradiol [48]. This hormone is present in much higher amounts than androgens in egg yolk [49] [32]. At the present stage, the interpretation of the mechanisms that mediate variations of yolk hormone levels is bound to be speculative. The regulatory mechanism for the production of yolk hormones might be at the level of the production of the follicular wall of the ovary or at the enzymatic level with more or less conversion of progesterone by side-chain cleavage. Our treatment could possibly have affected circulating hormones other than glucocorticoids like circulating prolactin or gonadotropins (luteinizing hormone, LH; follicular stimulating hormone, FSH). These hormones are related to ovarian function and their levels are known to vary when females are exposed to environmental challenges [50] [51] [52]. Although disparate, all the studies conducted so far point out that, despite selection and domestication, laying hens remain sensible to their environmental conditions. Previously we found higher concentrations of yolk testosterone and androstenedione in Japanese quail's (*Coturnix coturnix japonica*) eggs of females habituated to humans compared to females not exposed to humans [53]. Our present data comfort the hypothesis that the human-animal relationship during egg laying is at the origin of variation in yolk hormone levels in farm birds.

Times spent close to conspecifics in the runway and the discrimination tests did not differ significantly between H+ and H- chicks. This result shows that their motivation to seek the proximity of conspecifics did not differ significantly between H+ and H- chicks. However, in

the discrimination test, H+ chicks clearly preferred their familiar cage mate to the stranger whereas H- chicks showed no preference. The preference showed by H+ chicks corresponds to a pattern previously observed in young Leghorn chicks [36]. In addition, the capacity of domestic chicks to discriminate between strangers and familiar conspecifics and their preference for familiar companions is well documented, even in day-old chicks [54]. This preference for a familiar stimulus is explained by filial imprinting, the process by which chicks learn the characteristics of a stimulus and acquire a social preference for it [55] [56] [57]. Filial imprinting is crucial for young precocial birds that have to recognize their mother and flock members as soon as they hatch. As H+ and H- chicks were maintained in the same post-hatch environment, the absence of the typical preference for the familiar conspecific in H- chicks suggests that differences in the maternal environment and yolk hormone levels might be involved. Our results are in line with another study showing an absence of preference in chicks prenatally exposed to an experimental increase in yolk corticosterone levels [58]. Although speculative, yolk hormones could possibly have organizational or activational effects on neuronal circuits involved in the treatment of sensory information and memory. Increases in yolk progesterone levels were found to alter Bobwhite quail's (*Colinus virginiatus*), prenatal auditory learning of a maternal call [59], whereas increases in yolk testosterone levels were found to facilitate auditory learning [60]. In young songbirds that have to learn their song from adult tutors, œstradiol and testosterone levels in plasma or in the forebrain are known to play a key role in the consolidation of tutor song memories [61] [62]. The treatment applied to H- hens may have impaired the capacity of chicks to recognize their familiar cage mate. Encounters with strangers are stressful for chicks and may favour the expression of feather pecking [63], our study thus calls attention to the necessity to deepen our understanding of maternal effects on domestic chickens' social behaviours.

Contrarily to our expectation, our treatment did not affect chicks' fear of humans. When exposed to a human hand, no significant differences could be evidenced for any of the parameters observed between H+ and H- chicks. Although changes in the H+ hens' behaviour towards humans were observed, this adaptation to their environment was not transmitted to their offspring. According to the Predictive adaptive response hypothesis, early experience is a source of developmental plasticity that should be adaptive to the environmental conditions encountered later in life [64]. And, as demonstrated by Nätt et al. [15], behavioural adaptations of the parental population of domestic chickens can be transmitted to their offspring via maternal effects. As mentioned above, differences in the quality of the presence of the experimenter may not have been sufficiently stressful to engender transmission of adaptive plasticity to the offspring. The moderate intensity of our treatment could also explain the lack of differences between H+ and H- chicks'fear of novelty and cognitive abilities. Indeed, wild birds' neophobia appears to be plastic and more frequent in individuals experiencing high-risk environments [65]. Domestic chickens' neophobia (of food, objects, environment) and their capacity to perform a detour task were found to be plastic and influenced by their prehatch environment [35, 36]. More generally, Galliforms' fearfulness is commonly known to be regulated by maternal effects [31, 53, 66, 67]. Differences in the duration, intensity, nature of maternal stress and in the modifications engendered in egg quality might explain the discrepancies observed.

6.1 Conclusion

To conclude, more than duration of human presence, our study shows that the behaviour of the caretaker plays an important role in the environment of hens during egg formation. In addition to the welfare of hens, the human-animal relationship influenced yolk hormone levels and probably construction of offspring's crucial social skills like the capacity to discriminate between familiar and unfamiliar conspecifics. Additional studies are now required to

investigate the mechanisms mediating maternal effects. These results have broad implications for laboratory, commercial systems and conservatory programs where human-animal relationships can affect egg quality and the subsequent phenotypes of offspring.

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References

[1] Houdelier, C., Pittet, F., Guibert, F., De Margerie, E., Lumineau, S. Non-genetic Inheritance in Birds: transmission of behaviour from mother to offspring. Non-Genetic Inheritance. 2013,1:62-8.https://doi.org/10.1093/eep/dvy008

[2] Burton, T., Metcalfe, N. B. Can environmental conditions experienced in early life influence future generations? Proc. R. Soc. Lond. B. Biol. Sci. 2014,281.https://doi.org/10.1098/rspb.2014.0311

[3] Mousseau, T. A., Fox, C. W. Maternal effects as adaptations. New York: Oxford University Press; 1998.

[4] Pilz, K. M., Smith, H. G. Egg yolk androgen levels increase with breeding density in the European Starling, *Sturnus vulgaris*. Funct. Ecol. 2004,18:58-66.https://doi.org/10.1111/j.1365-2435.2004.00811.x

[5] Schwabl, H. The contents of maternal testosterone in house sparrow Passer domesticus eggs vary with breeding conditions. Naturwissenschaften. 1997,84:406-8.

[6] Kingma, S. A., Komdeur, J., Vedder, O., von Engelhardt, N., Korsten, P., Groothuis, T. G. G. Manipulation of male attractiveness induces rapid changes in avian maternal yolk androgen deposition. Behav. Ecol. 2009,20:172-9.https://doi.org/10.1093/beheco/arn130

[7] Müller, W., Heylen, D., Eens, M., Rivera-Gutierrez, H. F., Groothuis, T. G. G. An experimental study on the causal relationships between (ecto-)parasites, testosterone and sexual signalling. Behav. Ecol. Sociobiol. 2013,67:1791-8.https://doi.org/10.1007/s00265-013-1586-6

[8] Coslovsky, M., Groothuis, T., de Vries, B., Richner, H. Maternal steroids in egg yolk as a pathway to translate predation risk to offspring: Experiments with great tits. Gen. Comp. Endocrinol. 2012,176:211-4.https://doi.org/10.1016/j.ygcen.2012.01.013

[9] Dixon, L., Sparks, N., Rutherford, K. Early experiences matter: a review of the effects of prenatal environment on offspring characteristics in poultry. Poult. Sci. 2016,95:489-99.https://doi.org/10.3382/ps/pev343

[10] de Haas, E. N., Bolhuis, J. E., Kemp, B., Groothuis, T. G., Rodenburg, T. B. Parents and early life environment affect behavioral development of laying hen chickens. PLoS ONE. 2014,9:e90577.doi:10.1371/journal.pone.0090577

[11] Henriksen, R., Rettenbacher, S., Groothuis, T. G. G. Prenatal stress in birds: Pathways, effects, function and perspectives. Neurosci. Biobehav. Rev. 2011,35:1484-501.https://doi.org/10.1016/j.neubiorev.2011.04.010

[12] Bertin, A., Chanson, M., Delaveau, J., Mercerand, F., Möstl, E., Calandreau, L., et al. Moderate heat challenge increased yolk steroid hormones and shaped offspring growth and behavior in chickens. PLoS ONE. 2013,8:e57670.https://doi.org/10.1371/journal.pone.0057670

[13] Janczak, A. M., Torjesen, P., Rettenbacher, S. Environmental effects on steroid hormone concentrations in laying hens' eggs. Acta. Agric. Scand. Sect. A Anim. Sci. 2009,59:80-4.https://doi.org/10.1080/09064700903023348

[14] Janczak, A. M., Torjesen, P., Palme, R., Bakken, M. Effects of stress in hens on the behaviour of their offspring. Appl. Anim. Behav. Sci. 2007,107:66-77.doi:10.1016/j.applanim.2006.09.016

[15] Nätt, D., Lindqvist, N., Stranneheim, H., Lundeberg, J., Torjesen, P. A., Jensen, P. Inheritance of Acquired Behaviour Adaptations and Brain Gene Expression in Chickens. PLoS ONE. 2009,4:e6405.doi:10.1371/journal.pone.0006405

[16] Muller, W., Eising, C. M., Dijkstra, C., Groothuis, T. G. G. Sex differences in yolk hormones depend on maternal social status in Leghorn chickens (Gallus gallus domesticus). Proc. R. Soc. Lond. B. Biol. Sci. 2002,269:2249-55.doi:10.1098/rspb.2002.2159
[17] De Haas, E. N., Calandreau, L., Baéza, E., Chartrin, P., Palme, R., Darmaillacq, A. S., et al. Lipids in maternal diet influence yolk hormone levels and post- hatch neophobia in the domestic chick. Dev. Psychobiol. 2017,59:400-9.https://doi.org/10.1002/dev.21504
[18] Goerlich, V. C., Nätt, D., Elfwing, M., Macdonald, B., Jensen, P. Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. Horm. Behav. 2012,61:711-8.https://doi.org/10.1016/j.yhbeh.2012.03.006
[19] Barnett, J., Hemsworth, P., Hennessy, D., McCallum, T., Newman, E. The effects of modifying the amount of human contact on behavioural, physiological and production responses of laying hens. Appl. Anim. Behav. Sci. 1994,41:87-100.https://doi.org/10.1016/0168-1591(94)90054-X
[20] Hemsworthlt, P., Barnett, J., Coleman, G. The human-animal relationship in agriculture and its consequences for the animal. Anim Welf. 1993,2:33-51.
[21] Waiblinger, S., Boivin, X., Pedersen, V., Tosi, M.-V., Janczak, A. M., Visser, E. K., et al. Assessing the human–animal relationship in farmed species: a critical review. Appl. Anim. Behav. Sci. 2006,101:185-242.https://doi.org/10.1016/j.applanim.2006.02.001
[22] Zulkifli, I. Review of human-animal interactions and their impact on animal productivity and welfare. J. Anim. Sci. Biotechnol. 2013,4:25-.https://doi.org/10.1186/2049-1891-4-25
[23] Silvera, A., Wallenbeck, A., Butterworth, A., Blokhuis, H. Modification of the human–broiler relationship and its potential effects on production. Acta Agr. Scand. A. Anim. Sci. 2016,66:161-7.https://doi.org/10.1080/09064702.2017.1286379
[24] Jones, R. B., Waddington, D. Modification of fear in domestic chicks, gallus-gallus-domesticus, via regular handling and early environmental enrichment. Anim. Behav. 1992,43:1021-33.https://doi.org/10.1016/S0003-3472(06)80015-1
[25] Zulkifli, I., Gilbert, J., Liew, P., Ginsos, J. The effects of regular visual contact with human beings on fear, stress, antibody and growth responses in broiler chickens. Appl. Anim. Behav. Sci. 2002,79:103-12.https://doi.org/10.1016/S0168-1591(02)00135-1
[26] Cransberg, P., Hemsworth, P., Coleman, G. Human factors affecting the behaviour and productivity of commercial broiler chickens. Br. Poult. Sci. 2000,41:272-9.https://doi.org/10.1080/713654939
[27] Rettenbacher, S., Mostl, E., Hackl, R., Ghareeb, K., Palme, R. Measurement of corticosterone metabolites in chicken droppings. Br. Poult. Sci. 2004,45:704-11.doi:10.1080/00071660400006156
[28] Palme, R. Non-invasive measurement of glucocorticoids: Advances and problems. Physiol. Behav. 2019,199:229-43.https://doi.org/10.1016/j.physbeh.2018.11.021
[29] Palme, R., Touma, C., Arias, N., Dominchin, M., Lepschy, M. Steroid extraction: get the best out of faecal samples. Wien Tierärztl. Mschr. 2013,100:238-46.
[30] Lacassagne, L. Etude comparée des réserves vitellines et de la durée de la phase de grand accroissement de l'ovocyte chez la poule domestique. Influence de l'âge de l'animal et de la longueur de la série. Ann. Zootech. 1960,9:85-96.
[31] Guesdon, V., Bertin, A., Houdelier, C., Lumineau, S., Formanek, L., Kotrschal, K., et al. A Place to Hide in the Home-Cage Decreases Yolk Androgen Levels and Offspring Emotional Reactivity in Japanese Quail. PLoS ONE. 2011,6:e23941.doi:10.1371/journal.pone.0023941
[32] Mostl, E., Spendier, H., Kotrschal, K. Concentration of immunoreactive progesterone and androgens in the yolk of hens' eggs (*Gallus domesticus*). Wien Tierärztl. Mschr. 2001,88:62-5.

[33] Palme, R., Mostl, E. Biotin-Streptavidin enzyme-immunoassay for the determinant of estrogens and androgens in boar feces. Adv. Steroid. Anal. 1994:111-7.
[34] Hirschenhauser, K., Mostl, E., Kotrschal, K. Seasonal patterns of sex steroids determined from feces in different social categories of greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 1999,114:67-79.doi: 10.1006/gcen.1998.7236
[35] Bertin, A., Arnould, C., Moussu, C., Meurisse, M., Constantin, P., Leterrier, C., et al. Artificially Increased Yolk Hormone Levels and Neophobia in Domestic Chicks. Animals. 2015,5:1220-32.https://doi.org/10.3390/ani5040408
[36] Bertin, A., Calandreau, L., Meurisse, M., Georgelin, M., Palme, R., Lumineau, S., et al. Incubation temperature affects the expression of young precocial birds' fear-related behaviours and neuroendocrine correlates. Sci. Rep. 2018,8:1857.doi:10.1038/s41598-018-20319-y
[37] Forkman, B., Boissy, A., Meunier-Salaün, M. C., Canali, E., Jones, R. B. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. Physiol. Behav. 2007,92:340-74.
[38] Schweitzer, C., Poindron, P., Arnould, C. Social motivation affects the display of individual discrimination in young and adult Japanese quail (Coturnix japonica). Dev. Psychobiol. 2009,51:311-21.https://doi.org/10.1002/dev.20370
[39] Jones, R. B., Faure, J. M. The effects of regular handling on fear responses in the domestic chick. Behav. Processes. 1981,6:135-43.https://doi.org/10.1016/0376-6357(81)90032-2
[40] Suarez, S. D., Gallup Jr, G. G. Emotionality and fear in birds: A selected review and reinterpretation. Bird Behav. 1983,5:22-30.
[41] Hemsworth, P., Barnett, J., Coleman, G. J., Hansen, C. A study of the relationships between the attitudinal and behavioural profiles of stockpersons and the level of fear of humans and reproductive performance of commercial pigs. Appl. Anim. Behav. Sci. 1989,23:301-14.https://doi.org/10.1016/0168-1591(89)90099-3
[42] Jones, R. B. Reduction of the domestic chicks fear of human-beings by regular handling and related treatments. Anim. Behav. 1993,46:991-8.https://doi.org/10.1006/anbe.1993.1280
[43] Jones, R. B. Fear and adaptability in poultry: Insights, implications and imperatives. World's Poult. Sci. J. 1996,52:131-74.https://doi.org/10.1079/WPS19960013
[44] Hayward, L. S., Wingfield, J. C. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen. Comp. Endocrinol. 2004,135:365-71.https://doi.org/10.1016/j.ygcen.2003.11.002
[45] Henriksen, R., Groothuis, T. G., Rettenbacher, S. Elevated plasma corticosterone decreases yolk testosterone and progesterone in chickens: linking maternal stress and hormone-mediated maternal effects. PLoS ONE. 2011,6:e23824.https://doi.org/10.1371/journal.pone.0023824
[46] Hsu, B. Y., Dijkstra, C., Darras, V. M., de Vries, B., Groothuis, T. G. G. Maternal adjustment or constraint: differential effects of food availability on maternal deposition of macronutrients, steroids and thyroid hormones in rock pigeon eggs. Ecol. Evol. 2016,6:397-411.doi:10.1002/ece3.1845
[47] Henriksen, R., Rettenbacher, S., G.G. Groothuis, T. Maternal corticosterone elevation during egg formation in chickens (Gallus gallus domesticus) influences offspring traits, partly via prenatal undernutrition. Gen. Comp. Endocrinol. 2013,191:83-91.http://dx.doi.org/10.1016/j.ygcen.2013.05.028
[48] Huang, E. S., Nalbandov, A. V. Steroidogenesis of chicken granulosa and theca cells: in vitro incubation system. Biol. Reprod. 1979,20:442-53.10.1095/biolreprod20.3.442
[49] Hackl, R., Bromundt, V., Daisley, J., Kotrschal, K., Mostl, E. Distribution and origin of steroid hormones in the yolk of Japanese quail eggs (*Coturnix coturnix japonica*). J. Comp.

713 Physiol. B-Biochem. Syst. Environ. Physiol. 2003,173:327-31.doi:10.1007/s00360-003-0339-
714 7
715 [50] Rozenboim, I., Mobarky, N., Heiblum, R., Chaiseha, Y., Kang, S., Biran, I., et al. The
716 role of prolactin in reproductive failure associated with heat stress in the domestic turkey.
717 Biol. Reprod. 2004,71:1208-13.https://doi.org/10.1095/biolreprod.104.028167
718 [51] Johnson, A. Comparison of three serial blood sampling techniques on plasma hormone
719 concentrations in the laying hen. Poult. Sci. 1981,60:2322-
720 7.https://doi.org/10.3382/ps.0602322
721 [52] El Halawani, M., Silsby, J., Fehrer, S., Behnke, E. The influence of acute or repeated
722 immobilization on plasma prolactin levels in the turkey (Meleagris gallopavo). Gen. Comp.
723 Endocrinol. 1985,59:410-5.https://doi.org/10.1016/0016-6480(85)90398-3
724 [53] Bertin, A., Richard-Yris, M. A., Houdelier, C., Lumineau, S., Mostl, E., Kuchar, A., et al.
725 Habituation to humans affects yolk steroid levels and offspring phenotype in quail. Horm.
726 Behav. 2008,54:396-402.doi:10.1016/j.yhbeh.2008.04.012
727 [54] Zajonc, R. B., Wilson, W. R., Rajecki, D. W. Affiliation and social discrimination
728 produced by brief exposure in day-old domestic chicks. Anim. Behav. 1975,23:131-
729 8.https://doi.org/10.1016/0003-3472(75)90059-7
730 [55] Bateson, P. P. G. The characteristics and context of imprinting. Biological Reviews.
731 1966,41:177-217.
732 [56] Bolhuis, J. J. Mechanisms of avian imprinting: a review. Biological Reviews.
733 1991,66:303-45.
734 [57] Horn, G. Pathways of the past: the imprint of memory. Nat. Rev. Neurosci.
735 2004,5:108.https://doi.org/10.1038/nrn1324
736 [58] Nordgreen, J., Janczak, A. M., Bakken, M. Effects of prenatal exposure to corticosterone
737 on filial imprinting in the domestic chick, Gallus gallus domesticus. Anim. Behav.
738 2006,72:1217-28.http://dx.doi.org/10.1016/j.anbehav.2006.02.025
739 [59] Herrington, J., Vallin, C., Lickliter, R. Increased yolk progesterone interferes with
740 prenatal auditory learning and elevates emotional reactivity in bobwhite quail (Colinus
741 virginianus) chicks. Dev. Psychobiol. 2015,57:255-62.doi:10.1002/dev.21274
742 [60] Bertin, A., Richard-Yris, M. A., Mostl, E., Lickliter, R. Increased yolk testosterone
743 facilitates prenatal perceptual learning in Northern bobwhite quail (Colinus virginianus).
744 Horm. Behav. 2009,56:416-22. doi:10.1016/j.yhbeh.2009.07.008
745 [61] Chao, A., Paon, A., Remage-Healey, L. Dynamic variation in forebrain estradiol levels
746 during song learning. Dev. Neurobiol. 2015,75:271-86.doi:10.1002/dneu.22228
747 [62] Marler, P., Peters, S., Wingfield, J. Correlations between song acquisition, song
748 production, and plasma levels of testosterone and estradiol in sparrows. J Neurobiol.
749 1987,18:531-48.https://doi.org/10.1002/neu.480180605
750 [63] Riedstra, B., Groothuis, T. G. G. Early feather pecking as a form of social exploration:
751 the effect of group stability on feather pecking and tonic immobility in domestic chicks. Appl.
752 Anim. Behav. Sci. 2002,77:127-38.https://doi.org/10.1016/S0168-1591(02)00031-X
753 [64] Gluckman, P. D., Hanson, M. A. The developmental origins of the metabolic syndrome.
754 Trends Endocrinol. Metabol. 2004,15:183-7.https://doi.org/10.1016/j.tem.2004.03.002
755 [65] Greenberg, R. S. The role of neophobia and neophilia in the development of innovative
756 behavior of birds. In: Reader SM, Laland, K., ed. Animal innovation. Oxford: Oxford
757 University Press; 2003. p. pp. 175–96.
758 [66] Guibert, F., Richard-Yris, M.-A., Lumineau, S., Kotrschal, K., Bertin, A., Petton, C., et
759 al. Unpredictable mild stressors on laying females influence the composition of Japanese quail
760 eggs and offspring's phenotype. Appl. Anim. Behav. Sci. 2011,132:51-
761 60.doi:10.1016/j.applanim.2011.03.012

[67] Guibert, F., Richard-Yris, M.-A., Lumineau, S., Kotrschal, K., Guémené, D., Bertin, A., et al. Social Instability in Laying Quail: Consequences on Yolk Steroids and Offspring's Phenotype. PLoS ONE. 2010,5:e14069.doi:10.1371/journal.pone.0014069

Table 1: Frequency of behaviours (mean ± SE number of scans) of H+ and H- hens observed before and after the treatment. Different letters indicate significant differences within groups (Wilcoxon tests, $P < 0.05$).

	H+ hens		**H- hens**	
Behaviours	**before**	**after**	**before**	**after**
Maintenance	0.15 ± 0.08	0.52 ± 0.19	0.52 ± 0.22	0.42 ± 0.17
Feeding	0.31 ± 0.13[a]	1.05 ± 0.41[b]	1.79 ± 0.66	1.47 ± 0.44
Locomotion	4.84 ± 0.92	3.10 ± 0.84	4.68 ± 1.14	6.89 ± 1.26
Exploring	0.37 ± 0.17[a]	0.84 ± 0.32[b]	1.37 ± 0.49	1.73 ± 0.46
Resting	2.42 ± 0.87[a]	9.89 ± 1.71[b]	5.58 ± 1.40	5.52 ± 1.67
Observe	23.89 ± 1.18[a]	16.58 ± 1.49[b]	18.05 ± 1.49	15.95 ± 1.55

804 Table 2: Mean (± SE) weight (g) of H+ and H- hens before and during the five-week long treatment.

	Mass (g)					
	Before	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
H+ hens	1863 ± 63	1896 ± 67	1910 ± 60	1927 ± 60	1965 ± 64	1968 ± 64
H- hens	1782 ± 66	1812 ± 52	1839 ± 53	1820 ± 45	1858 ± 54	1851 ± 58

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Table 3: Mean (± SE) egg mass (g) and relative proportions of yolk, albumin and eggshell mass in H+ and H- eggs collected at the end of the treatment.

	H+ eggs	**H- eggs**
Egg mass (g)	60.62 ± 0.87	60.68 ± 1.37
Yolk mass / egg mass	0.28 ± 0.05	0.28 ± 0.04
Albumin mass / egg mass	0.62 ± 0.05	0.62 ± 0.05
Eggshell mass / egg mass	0.08 ± 0.02	0.08 ± 0.02

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833 Table 4: Mean (± SE) body mass (g) of H+ and H- chicks at post-hatch days 1, 11, 18, 25 and 32.

		Body mass (g)				
		Day 1	**Day 11**	**Day 18**	**Day 25**	**Day 32**
H+ chicks	**Females**	42 ± 0.6	99 ± 2.3	180 ± 3.7	268 ± 4.9	368 ± 6.6
	Males	43 ± 0.8	109 ± 1.9	198 ± 3	302 ± 4.6	420 ± 6.2
H- chicks	**Females**	43 ± 0.7	102 ± 2.3	183 ± 3.5	273 ± 4.4	374 ± 5.3
	Males	43 ± 0.8	106 ± 2	198 ± 4.2	302 ± 5.7	425 ± 7.6

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841 Table 5: Mean (± SE) latencies to eat (s) and time spent eating (s) in neophobia tests. Mean (±
842 SE) latency of first step, number of lines crossed, latency to distress call and number of calls
843 of H+ and H- chicks in novel environment tests.

		H+ chicks	**H- chicks**
Food neophobia	Latency to eat (s)	27.52 ± 9.46	13.66 ± 2.56
	Time spent eating (s)	63.84 ± 8.61	67.37 ± 7.38
Object neophobia	Latency to eat (s)	123.27 ± 14.31	111.00 ± 13.11
	Time spent eating (s)	25.64 ± 7.84	36.20 ± 7.20
Novel environment	Latency of first step (s)	40.12 ± 4.74	31.91 ± 4.83
	Number of lines crossed	5.62 ± 0.87	5.16 ± 0.72
	Latency to distress call (s)	16.74 ± 1.78	17.43 ± 1.88
	Number of calls	218.62 ± 11.56	231.25 ± 10.86

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856 **Figure captions**

857 Fig. 1: Mean (±SE) concentrations (ng/g of yolk) of yolk progesterone, testosterone,
858 androstenedione, and œstradiol, in the eggs from H+ and H- hens. $^{**}P < 0.01$; $^{*}P \leq 0.05$.

859 Fig. 2: Mean (± SE) time (s) spent close to the familiar or the unfamiliar conspecific of H+
860 and H- chicks in the discrimination test. ** $P < 0.01$.

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866 - humans' behaviour during egg formation engenders maternal effects in chickens

867 - humans' behaviour influences yolk progesterone and estradiol levels

868 - humans' behaviour does not influence offspring emotional reactivity

869 - humans' behaviour influences offspring social discrimination skills