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1 **Human behaviour at the origin of maternal effects on offspring behaviour in laying hens**  
2 **(*Gallus gallus domesticus*)**

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28

29

30 **Abstract**

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

53

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

55

## 56 1. Introduction

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

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

78 synthesis of steroid hormones which accumulate in the yolk [11]. However, moderate  
79 environmental challenges such as moderate heat also trigger variations in yolk hormone levels  
80 and engender maternal effects on offspring independently of maternal plasma corticosterone  
81 levels [12]. These results show that the physiological mechanisms at the origin of variation in  
82 yolk hormone levels remain unclear. In addition, the environmental factors triggering  
83 maternal effects are barely explored in domestic chicken. So far, housing conditions [13],  
84 unpredictable access to food [14, 15], maternal social status [16], thermal environment [12] or  
85 maternal diet [17] are all factors identified as potential sources of maternal effects mediated  
86 by yolk hormone variations in domestic chickens. These environmental factors caused  
87 modifications in growth, feeding behaviour, or emotional reactivity of the progeny [18] [15]  
88 [12] [17]. As these behaviours are implicated in chicks' capacity to adapt to their  
89 environment, deepening our understanding of maternal effects is of importance to contribute  
90 to a better management of layer breeders.

91 Research has aimed to understand effects of human presence on hens' fearfulness and  
92 productivity but the potential transgenerational effects engendered by human presence during  
93 egg formation have never been investigated. In most poultry production systems, due to the  
94 large size of flocks, the birds generally have very limited physical contact with the  
95 stockperson. And, fear of humans can be detrimental for welfare and productivity [19] [20,  
96 21]. Regular visual contact of domestic chicken with humans and gentle tactile contact are  
97 largely known to effectively reduce the expression of fear related behaviours in layers or  
98 broilers [reviewed in 22]. For example, regular visual contact with a human's slow  
99 movements or static presence reduced subsequently the avoidance of the experimenter in  
100 adult layers [19] or broilers [23]. In layers, gentle daily handling and stroking facilitate  
101 chicks' habituation to human beings [24]. In broilers, the presence of a static experimenter  
102 twice a day for 10 min reduced significantly chicks subsequent fear of humans [25]. On the

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

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

## 126 **2. Materials and methods**

### 127 **2.1 Adult hens housing conditions and treatment**

128 Thirty-six one-year old White Leghorn hens (*Gallus gallus domesticus*) from the PEAT  
129 experimental unit (INRA, Nouzilly) were split into two groups. The groups were balanced for  
130 mass of the hens. Both groups were housed in two similar 60-m<sup>2</sup> thermo-regulated rooms. For  
131 the needs of the experiment (egg identity and individual behaviour), subjects were placed in  
132 individual wire home-pens (100 cm × 100 cm × 50 cm) with wood shavings on the floor, a  
133 nest, a perch, a drinker and a trough. Cages were adjacent to one another so that all birds had  
134 tactile, visual, and vocal contacts with one another. All the birds were maintained at a  
135 temperature of 21 ±1°C for the duration of the experiment. Water and food were available *ad*  
136 *libitum* during a 14-h light/10-h dark cycle.

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

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

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

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

166

### 167 **2.3 Hens' behaviour**

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

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

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

## 224 2.7 Offspring's fear of humans

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

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

## 232 **2.8 Food and object neophobia**

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

## 250 **2.9 Open-field test**

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

### 259 **2.10 Detour task**

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

### 269 **2.11 Social discrimination**

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

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

## 281 **2. 12 Social motivation**

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

## 292 **3. Statistics**

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

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

#### 316 **4. Ethics statement**

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

#### 322 **5. Results**

### 323 **5. 1 Behaviour of hens**

324 H+ hens' behaviour differed significantly between before and after the treatment. The  
325 numbers of scans with hens observed close to the experimenter increased significantly  
326 between before and after the treatment ( $15.95 \pm 2.93$  vs.  $23.22 \pm 2.19$  scans,  $z = -2.58$ ,  $P <$   
327  $0.01$ ) and they were observed significantly more frequently feeding, exploring and resting and  
328 less in observation after than before the treatment (Table 1). Neither the numbers of scans  
329 with H- hens close to the experimenter ( $18.13 \pm 2.86$  vs.  $14.87 \pm 2.78$  scans,  $z = -1.44$ ,  $P =$   
330  $0.15$ ) nor their behaviour differed significantly between before and after the treatment (Table  
331 1).

332 [table 1 approximately here]

### 333 **5. 2 Morpho-physiological measurements on hens**

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

338 [table 2 approximately here]

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

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

### 343 **5. 3 Egg characteristics**

344 The masses of eggs and egg constituents did not differ significantly between H+ and H- hens  
345 (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 oestradiol 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 ( $6.20 \pm 1.09$   
365 scans and  $7.00 \pm 1.09$  scans respectively; treatment effect: Mean Square(MS) = 4.96,  $P =$   
366  $0.84$ ; sex effect:  $5.71 \pm 0.99$  scans for females vs.  $7.76 \pm 1.19$  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.**

369 Similarly, no significant differences were found between H+ and H- chicks for the neophobia  
 370 (food and object) or novel environment tests (Table 5: Food neophobia: latency to eat,  
 371 treatment effect:  $MS = 2234.81, P = 0.19$ ; sex effect:  $22.86 \pm 6.41$  s for females *vs.*  $17.90 \pm$   
 372  $8.22$  s for males,  $MS = 104.57, P = 0.98$ ; treatment\*sex effect:  $MS = 74.49, P = 0.71$ ; time  
 373 spent eating, treatment effect:  $MS = 289, P = 1$ ; sex effect:  $65.43 \pm 7.97$  s for females *vs.*  
 374  $65.76 \pm 7.97$  s for males,  $MS = 1.65, P = 0.98$ ; treatment\*sex effect:  $MS = 1127.04, P = 0.36$ ;  
 375 Object neophobia: latency to eat, treatment effect:  $MS = 1622.9, P = 0.53$ ; sex effect:  $125.14$   
 376  $\pm 13.13$  s for females *vs.*  $106.71 \pm 14.24$  s for males,  $MS = 3298, P = 0.29$ ; treatment\*sex  
 377 effect:  $MS = 2063.8, P = 0.30$ ; time spent eating, treatment effect:  $MS = 1234.2, P = 0.37$ ; sex  
 378 effect:  $25.07 \pm 6.64$  s for females *vs.*  $38.47 \pm 8.65$  s for males,  $MS = 1676.1, P = 0.28$ ;  
 379 treatment\*sex effect:  $MS = 1092.3, P = 0.25$ ; Novel environment: latency of first step,  
 380 treatment effect:  $MS = 1570.08, P = 0.10$ ; sex effect:  $34.98 \pm 4.54$  s for females *vs.*  $37.59 \pm$   
 381  $5.16$  s for males,  $MS = 141.44, P = 0.52$ ; treatment\*sex effect:  $MS = 3.30, P = 0.98$ ; number  
 382 of lines crossed, treatment effect:  $MS = 2.56, P = 0.82$ ; sex effect:  $5.61 \pm 0.72$  for females *vs.*  
 383  $5.12 \pm 0.91$  for males,  $MS = 5.36, P = 0.47$ ; treatment\*sex effect:  $MS = 22.03, P = 0.27$ ;  
 384 latency to distress call, treatment effect:  $MS = 12.66, P = 0.44$ ; sex effect:  $18.37 \pm 2.11$  s for  
 385 females *vs.*  $15.36 \pm 1.03$  s for males,  $MS = 213.44, P = 0.42$ ; treatment\*sex effect:  $MS =$   
 386  $11.45, P = 0.96$ ; number of calls, treatment effect:  $MS = 2874.6, P = 0.62$ ; sex effect:  $212.39$   
 387  $\pm 11.01$  for females *vs.*  $241.36 \pm 10.90$  for males,  $MS = 19928, P = 0.11$ ; treatment\*sex  
 388 effect:  $MS = 6451.3, P = 0.40$ ).

389 [table 5 approximately here]

### 390 **5.8 Detour task**

391 Latencies to go round the barrier did not differ significantly between H+ and H- chicks  
 392 ( $294.40 \pm 51.43$  s and  $274.87 \pm 47.31$  s respectively, treatment effect,  $MS = 30036, P = 0.49$ )  
 393 Latencies were significantly longer for males ( $383.76 \pm 52.75$  s) than for females ( $210.64 \pm$

394 41.45 s) (sex effect:  $MS = 382307$ ,  $P = 0.03$ ) and there was no significant interaction between  
395 treatment and sex (treatment\*sex effect:  $MS = 20009$ ,  $P = 0.62$ ).

## 396 5. 9 Social discrimination and social motivation

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

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

405 [Fig.2 approximately here]

406 Latencies to reach their cage mate in the runway test did not differ significantly between H+  
407 and H- chicks ( $24.00 \pm 7.51$  s and  $9.00 \pm 2.66$  s respectively, treatment effect:  $MS = 3404.2$ ,  $P$   
408  $= 0.09$ ; sex effect:  $14.64 \pm 6$  s for females *vs.*  $19.33 \pm 5.55$  s for males,  $MS = 596.2$ ,  $P = 0.33$ ;  
409 treatment\*sex effect:  $MS = 624.4$ ,  $P = 0.36$ ). The proportions of time spent in the social zone  
410 did not differ significantly between H+ and H- chicks ( $0.83 \pm 0.03$  and  $0.89 \pm 0.03$   
411 respectively, treatment effect:  $MS = 0.05$ ,  $P = 0.21$ ; sex effect:  $0.84 \pm 0.04$  for females *vs.*  
412  $0.88 \pm 0.03$  for males,  $MS = 0.01$ ,  $P = 0.88$ ; treatment\*sex effect:  $MS = 0.07$ ,  $P = 0.11$ ).

## 413 6. Discussion

414 In this study we show that the quality of human presence during egg formation induced  
415 variations in yolk hormonal levels and that the capacity to discriminate in the offspring was  
416 plastic. H- hens laid eggs with significantly lower yolk progesterone and oestradiol 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 oestradiol concentrations than  
464 H- hens' eggs. Previously we observed that exposure to a moderate heat challenge  
465 significantly increased hens' yolk progesterone, testosterone, and oestradiol levels [12]. The  
466 quality of polyunsaturated acids in hens' diet modulated concentrations of yolk progesterone,

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

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

492 the discrimination test, H+ chicks clearly preferred their familiar cage mate to the stranger  
493 whereas H- chicks showed no preference. The preference showed by H+ chicks corresponds  
494 to a pattern previously observed in young Leghorn chicks [36]. In addition, the capacity of  
495 domestic chicks to discriminate between strangers and familiar conspecifics and their  
496 preference for familiar companions is well documented, even in day-old chicks [54]. This  
497 preference for a familiar stimulus is explained by filial imprinting, the process by which  
498 chicks learn the characteristics of a stimulus and acquire a social preference for it [55] [56]  
499 [57]. Filial imprinting is crucial for young precocial birds that have to recognize their mother  
500 and flock members as soon as they hatch. As H+ and H- chicks were maintained in the same  
501 post-hatch environment, the absence of the typical preference for the familiar conspecific in  
502 H- chicks suggests that differences in the maternal environment and yolk hormone levels  
503 might be involved. Our results are in line with another study showing an absence of  
504 preference in chicks prenatally exposed to an experimental increase in yolk corticosterone  
505 levels [58]. Although speculative, yolk hormones could possibly have organizational or  
506 activational effects on neuronal circuits involved in the treatment of sensory information and  
507 memory. Increases in yolk progesterone levels were found to alter Bobwhite quail's (*Colinus*  
508 *virginiatus*), prenatal auditory learning of a maternal call [59], whereas increases in yolk  
509 testosterone levels were found to facilitate auditory learning [60]. In young songbirds that  
510 have to learn their song from adult tutors, oestradiol and testosterone levels in plasma or in the  
511 forebrain are known to play a key role in the consolidation of tutor song memories [61] [62].  
512 The treatment applied to H- hens may have impaired the capacity of chicks to recognize their  
513 familiar cage mate. Encounters with strangers are stressful for chicks and may favour the  
514 expression of feather pecking [63], our study thus calls attention to the necessity to deepen  
515 our understanding of maternal effects on domestic chickens' social behaviours.

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

## 535 **6.1 Conclusion**

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

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

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

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

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

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

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816 Table 3: Mean ( $\pm$  SE) egg mass (g) and relative proportions of yolk, albumin and eggshell

817 mass in H+ and H- eggs collected at the end of the treatment.

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	<b>H+ eggs</b>	<b>H- eggs</b>
<b>Egg mass (g)</b>	60.62 $\pm$ 0.87	60.68 $\pm$ 1.37
<b>Yolk mass / egg mass</b>	0.28 $\pm$ 0.05	0.28 $\pm$ 0.04
<b>Albumin mass / egg mass</b>	0.62 $\pm$ 0.05	0.62 $\pm$ 0.05
<b>Eggshell mass / egg mass</b>	0.08 $\pm$ 0.02	0.08 $\pm$ 0.02

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

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

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841 Table 5: Mean ( $\pm$  SE) latencies to eat (s) and time spent eating (s) in neophobia tests. Mean ( $\pm$   
 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.

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

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856 **Figure captions**

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

859 Fig. 2: Mean ( $\pm$  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

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