



## Habituation to humans affects yolk steroid levels and offspring phenotype in quail

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### ARTICLE INFO

#### Article history:

Received 23 January 2008

Revised 21 March 2008

Accepted 23 April 2008

Available online 10 May 2008

#### Keywords:

*Coturnix japonica*

Human disturbances

Maternal effects

Eggs' quality

Yolk steroids

Androgens

Progesterone

Growth

Behavior

### ABSTRACT

In the field as well as in the laboratory, human-generated stress responses are reduced in adult animals previously habituated to humans in comparison to non-habituated individuals. In birds, yolk steroid levels vary with maternal environment and condition. We tested the hypothesis that the experience of female birds with humans could affect yolk steroids levels and offspring phenotype. Two groups of Japanese quail, one habituated to humans (H) and a second non-habituated (NH), were exposed daily to brief human disturbances. We analysed egg quality, offspring growth, and offspring emotional reactivity. NH females produced eggs with less androgens (testosterone and androstenedione) and more immunoreactive progesterone compared to birds habituated to humans. NH females produced eggs with less yolk, heavier shell and chicks hatching later and being smaller as compared to habituated individuals. A lower emotional reactivity was found in young of NH females compared to young of H females. Thus, human disturbance of the mother triggered different effects on chick phenotype depending on previous experience of mother birds with humans. In addition, we describe for the first time the influence of environmental stimuli on yolk immunoreactive progesterone levels. Our results show that a relatively minor difference in behavioral habituation may have substantial effects on eggs and offspring. This has obvious implications for keeping and handling laboratory animals, for conservation biology and for animal welfare.

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In birds, including domestic species (e.g. domestic fowl), the main response to humans is fear, associated with cardiac acceleration and adrenocortical activation (Jones et al., 1981; Suarez and Gallup, 1983). However, birds may be habituated to humans (e.g. Bertin and Richard-Yris, 2004; Jones et al., 1991; Jones, 1996 for a review) and the amount and quality of human contact may have profound behavioral consequences. For example, in hens, visual contact with humans in addition to routine husbandry decreases withdrawal and corticosterone response to handling and enhances egg production (Barnett et al., 1994). In the field, human disturbances during the laying period may reduce hatchability of eggs and growth of offspring (Rodgers and Smith, 1995). However, in some cases a lower impact of anthropogenic disturbance has been found in populations with a previous history of human contact, for example, in Magellan Penguins (*Spheniscus magellanicus*, Walker et al., 2006).

Fear of humans or other potential predators may thus affect birds behaviorally and physiologically. Still, the mechanism responsible for the inverse fear-productivity relationship in lab and field remain unknown. Recent investigations mainly focus on anthropogenic disturbances effects on the activation of the HPA axis and corticoster-

one secretion in birds (Müllner et al., 2004; Walker et al., 2006). On the other hand, yolk steroids levels may vary with the laying birds' living condition and these, in turn, have the potential to affect offspring phenotype and growth. Breeding density (e.g. Schwabl, 1997; Groothuis and Schwabl, 2002), frequency of social intrusions (Mazuc et al., 2003), maternal social status (Müller et al., 2002), diet (Verboven et al., 2003) and male attractiveness (Gil et al., 1999) are all environmental factors that may potentially affect offspring embryonic development and the later morphological, physiological and behavioral characteristics (Groothuis et al., 2005 for a review). Experimentally enhanced testosterone concentrations were found to decrease fearful performance in the precocial Japanese quail (Daisley et al., 2005). Therefore, mothers may influence offspring emotional reactivity (i.e. propensity to express fear responses, Bouissou et al., 1994; Jones, 1996) by an epigenetic mechanism across multiple generations.

We investigated human disturbances as a potential factor influencing yolk steroids levels and the subsequent development and behavior of Japanese quail chicks. We predicted that yolk hormones levels would vary according to laying birds' habituation history. To this end, two groups of quail from the same breeding line, one previously habituated to humans and a second non-habituated were exposed daily to brief human disturbances. As stressful conditions have been found to enhance androgen levels in other species (e.g. Mazuc et al., 2003, Gil et al., 2004), we predicted that we would find higher

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androgens and less fearful birds in eggs and chicks of mothers non-habituated to humans as compared to eggs and chicks from habituated mothers. As the production of progesterone may also be influenced by human-caused stress in mammals (Braastad, 1998), we also analysed levels of yolk immunoreactive progesterone (precursor of androgens). Although often ignored in the avian literature, this hormone is present in large amounts in eggs' yolk (Möstl et al., 2001; Hackl et al., 2003), and therefore, may potentially affect offspring phenotype.

## Materials and methods

### Housing and treatment of laying birds

Thirty six 35 day-old female quails from a commercial farm were used. Each bird was identified by a numbered ring and housed in an individual cage (94×64×69 cm) with opaque lateral walls so that they could hear, but not see, one another. Water and food were available *ad libitum* during a 13:11 h light:dark cycle and an ambient temperature was maintained at approximately 19±2 °C. To estimate the propensity of our females to express “fear” responses towards humans we applied behavioral tests commonly described in the literature (see below). Birds were tested when they were 37 days-old. They were then divided into two groups (weights and frequencies of “fear” behaviors were balanced between the two groups). An effective “habituation” treatment as described in (Bertin and Richard-Yris, 2004 and see below) was applied to the group H until 60 days of age (habituated birds), whereas the NH group (non-habituated birds) had no contact with humans except for approximately 10 min a week during routine maintenance. Birds of both groups were housed in the same room but were separated by an opaque plastic curtain so that birds of NH group can not have any visual contact with the experimenter during the habituation treatment. During all of these procedures and until the end of the experiment, male quail were randomly introduced every 2 days into each female's cage as to fertilize eggs.

### Habituation

The experimenter interacted daily with quail of H group in two different ways: the experimenter's open hand was placed palm down for 30 s in the home cage and no attempt was made to initiate contact with the quail. This was carried out four times in the morning between 9 and 11 p.m. with a 30-min periodicity. In the afternoon, each quail was picked up, put on the sitting experimenter's knees, stroked for 30 s, maintained with one hand and offered attractive food (two meal worms, oyster shells and birdseed) on the handler's palm for 10 s and then returned to the home cage. This treatment was carried out twice between 2–4 p.m. with 1 h between two sessions. Quail of NH group received the same quantity of food that the experimenter (out of sight of the quail) introduced each day into their cages by aid of tweezers.

### Human disturbances

After the habituation treatment, when birds were 61 days-old and for a 15 day period, both NH and H birds were exposed to identical human disturbances. We chose to apply a combination of actions known to induce stressor-effects in this species (Jones, 1996) and in animals in general. That is: unpredictability, sharp movements, and contact with humans. Birds were stressed three times a day at unpredictable times. The order of stress-inducing actions changed for each exposure. Each day, the experimenter ran back and forth past the cages, caught and carried each bird for 30 s, opened and closed each home cage door, or waved one hand for 30 s inside each home cage.

### Egg sampling and analysis

As the formation of individual yolks lasts 7 days (Sauveur and Picard, 1987), egg collecting only started 9 days after the beginning of the human disturbances. Eggs from each hen were then collected every day for six consecutive days. All eggs were stored frozen at -20 °C until extraction. Eggs were weighed on electronic scales. As described by Lipar et al. (1999) and Hackl et al. (2003), the frozen yolk was separated from the albumin and weighed. Eggshells were also separated, washed with water to remove the albumin, dried and weighed. The weight of the albumin was determined by subtracting the weight of the eggshell and the yolk from the total mass of the egg. For measuring the concentrations of progesterone and androgens in yolk we used a method similar to that described in Möstl et al. (2001) and Hackl et al. (2003). As the distribution of hormones vary within egg layers (Hackl et al., 2003; Möstl et al., 2001) the entire mixed yolk was assayed. To extract steroids, after thawing, each yolk was suspended in 10 ml of water and vortex twice for 30 s. Samples were then stored overnight at 4 °C. Samples were then vortexed and 1 ml of the suspension was transferred into a new vial. The suspension was then diluted with 4 ml methanol, vortexed for 30 min and stored at -20 °C overnight to precipitate apolar lipids. After centrifugation (-15 °C, 2500 g, 10 min) 10 µl of the supernatant were used for enzyme immunoassays (for full descriptions of antibodies and validation see Palme and Möstl, 1993; Hirschenhauser et al., 1999; Möstl et al., 2001). We measured yolk testosterone (T) in five assays, and androstenedione (A<sub>4</sub>) and progesterone in six assays. The inter-assay coefficients of

variation were 19.5%, 10.4% and 15.9% respectively for the low level pool and 12.3%, 5.5% and 15.3% for the high level pool. The intra-assay variation was 8.5%, 4.2% and 9.2% respectively.

### Chicks rearing

A total of 63 eggs from the H females and 54 eggs from the NH females (on average 3.16±1.8 eggs per female) were placed in an incubator and maintained at 37.6±0.1 °C and 60% humidity while being turned automatically and continuously. Three days before hatching (day 14), the rotation was stopped and the humidity increased. Eggs were placed in a grid constructed of wire mesh and cardboard dividers so that chicks from both sets could be identified. Chicks hatched between days 18 and 19. Non-hatched eggs remaining in the incubator after day 20 were opened so as to determine if they were fertilized or not (i.e. presence or absence of an embryo). 37 out of the 63 eggs of the H group (62%) and 34 out of the 54 eggs of the NH group (63%) hatched. Each chick was marked with a leg numbered ring. They were maintained in two separated but identical home cages (94×64×69 cm until 2 weeks of age and 282×64×69 cm after), with sawdust on the floor and equipped with dull-emitter heating lamps (250 W). Water and food (starter) were available *ad libitum* during a 13:11 h light:dark cycle. To assess weight gain, young birds were weighed four times: a few hours after hatching and at 3, 7 and 25 days-old. Sex was determined at 15 days of age via sexual dimorphic plumage.

### Characterization of animals

To assess the emotional reactivity of adult females and their offspring, various ethological tests with different potentially fearful situations commonly described in the literature were presented. The same person performed all tests and wore similar clothing (i.e. a white laboratory coat) at all times.

Human observer tests: the experimenter passed in front of each home cage (at approximately 40 cm, separated from the bird by wire mesh) every 4 min the experimenter instantaneously noted behaviors known to reflect fear: withdrawal (quail ran away from the experimenter) and/or violent attempts to escape (jumped violently) but also behavioral inhibition (freezing, crouching on the ground) (Jones, 1996; Mills et al., 1997). The experimenter also noted moving (apparently non-oriented walking), standing still and observing, lying and maintenance behaviors (eating, pecking, drinking, preening and dust bathing). This test was carried out on all adult females before and after habituation when birds were respectively 37 and 60 days-old.

Cage-plus-experimenter-tests: this test was a modification of the protocol described by Jones and Waddington (1992). Animals were captured and were gently carried in a transport box approximately 5 m from their home cage in the same room and put in a cage similar to their own (94×64×69 cm). The experimenter sat facing the wire mesh wall so that her head and upper body were approximately 30 cm from the wire mesh cage door. The cage was divided by imaginary lines into two areas (close to the door and far from the door). Quail position in the cage, “fear” behaviors, moving, standing still and observing, sitting posture and maintenance behaviors were recorded every 10 s over the 4 min test period (that is to say a total amount of 24 statements per test). This test included all laying females after habituation and all young when they were 27 days old.

Tonic-immobility tests: this protocol was similar to that described by Jones, 1986). Animals were caught and gently carried individually in a transport box (10×10×10 cm). Quail were placed on their backs and restrained for 10 s prior to release. The experimenter remained in the same room, approximately 1 m away, but out of sight of the birds. If more than 10 s lapsed between the release of the quail and their escape, duration of TI was noted. If not, the experimenter immediately reattempted to induce TI. If TI had not been induced after five induction attempts, birds scored 0 s for TI duration. If quail had not yet attempted to stand up after 5 min, the test was stopped and a maximum of 300 s was allocated. Young were tested when they were 11 days old.

Novel object tests: each animal was introduced in the middle of a cage similar to their own. The cage was divided by imaginary lines dividing the home cage into two equal size zones (near the door and away from the door) and contained a moss-coloured ball (10 cm diameter) unknown from the birds and placed close to the door. Hidden behind a curtain with small observation windows (1 cm×1 cm), the experimenter noted the position of the birds in the cage and “fear” behaviors every 10 s over the 4 min test period. Fear behaviors were defined as previously as: withdrawal (quail ran away from the experimenter) and/or violent attempts to escape (jumped violently) but also behavioral inhibition (freezing, crouching on the ground) (Jones, 1996; Mills et al., 1997). Moving, standing still and observing, sitting posture and maintenance behaviors were also recorded. Young were tested when they were 26 and 68 days of age.

All work was carried out in accordance with Upper Austrian guidelines on animal research.

### Data analysis

Kolmogorov–Smirnov test was used to determine whether the data sets were normally distributed. The means per female were used for analysing egg components

and hormonal contents. As some females failed to lay eggs or laid eggs with abnormal eggshells or egg sizes, the actual sample size was 14 eggs from the 18 NH females and 17 eggs from the 18 H females. The frequency of behaviors (number of scans), tonic immobility scores, and the relative proportions of hormones were not normally distributed, so the Mann–Whitney *U*-test and Bonferroni corrections for multiple comparisons were used. Throughout the text, corrected *P*-values are reported. Hatching success and survival were tested using the Chi-square test. Laying rate conformed to the assumptions of normality and was therefore analysed using one-way analyses of variance (Student *t*-tests). Data on the weight of young were analysed using a one-way repeated measures ANOVA (treatment×time). The relative proportions of egg components (yolk and eggshell) were determined and analysed using Multivariate Analysis of Variance (MANOVA). If Wilks' Lambda tests showed a significant multivariate effect, individual one-way ANOVAs were performed for each dependant variable. Yolk hormone (T, A<sub>4</sub> and progesterone) data were transformed by a logarithmic (*X*+1) transformation to meet the variance and covariance homogeneity requirements and analysed with a MANOVA and individual one-way ANOVAs. Data are presented as mean±SEM. All analyses were performed using Statview software (SAS, Cary, NC), with significance accepted at *P*≤0.05.

## Results

### Behavioral differences between H and NH laying birds

The behavior of NH and H females toward humans clearly diverged following the habituation treatment. In the human observer test, NH birds had significantly higher frequencies of fear behaviors, lower frequencies of maintenance behaviors, standing still and observing or walking (Table 1). They also tended to maintain less proximity to the experimenter.

Birds also differed when facing a static human in the cage-plus-experimenter test (Table 1). We observed significantly higher frequencies of fear behaviors in NH birds than in H birds, lower frequencies of maintenance behaviors, they also tend to walk less. Furthermore, NH quail spent significantly less time in the area near the experimenter than H birds. All these results indicate a higher emotional reactivity in NH birds when exposed to humans.

### Egg laying and eggs quality

Laying rates did not differ between NH and H birds during the six consecutive days preceding human disturbances (mean±SEM number of egg per female and per day: NH females: 0.87±0.44 versus 0.88±0.05 for H birds; *t*=0.28, *P*=0.78). The laying rate was lower in NH birds than in H birds during the six consecutive days of eggs sampling following human disturbances (*T*-test; *t*=2.06, *P*=0.046) (Table 2). Egg composition differed between the two groups of females. We found a significant overall effect on egg components masses (MANOVA, *F*<sub>1,27</sub>=9.96, *P*<0.01). One way ANOVAs showed a significant effect of the habituation treatment on yolk mass (*F*<sub>1,28</sub>=6.76, *P*=0.015) and eggshell mass (*F*<sub>1,28</sub>=10.68, *P*<0.01) with less yolk and heavier eggshell in eggs of NH females (Table 3).

**Table 1**

Females NH and H mean±SEM behavior frequencies (expressed in % of scans) in the human observer and cage-plus-experimenter tests after the habituation treatment (60 days of age)

Tests	Parameters measured	NH females	H females
Human observer	Fear behaviors	79.2±5.2	3.5±2.3**
	Maintenance behaviors	0	16±3.5**
	Standing still and observing	16.7±4.7	60.8±4.5**
	Walking	4.2±1.6	12.2±2.9*
	Lying	0	7.6±5
	Zone proximate to the door	59.4±6.6	76.7±6.1°
	Cage-plus-experimenter-test	Fear behaviors	50.7±8.8
Maintenance behaviors		8.8±2.9	28.5±5.3*
Standing still and observing		27.7±5.9	41.7±4.7
Walking		12.7±3.6	28±4.2°
Lying		0	0
Zone proximate to the door		16.9±6.1	54.2±6.8**

Mann–Whitney *U*-test 0.05<*P*<0.1; \**P*<0.05; \*\**P*<0.01.

**Table 2**

Mean±SEM laying rate, hatching success, and number of days until hatching of chicks from the NH and H treatment groups

Treatment	Laying rate	Number hatched	Failed	Number of days until hatching	Survival the fist week	Total
NH	0.69±0.09	34	1	18.42±0.09	85.29 %	29
H	0.89±0.04	37	6	18.05±0.04	83.78 %	31

We found a significant overall effect on yolk hormone concentrations (Fig. 1) (MANOVA, *F*<sub>3,27</sub>=6.36, *P*<0.01). The yolk of NH females had lower concentrations of both T (*F*<sub>1,29</sub>=4.45, *P*=0.04) and A<sub>4</sub> (*F*<sub>1,29</sub>=5.83, *P*=0.02) compared to the yolk of H females. The quantity of the three hormones per yolk also differed (MANOVA, *F*<sub>3,27</sub>=5.32, *P*<0.01). Yolk of NH females contained both less T (41.6±5 ng/yolk versus 62.6±8 ng/yolk, *F*<sub>1,29</sub>=4.29, *P*=0.047) and less A<sub>4</sub> (574.75±55.33 ng/yolk versus 745.75±43.97, *F*<sub>1,29</sub>=5.85, *P*=0.02) compared to yolk of H females.

The total quantity of hormones found in the yolk of eggs from NH and H birds did not differ significantly (5652.28±582.15 ng/yolk for NH birds versus 5384.5±3.88,4 ng/yolk for H birds; Mann–Whitney *U*-test, *U*=119, *P*>0.05) but the relative proportions of hormones did (% of the total amount of hormones). The yolk of NH eggs contained a significantly higher proportion of progesterone than H eggs (89±0.5 % and 84±1 % respectively, *U*=37, *P*=0.01). On the other hand, the yolk of NH eggs contained lower proportions of androgens (T+A<sub>4</sub>) than H eggs (11±0.5 % and 16±1 % respectively, *U*=37, *P*=0.01). If instead of total yolk hormone content (ng/yolk) we analyse hormone concentrations (ng/ml), the results remained exactly the same. Within both NH and H sets, the amount of T correlated positively with the amount of A<sub>4</sub> (*r*=0.63, *P*=0.02 for NH birds; *r*=0.67, *P*=0.007 for H birds). Within the NH group, the amount of progesterone correlated positively with the amount of both T (*r*=0.55; *P*=0.04) and A<sub>4</sub> (*r*=0.83; *P*=0.002) but it was not significant in the H group (T: *r*=0.22; *P*=0.37; A<sub>4</sub>: *r*=0.45; *P*=0.07).

### Offspring growth

Fertile eggs laid by NH females tended to be more likely to hatch than fertile eggs laid by H females (Chi-2 test, *P*=0.08) (Table 2) but chicks hatched significantly later (Mann–Whitney *U*-test, *U*=382, *P*=0.001). We found no difference in the survival rate of chicks during the first week of life (Chi-2 test, *P*=0.8). There was a significant effect of the habituation treatment on chick growth (*F*<sub>1,56</sub>=4.065, *P*=0.048), as well as an effect of time (*F*<sub>3,168</sub>=14507.61, *P*<0.001) but no significant interaction between treatment and time (*F*<sub>3,168</sub>=2.43, *P*=0.067). Growth of NH chicks appeared delayed during the first week of life compared to H chicks (Table 4). H and NH hatchlings did not differ in sex ratio.

### Behavior of offspring

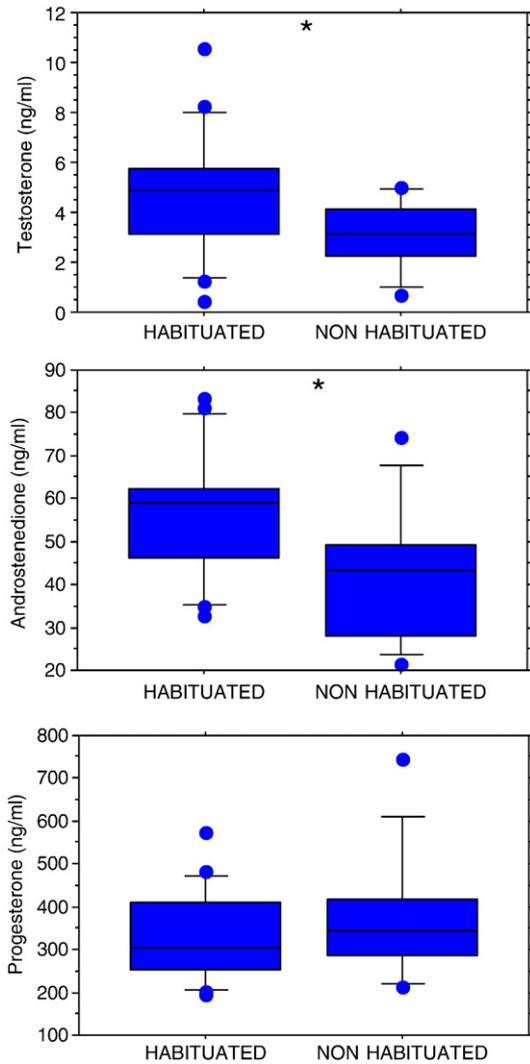
Tonic immobility tests revealed significant differences between the two groups of chicks. Significantly more induction attempts were required for NH than for H chicks (2.96±0.3 versus 2±0.2, Mann–

**Table 3**

Egg composition in the NH and H females

Trait	Groups	
	Non-habituated	Habituated
Egg weight (g)	12.16±0.21	12.66±0.17
Ratio yolk/ egg weight %	29.4±0.5	31.1±0.4**
Ratio albumen/ egg weight %	62.8±0.6	61.5±0.4
Ratio shell/ egg weight %	8±0.1	7.4±0.1*

\*Indicate significant differences at *P*<0.05; \*\**P*<0.01.



**Fig. 1.** Boxplots of the testosterone, androstenedione and immunoreactive progesterone levels (ng/yolk) in eggs of the H and NH females. \* indicate significant differences at  $P < 0.05$ .

Whitney  $U$ -test,  $U = 274.5$ ,  $P = 0.019$ ). TI durations did not differ between the two groups ( $32.9 \pm 4.5$  s for H chicks versus  $31.3 \pm 6$  s for NH chicks, Mann-Whitney  $U$ -test,  $U = 342.5$ ,  $P = 0.22$ ).

In front of a static human, NH young were found to express significantly less fear behaviors, more standing still and observing, less distress calling, and were more proximate to the experimenter than H young (Table 5).

Offspring of NH and H females also differed in novel object tests (Table 5). At 26 days of age, NH young were found to spend more time close to the object, more time standing still and observing and less time distress calling or moving. NH young also tend to spent more time in exploratory pecking compared to H young. In young animals, no sex differences were observed in all tests.

**Table 4**  
Body weight (g) of chicks from the NH and H treatment groups at hatch, days 3, 7 and 27 of life

Young	Weights (g)			
	Hatch	Day 3	Day 7	Day 27
NH	$9.01 \pm 0.16$	$11.12 \pm 0.26$	$26.8 \pm 0.7$	$185.37 \pm 3.82$
H	$9.43 \pm 0.13$	$11.98 \pm 0.24$	$28.62 \pm 0.62$	$184.63 \pm 2.55$

**Table 5**

Young NH and H mean  $\pm$  SEM behavior frequencies (expressed in % of scans) in the novel object and the cage-plus-experimenter tests

Test	Parameters measured	NH young	H young
Cage-plus-experimenter-test	Fear behaviors	0	$2.5 \pm 0.9^{**}$
	Maintenance behaviors	0	0
	Standing still and observing	$57.9 \pm 4.5$	$41.1 \pm 4.3^{**}$
	Moving	$35.5 \pm 4.6$	$42.2 \pm 4.1$
	Lying	0	0
	Distress calling	$4.2 \pm 1$	$13.9 \pm 2.6^{**}$
Novel Object test	Zone proximate to the door	$22.2 \pm 4.5$	$13.5 \pm 4.7^*$
	Fear Behaviors	$0.1 \pm 0.1$	$0.7 \pm 0.5$
	Maintenance behaviors	0	0
	Standing still and observing	$72.3 \pm 4.5$	$36.5 \pm 3.6^{**}$
	Moving	$17.4 \pm 3.2$	$46.5 \pm 3.8^{**}$
	Lying	0	0
	Distress calling	$8 \pm 1.9$	$15.8 \pm 2.4^*$
	Exploratory pecking	$2.1 \pm 0.8$	$0.3 \pm 0.2^*$
%Time spent close to the object		$67.9 \pm 6.7$	$45.6 \pm 6^*$

Mann-Whitney  $U$ -test  $0.05 < P < 0.1$ ;  $*P < 0.05$ ;  $**P < 0.01$ .

Once adult and sexually mature, when birds were 68 days of age, differences between NH and N animals still persisted, although they were less pronounced. In a novel object test, NH females were observed more often engaged in exploratory pecking compared to H females ( $4.5 \pm 2.3$  % of scans versus 0 %, Mann-Whitney  $U$ -test,  $U = 52.5$ ,  $P = 0.05$ ). NH males were observed more often standing still and observing than H males ( $56.9 \pm 7.4$  % of scans versus  $26.5 \pm 4.9$  %, Mann-Whitney  $U$ -test,  $U = 42$ ,  $P = 0.05$ ).

**Discussion**

In the current study we demonstrated that daily brief exposure of hens to humans can differentially influence the quality of the pre-hatch environment and the subsequent embryonic development, weight gain and behavior of offspring. The offspring of non-habituated mothers developed in a pre-hatch environment with less yolk, a heavier shell, more progesterone and in contrast to our predictions, less androgen. As expected, the embryonic development was found to be delayed and a lower emotional reactivity was found in the offspring of non-habituated mothers compared to chicks of habituated mothers.

Our habituation treatment was found to affect both laying rate and the relative weight of egg components. In the Japanese quail previous studies have reported lower laying rates in birds selected for high fearfulness (Richard-Yris et al., 2005; Minvielle et al., 2002). Differences in the endocrine control of ovulation and egg production have probably been induced by divergent fear-eliciting effects of disturbances between our fearful (NH) and relatively tame animals (H). Quail selected for a high inherent fearfulness were found to have a relative lower shell content compared to less fearful birds (Minvielle et al., 2002). In the present study, we observed a higher ratio of eggshell to egg mass in NH birds compared to H birds. This may be explained by a difference in the endocrine regulation of calcium mobilisation or time spent in the uterus (Sauveur and Picard, 1987).

Interestingly, chicks from eggs laid by NH mothers were exposed to less androstenedione ( $A_4$ ) and testosterone (T) of maternal origin compared to chicks hatched from eggs laid by H mothers.  $A_4$  can serve as a source of biologically potent androgens for embryos (Groothuis and Schwabl, 2002) and may affect nestling growth and competitiveness (Carriello et al., 2006; Sockman and Schwabl, 2000; Eising and Groothuis, 2003). Stressful conditions experienced by mothers, such as poor food conditions or high social density seem to enhance T in the yolk (Groothuis et al., 2005 for a review). In our case, birds expressing greater “fear” responses toward humans showed less T in the yolk.

Although present in much higher amounts than androgens in egg yolk (Hackl et al., 2003; Möstl et al., 2001), role of progesterone in

mediating maternal effects remains unclear. Progesterone is the precursor of androgens and is produced in the granulosa cells of the pre-ovary follicles (Huang and Nalbandov, 1979). In quail and other bird species, much higher amounts of progesterone than T or A<sub>4</sub> were found around the germinal disc (outer layer of the yolk) (Hackl et al., 2003). However, the potential influence of this hormone on embryonic growth and the development of behavior remains unknown. In mammals, progesterone has been found to influence embryonic growth (Lonergan et al., 2007; Ghosh et al., 1997) and is considered to be an anxiolytic steroid (Reddy et al., 2005). Due to the similarities of steroid molecules across species, it is conceivable that this hormone may play a role in the emergence of avian phenotypes. The difference observed in correlations between the different steroid hormones in our study may be explained by differences in the metabolism of steroids between H and NH birds. Higher androgen concentrations in H eggs may be a result of a higher production of gonadal steroids in total as compared to NH eggs, or that the conversion of progesterone/pregnenolone into androgens in the follicular wall decreases later as demonstrated by the fact that progesterone concentration is high in the outer layers of the yolk ball, whereas the androgen concentration is low in this part of the yolk (Möstl et al., 2001). These variations in progesterone and A<sub>4</sub> levels confirmed that focusing only on T variations is no longer justified (Groothuis and Schwabl, 2008).

The profile of embryonic growth, weight gain and hatching is in accordance with previous reports showing a positive correlation of growth with yolk androgens (Eising et al., 2001; Eising and Groothuis, 2003) but also lower hatching success (Sockman and Schwabl, 2000). However the higher weights observed in chicks from H females may also be explained by the higher mass of yolk they were deposited.

The behavioral profiles of NH and H adults confirmed the effectiveness of habituation treatments and previous findings in quail and other birds (Jones, 1996) but the behavioral profiles observed in our chicks contrasted with previous findings. Indeed, quail chicks from high androgen eggs were previously shown to develop a more “proactive” (sensus Koolhass et al., 1999) behavioral phenotype than chicks from low-androgen eggs (Daisley et al., 2005); being less fearful, less socially dependant and approaching novel objects more rapidly. However, despite the lower androgens levels observed in eggs of the NH groups, chicks developed a “proactive” like profile compared to H chicks. Indeed, in tonic immobility tests, NH chicks required a higher number of inductions, indicating a lower sensitivity to this test (Mills et al., 1994; Jones et al., 1991) and thus a lower general underlying fearfulness. Furthermore, NH chicks had a lower frequency of “fear” behaviors in cage-plus-experimenter tests, they were also more quiet (standing still and observing) and more proximate. Thus, the level of fear was lower in NH than in H chicks, which seemed to perceive the experimenter as a more frightening stimulus.

Domestic quail chicks can present avoidance responses within 1 h after hatching, and studies indicate that fear responses are to some degree genetically determined (Mills and Faure, 1991). As eggs and chicks of both groups came from the same population and were raised in identical conditions we hypothesized that the behavioral difference observed could be due to differences in eggs quality and prenatal development. The hypothesis of a lower level of emotional reactivity in NH chicks is supported by the results in novel object tests. Indeed, NH chicks were found to be quieter and maintained closer proximity to the novel items, and also showed less distress calling and more exploratory behavior. Furthermore, this difference in reactivity to novel objects persisted in older birds of both sexes (68 days of age), indicating a stable temperamental trait. The implication of hormones other than T (e.g. A<sub>4</sub> and/or progesterone) and general egg quality may explain the difference between our results and those of Daisley et al. (2005). But Okuliarová et al. (2007) also found more fearful performance in quail chicks exposed to higher T concentrations. A dose-dependant effect may also explain the contradictions in T effects on the behavior of quail chicks. For example, in Daisley et al. (2005)

and Okuliarová et al. (2007) studies, 50 ng and 25 ng of T were respectively injected into egg yolks.

Our results show that hormone-mediated maternal effects and egg quality contribute to shape the behavior of the next generation toward humans. These findings extend the results of a previous study where we demonstrated that young Japanese quail reared by adoptive mothers previously habituated to humans were less fearful toward humans than young reared by adoptive mothers non-habituated to humans (Bertin and Richard-Yris, 2004). The behavior of young Japanese quail also mirrors, to a certain extent, the general emotional reactivity of adoptive mothers (Richard-Yris et al., 2005). All this work demonstrates the importance of the mother as a modulator in the human-animal relationship. Habituation treatments could have direct effects on adult animals but also indirect effects on offspring via a modification of egg quality and of the postnatal environment. Although the offspring of NH females appeared less fearful compared to offspring of H females, it could be thought that the possibility to interact with adoptive H or NH adoptive mothers would have modify their reactivity. As demonstrated in rodents (e.g. Francis et al., 2003, Champagne and Curley, 2005), in birds, the postnatal experience probably interacts with the prenatal experience to shape the development of behavior. Young quail coming from T injected eggs were found to be less socially dependent compared to control birds (Daisley et al., 2005). Although further investigation is necessary, prenatal experience may also modify the sensitivity of animals to the postnatal social environment.

In mammals such as rodents, primates and humans, prenatal maternal stress involves the activation of the hypothalamo-pituitary-adrenal (HPA) axis and the release of glucocorticoids that have in turn a large range of effects on the general development of offspring and their later behaviour (for reviews: Welberg and Seckl, 2001; Edwards and Burnham, 2001; Seckl, 2004). In birds, exposure to stressors such as predation density, habitat quality or human disturbances can cause an increase in circulating glucocorticoids (e.g. Wasser, 1997; Silverin, 1998; Müllner et al., 2004; Wingfield et al., 1997). It was also reported that stress in hens (White Lohmann Selected Leghorns) influences the behavioural development of offspring (Janczak et al., 2006). It can thus be thought that glucocorticoids may have contributed to the differences in growth and behaviors we observed. However, we still know little about the transfer of plasma corticosterone into the egg yolk. Such a transfer was observed by using unphysiological levels of circulation corticosterone (implants: Hayward and Wingfield, 2004; feeding: Rettenbacher et al., 2005) and these high concentrations only result in a minor transfer (Rettenbacher et al., 2005). Although some authors report a transfer of corticosterone into eggs and natural variations within- and between-clutch (e.g. European starling, *Sturnus vulgaris*, Love and Williams, in press; Love et al., 2008; Barn swallow, *Hirundo rustica*, Saino et al., 2005), other authors do not. Administration of ACTH increased adrenocortical activity but does not influence corticosterone concentration in hens' yolk (Rettenbacher et al., 2005). Janczak et al. (2007) reported an influence of pre-hatch stress on offspring behavior without any effect on the concentration of corticosterone in eggs. Although this hypothesis cannot be totally rejected, we argue like Rettenbacher et al. (2005) and Janczak et al. (2006) that the effects of maternal stress were probably mainly mediated in an indirect way. Furthermore, the presence of high concentrations of various gestagens and gestagen metabolites in egg yolk (which are structurally similar to corticosterone), could also cause immunoreactive “corticosterone” as the concentrations of corticosterone measured in yolk are low compared to, for example, progesterone. As suggested by Rettenbacher et al. (2005), HPLC separations should be used to characterize these immunoreactive substances.

Our study revealed complex mechanisms of maternal effects including both egg components and hormones. In the lab as well as in the field, human disturbances in interaction with the history of birds have the potential to modify egg quality and engender phenotypic

variability in subsequent generations. For example, the way breeding hens are treated in the poultry industry may affect performance of broiler chickens and of laying hens. Exposing laying birds to human beings may influence the subsequent generation by an epigenetic influence on egg size and egg content. For the first time we documented the environmental influence on yolk immunoreactive progesterone levels. These results may have broad implications for the field of hormone-mediated maternal effects in laboratory animals and in conservation biology where the effects of anthropogenic disturbances must be monitored on multiple levels.

## Acknowledgments

The work reported here was funded by the Foundation Fyssen and by FWF Project P 18601-017. We are very grateful to Gregory Loucougaray for his help with data analysis, to Christopher Harshaw and Susan Schneider for improving the English of our manuscript and to the two anonymous reviewers for their constructive comments.

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