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RESEARCH ARTICLE

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Lipids in maternal diet influence yolk hormone levels and post-hatch neophobia in the domestic chick

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Abstract

We assessed whether the ratio of dietary n-6/n-3 polyunsaturated fatty acids (PUFA) during egg formation engenders transgenerational maternal effects in domestic chicks. We analyzed yolk lipid and hormone concentrations, and HPA-axis activity in hens fed a control diet (high n-6/n-3 ratio) or a diet enriched in n-3 PUFAs (low n-6/n-3 ratio) for 6 consecutive weeks. Their chicks were tested for neophobia during the first week of life. We found higher corticosterone metabolites in droppings of hens fed the diet enriched in n-3 and significantly higher concentrations of yolk progesterone, androstenedione, and estradiol in their eggs compared to controls. Chicks of hens fed the n-3 enriched diet showed a lower body mass at hatch than controls and expressed higher neophobia when exposed to a novel object. These results add support to the hypothesis that the nutritional state of female birds produces variation in yolk hormone levels and engender maternal effects.

KEYWORDS

Gallus gallus domesticus, maternal effects, neophobia, poly unsaturated fatty acids, yolk hormones

1 | INTRODUCTION

Understanding the reasons for phenotypic variation is one of the cornerstones of behavioral biology. Non-genetic maternal effects play a key role in generating phenotypic variation in many taxa (Ledón-Rettig, Richards, & Martin, 2013). In birds, prenatal maternal effects can affect the morphology, behavior, and physiology of offspring (Ruuskanen, 2015). Such effects are partly mediated via

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variation in yolk-hormone concentrations of maternal origin according to different environmental conditions (Groothuis, Muller, von Engelhardt, Carere, & Eising, 2005). Yolk androgens (mainly testosterone and its precursor androstenedione) have been widely studied in wild bird populations. Differential allocation of androgens into eggs is presumed to be a way to adapt offspring to their hatching environment (Gil, 2008; Groothuis et al., 2005). Androgen concentrations can vary substantially within and between egg clutches as a consequence of the maternal environment; factors such as social conditions (density, aggressive interactions, mate quality) (Kingma et al., 2009; Pilz & Smith, 2004; Schwabl, 1997), parasitic infection (Müller, Heylen, Eens, Rivera-Gutierrez, & Groothuis, 2013), and predation risk (Coslovsky, Groothuis, de Vries, & Richner, 2012) can influence hormone production. Because yolk hormone levels show such a strong context-dependency within species (Ruuskanen, 2015), the specific environmental cues triggering maternal effects must be elucidated further.

Recently, food resource availability has been identified as an environmental factor engendering differential maternal hormone allocation. In the lesser black-backed gull (*Larus fuscus*), pied flycatcher (*Ficedula hypoleuca*), and black-legged kittiwake (*Rissa tridactyla*) food supplementation prior to, and during oviposition affected androgen quantity in the yolk (Verboven et al., 2003; Verboven, Monaghan, Nager, & Evans, 2010). While in great tits (*Parus major*), no effect of food supplementation on yolk androgens has been recorded (Ruuskanen, Darras, de Vries, Visser, & Groothuis, 2016). Manipulation of food resources also shows to increase the within clutch variation of yolk-hormones in canaries (*Serinus canaria*) (Vergauwen, Goerlich, Groothuis, Eens, & Müller, 2012) and zebra finches (*Taeniopygia guttata*) (Sandell, Adkins-Regan, & Ketterson, 2007). These studies demonstrate that the nutritional state of maternal birds during oviposition contributes to differences in yolk hormone concentrations between or within a clutch. In the reported studies, enrichment of the maternal diet was achieved by providing additional protein and/or lipids from different sources (e.g., seeds, boiled eggs). This procedure leaves open the question on the influence of the nutritional composition of the food provided to the mother. To our knowledge, the effect of the quality of maternal diet on egg hormone levels and offspring phenotype has not been investigated so far in birds.

This question is of particular importance for factory-farmed birds which are fed an unique diet. The domestic chicken (*Gallus gallus domesticus*) is the most abundant bird species on the earth but, despite this importance, many avenues of research remained to be explored in order to advance our knowledge on maternal effects and behavioral development (Dixon, Sparks, & Rutherford, 2016). Housing conditions (Janczak, Torjesen, & Rettenbacher, 2009), unpredictable access to food (Janczak, Torjesen, Palme, & Bakken, 2007; Nätt et al., 2009), maternal social status (Muller, Eising, Dijkstra, & Groothuis, 2002), early social stress (Goerlich, Nätt, Elfwing, Macdonald, & Jensen, 2012), and thermal environment (Bertin et al., 2013) cause variation in yolk androgens and progesterone levels in domestic hens. This caused modifications in growth, feeding behaviors, and emotional reactivity of their chicks (Goerlich, Nätt, Elfwing, Macdonald, & Jensen, 2012; Nätt et al., 2009; Bertin et al., 2013). In domestic hens, maternal stress during egg formation—mimicked with subcutaneous corticosterone implantation—increased plasma corticosterone levels and decreased the synthesis of reproductive hormones which accumulate in the yolk (Henriksen, Groothuis, & Rettenbacher, 2011). In addition, on-farm longitudinal observations showed that parental stress physiology correlate with offspring's anxiety and expression of damaging behavior (de Haas, Bolhuis, Kemp, Groothuis, & Rodenburg, 2014; Rodenburg & de Haas, 2016).

In domestic hens, we previously showed that adding fish oil to the maternal diet induced food neophobia in the offspring (Aigueperse, Calandreau, & Bertin, 2013). As fish oil is particularly rich in n-3 poly-unsaturated fatty acids (PUFA), this result suggested that PUFA in the maternal diet may directly affect the development of behavior in the offspring. The aim of the present study was to test this hypothesis and assess whether the ratio between n-6 and n-3 PUFA in the maternal diet during egg formation could engender maternal effects. N-3 and n-6 PUFA unambiguously influence the development of the central nervous system in both mammalian and avian species (Noble & Cocchi, 1989). But these effects are better-understood for mammals. In mammals, PUFAs and their mediators are known to regulate many processes within the brain, such as neurotransmission, cell survival, and neuro-inflammation, and also affect hypothalamic–pituitary–adrenal (HPA) axis responses, thereby possibly playing a role in anxiety-like behavior and cognition (e.g., Bazinet & Layé, 2014; Lafourcade et al., 2011). In the domestic chicken, PUFA ratios in the maternal diet are transferred to the egg yolk (Noble & Cocchi, 1990) and subsequently to the tissues of the developing chick embryo (Maldjian, Farkas, Noble, Cocchi, & Speake, 1995). Numerous studies show that the PUFA profile of the developing chick brain reflects the PUFA composition in the yolk (Cherian & Sim, 1993; Anderson, Connor, Corliss, & Lin, 1989) but the consequences on behavioral development remain to be investigated.

We analyzed yolk lipid and hormone concentrations, and HPA-axis activity in hens fed a control diet (high n-6/n-3 ratio) or a diet enriched in n-3 PUFAs (low n-6/n-3 ratio). Development and neophobia (i.e., fear of novelty) of the hatchlings were analyzed. Neophobia is considered to be a temperament trait which can impair an animal's capacity to adapt to new resources, new habitats, and express behavioral innovation (Greenberg & Mettke-hofmann, 2001; Webster & Lefebvre, 2001). As a high level of neophobia can reduce fitness (Brown, Ferrari, Elvidge, Ramnarine, & Chivers, 2013), this behavioral trait is a growing topic of interest for behavioral biologists (Camín, Martín-Albarracín, Jefferies, & Marone, 2016; Greggor, Thornton, & Clayton, 2015). The precocial domestic chick is a particularly appropriate model in which to analyze developmental influences on neophobia since chicks start food selection soon after hatching (Bertin et al., 2010). In addition, under commercial conditions the refusal to accept new foods leads to a major reduction in feed intake and, subsequently, in growth and animal welfare (Murphy, 1977).

2 | METHODS

2.1 | Ethics statement

All birds were maintained at the Experimental Unit PEAT of INRA (Nouzilly, France). The Experimental Unit is registered by the ministry of Agriculture with the license number B-37-175-1 for animal experimentation. All experiments were approved by the Ethic Committee in Animal Experimentation of Val de Loire, CEEA Vdl (reference number 02153.02). The CEEA vdl is registered by the National Committe "Comité National de Réflexion Ethique sur l'Expérimentation Animale" under the number 19. All experiments were performed in accordance with the European Communities Council Directive 2010/63/UE. All animals were sold for rehabilitation at the end of the experiment.

2.2 | Maternal hens and diets

Hens ($n = 40$) and diets were provided by the experimental unit PEAT of INRA (Nouzilly, France). Two groups of 20 1-year old White Leghorn laying hens were matched for body weight, and divided into two dietary treatment groups, a control diet and a diet enriched in n-3 FA. Diets were administered for 6 consecutive weeks. The control diet contained soya-oil (3%) with a n-6:n-3 ratio of 7.99, a value within the range recommended for commercial laying hens (Van Elswyk, 1997). The n-3 diet contained 3% menhaden fish-oil (Sigma Life Science) having an n-6:n-3 ratio of 0.82. Diets were iso-caloric (2,820 kcal ME/kg) and iso-nitrogenous (170 g CP/kg), differing only in the composition of PUFAs (electronic supplementary material, Table S1). Hens were housed individually in wire-mesh cages of $100 \times 100 \times 50\ cm^3$ ($l \times w \times h$), with the possibility of tactile, visual, and vocal contact with neighboring hens. Cages contained wood shavings and a wooden perch on the floor, a nest enclosed by red curtain flaps, a nipple drinker and a feed trough. Water and food were provided ad libitum. Four hens were excluded from the experiment, three due to a lack of habituation to the experimental diets (food conservatism) and one death unrelated to the diet treatment, resulting in $n = 18$ hens per diet group.

2.3 | Morpho-physiological measurements of hens

All hens were weighed once per week. A total of 24 hr-feed intake per hen was measured once a week by calculating the difference in weight of the feeder before and after 24 hr. The number of eggs laid was recorded daily. Laying rate was calculated as a percentage of the number of eggs laid per female per day.

In order to evaluate HPA activity in chickens, fecal corticosterone metabolite (FCM) concentrations were measured (Rettenbacher, Mostl, Hackl, Ghareeb, & Palme, 2004). In the sixth week of diet treatment, one fresh fecal dropping per hen was collected from the home cage. Urine was removed from the sample as the concentrations of FCM in the liquid portions of droppings are more likely to represent acute HPA activity caused by handling or disturbing the animals during collection (Rettenbacher et al., 2004). Each sample was homogenized and stored at −20°C. From each sample an aliquot (0.5 g) was extracted with 60% methanol (Mostl, Spendier, & Kotrschal, 2001) and analyzed by a cortisone enzyme immunoassay (EIA) validated for chickens and fully described in Rettenbacher et al. (2004).

2.4 | Analysis of egg quality, yolk hormones, and yolk lipids

As the vitellogenesis has an average length of 8 days (Lacassagne, 1960), we collected eggs for hormonal and lipid assays during the fifth and sixth weeks of treatment to ensure hens were fully habituated to the diets, and that differences in egg components were a result of the maternal diet treatments. One egg per female was weighed and stored at −20°C for hormonal assay. Eggshells were separated and dried for 24 hr and weighed. Frozen yolk was separated from the albumen and weighed. The weight of albumen was calculated by subtracting the weight of the eggshell and yolk from that of the whole egg. We then determined the ratio of each component relative to the egg mass (yolk mass/egg mass; albumen mass/egg mass; shell mass/egg mass) for each female. A total of 18 eggs were collected per group. The concentrations of immunoreactive progesterone, testosterone, androstenedione, and estrogens were analyzed by EIAs. Details of the extraction protocol are found in (Guesdon et al., 2011). For a full description of the assays including specific antibodies (Palme, Touma, Arias, Dominchin, & Lepschy, 2013). Intra- and inter-assay coefficients of variation were less than 10% and 15%, respectively.

In order to measure yolk lipid content, three yolks per female were pooled and homogenized. We then determined the total lipid content of the yolk and the percentages of saturated, mono-unsaturated, and poly-unsaturated FAs within the lipid fraction. Yolk lipid analysis was conducted by trans-methylation with gas chromatography (Perkin Elmer Autosystem, St. Quentin en Yvelines, France) following the protocol described by Chartrin, Berri, Lebihan-Duval, Quentin, and Baéza (2005).

During the fourth and fifth week of treatment, hens were artificially inseminated three times (same mix of sperm for all the hens). Two hundred and thirty-nine eggs (mean of 5.6 ± 0.4 eggs per female) produced offspring. Eggs were incubated at 37.8°C with 56% relative humidity for 21 days.

2.5 | Chicks and housing conditions

Forty-eight chicks from hens fed the n-3 enriched diet (n-3 chicks) and 50 control chicks (C chicks) were used in this experiment. Chicks were identified with a metal leg-ring. Chicks were housed in non-sibling pairs but with birds from the same diet treatment. We chose not to mix treatments within each cage as potential unknown differences in chicks' social or emotional behavior between treatments may mask prenatal effects. Chicks were housed in plastic cages ($50 \times 40 \times 30\ cm^3$) placed in rows with treatments in alternation within a row (two adjacent cages were from different treatments). All the cages were equally distributed between two identical rooms. Cages had wood shavings on the floor and a wire-mesh lid. Food and water were provided ad libitum. Chicks were fed a postnatal starter mash with a PUFA ratio similar to the maternal diet. We used this diet so that the PUFA ratio of the chick's diet would not counteract the

effects induced by the maternal diet on offspring's brain PUFA levels. This is especially important since precocial birds adapt their feeding behavior to their mother's diet choices (Hess, 1964). Moreover, when the chick's brain experiences a deficiency in PUFAs due to restriction of the maternal diet, it is able to compensate for this deficiency by altering lipid metabolism and demonstrating preferential uptake of the PUFA lacking in the diet (Anderson, Van Winkle, & Connor, 1992). As chicks rely on the vitelline sac reserve for 2/3 days after hatching, any phenotypic differences during the first day of life between chicks of both maternal diets could, therefore, be attributed to maternal diet effects. The n-3 diet administered to experimental chicks had an n-6:n-3 ratio of 1.03, and the control diet had an n-6:n-3 ratio of 8.15. Both diets were iso-caloric (2,900 kcal ME/kg) and iso-nitrogenous (207.2 g CP/kg) differing solely in PUFA composition (electronic supplementary material, Table S1). To determine the sex we looked for the development of the comb at 23 days of age. The body weight of each chick was measured at hatching and at 7 days of age.

2.6 | Behavioral characterization of chicks

To characterize neophobia, behavioral responses to two novel foods and a novel object were assessed with a protocol previously described by (Bertin et al., 2015). Each test was executed at the same age for all chicks. All tests lasted 180 s.

Because chicks become distressed when socially isolated (Hocking, Haldane, Davidson, Sandøe, & Kristensen, 2015), we tested cage mates together (n = 24 pairs of n-3 chicks and n = 25 pairs of C chicks). Testing took place in a separate room within an experimental cage that had the same features as their home cage. Testing commenced 90 min after the feeder was removed from their home cage. Pairs were transported to the test room in a $15 \times 15 \times 15\ cm^3$ plastic container, and were gently deposited in an opaque enclosure ($20 \times 6 \times 20\ cm^3$) within the testing cage, opposite the feeding trough. After 30 s, the enclosure was lifted and removed while chicks remained inside the testing cage. The behavior of one marked focal chick of each pair was recorded by an unseen observer. Focal chicks were chosen randomly at 2 days of age and were tagged with a blue-colored mark on the head. The latency (s) to eat (the moment swallowing was observed) and time spent eating (s) were recorded.

1. *Habituation test/familiarization*: at 3 days of age, chicks were familiarized with the testing cage and handling procedure. As chicks rely on their vitelline sac reserve the first days of life, this test also measured food motivation. The metallic feeding trough of their home cage was placed in the test cage, filled with 200 g of their usual diet.
2. *Food neophobia tests*: we used these tests to assess the immediate reaction to novel foods. Chicks were exposed to changes in sensory properties of the food. At 4 days of age, the first test was done with cracked corn-wheat (cereals contained in familiar pellets). Millet seeds (a novel food) was used at 5 days of age for the second test.
3. *Object neophobia test*: chicks were tested at 6 days of age. The novel object was an unfamiliar feeder containing the familiar diet of the test pair.

2.7 | Statistical analysis

Data were analyzed with Statview (SAS Institute Inc., Cary, NC). The distribution of variables was tested with a Kolmogorov–Smirnov test. Hen and egg data were normally distributed. Groups were compared using a t-test (FCM, mass of egg components, yolk-hormones, yolk-PUFA concentrations) or repeated measures ANOVA (hen body weight, food intake, and laying rate) with the main effect of diet, time, and the interaction between time and diet. The number of chicks hatched (hatching success) and sex ratio between diet groups were compared with a χ^2 test. The weight of chicks at hatching and their growth rate during the first week were analyzed via ANOVA. Offspring behavioral data did not follow a normal distribution even after transformation, so the non-parametric Mann–Whitney U-test was used to compare groups. Data are presented as mean ± SEM, with significance accepted at $p < 0.05$.

3 | RESULTS

3.1 | Dietary effects on laying performance of hens

After 5 weeks of consuming their respective diets, n-3 enriched hens had significantly higher FCM levels than hens of the C diet (398 ± 57 vs. 99 ± 19 ng/g, t = 2.98, p = 0.006).

No effect of diet or interaction between diet and time were observed for body weight, 24 hr-food intake, or laying rate (see Table 1). For food intake, a time effect was observed (time: $F_{4,33} = 15.3$, $p < 0.01$). Hen food intake was lower during the first week compared to the second week for both diets (Table 1).

3.2 | Dietary effects on egg components, yolk hormones, and yolk lipids

The eggs of n-3 hens were significantly lighter than those of C hens (54.9 ± 1.25 vs. 58.0 ± 0.86 g, t= −2.11, p = 0.04). N-3 eggs had a significantly lower yolk mass/egg mass ratio (0.28 ± 0.04 vs. 0.29 ± 0.06, t = 2.47, p = 0.02), and higher albumin mass/egg mass ratio (0.64 ± 0.05 vs. 0.62 ± 0.04, t = −2.07, p = 0.05) than C eggs. Eggshell mass/egg mass ratio did not differ between diets (0.09 ± 0.02 for both diets, t = − 0.41, p = 0.68).

Compared to C hens, the egg yolk of n-3 hens contained a significantly higher concentration of immunoreactive progesterone (t = 2.96, p = 0.006), estrogens (t = 5.97, $p < 0.0001$) and androstenedione (t = 2.01, p = 0.05) with a trend in the same direction for testosterone (t = 1.68, p = 0.10; Figure 1). If instead of yolk hormone concentrations (ng/g) we analyze hormone contents (ng/yolk), the results remained the same except for androstenedione (n-3 eggs vs. n-6 eggs: progesterone: 17897.2 ± 2241.07 vs. 12042.04 ± 1635.8 ng, t = 2.1, p = 0.04; estrogens: 162.5 ± 4.9 vs. 134.05 ± 5.4 ng, t = 3.85, p = 0.0005; androstenedione: 785.6 ± 75.06 vs. 641.7 ± 94.16 ng, t = 1.2, p = 0.23; testosterone: 42.7 ± 4.7 vs. 30.4 ± 5.2 ng, t = 1.77, p = 0.08).

TABLE 1 Mean (±SE) body mass, food intake, and laying rate of adult n-3 hens (N = 18) and control hens (N = 18) during dietary treatment

Parameters	Hens	Week 1	Week 2	Week 3	Week 4	Week 5
Body mass (g)	n-3 enriched	1,958.7 ± 33.9	1,854.5 ± 40.6	1,863.8 ± 37.3	1,835.3 ± 35.3	1,856.6 ± 36.5
	Controls	1,966.6 ± 67.2	1,880.9 ± 60.9	1,974.1 ± 62.5	1,865.4 ± 60.8	1,873.5 ± 62.3
24th feed intake (g)	n-3 enriched	1,54.7 ± 23.4	144.7 ± 7.4	173.7 ± 16.7	194.6 ± 22.8	224.4 ± 28.1
	Controls	1,33.7 ± 11.8	169.6 ± 21.1	161.8 ± 20.5	219.0 ± 24.5	265.4 ± 24.3
Laying rate (number per day)	n-3 enriched	0.78 ± 0.07	0.89 ± 0.03	0.91 ± 0.02	0.78 ± 0.04	0.69 ± 0.06
	Controls	0.71 ± 0.07	0.82 ± 0.06	0.77 ± 0.07	0.69 ± 0.07	0.65 ± 0.06

The percentage of total lipid content in the yolk did not differ between diets (34.7 ± 0.6% vs. 33.1 ± 1.0%, t = 1.36, p = 0.18). Within the lipid fraction of the yolk, the percentage of saturated fatty acids did not significantly differ between diets (33.6 ± 0.47% vs. 33.7 ± 0.37%, t = −0.28, p = 0.78). For n-3 hens, the percentage of mono-unsaturated fatty acids was higher, while the percentage of PUFAs was lower in comparison to eggs from the C hens (mono: 49.5 ± 0.62% vs. 44.0 ± 0.65%, t = −6.2, p < 0.001, PUFA: 16.7 ± 0.36% vs. 22.5 ± 0.60%, t = 8.22, p < 0.001). The yolk of eggs from n-3 hens had significantly higher percentages of n-3 PUFAs with a significantly lower n-6/n-3 ratio, while the yolk of eggs from C hens had significantly higher percentages of n-6 PUFAs and a significantly higher n-6/n-3 ratio (Table 2).

3.3 | Dietary effects on hatching success and weight of chicks

Hatching success did not differ between diets (98 hatched out of 111 fertile eggs for the n-3 group vs. 125 out of 138 fertile eggs for the n-6 group; χ^2-test, p = 0.67), neither did sex-ratio (25 females and 23 males in the n-3 group and 28 females and 22 males in the C group, χ^2-test, p = 0.69). N-3 chicks were lighter at hatching than C chicks (39.4 ± 0.4 g vs. 40.9 ± 0.5 g, t = −2.29, p = 0.02) but did not differ significantly in growth rate during the first week of life (8.60 ± 0.16 vs. 9.72 ± 1.86 g/week, $F_{3,93}$ = 0.66, p = 0.57).

3.4 | Dietary effects on neophobic behavior in the chicks

Latencies to eat and the time spent eating did not differ significantly between n-3 and C chicks in the habituation/familiarization test and both food neophobia tests (Table 3). In the novel object test, n-3 chicks demonstrated a longer latency to eat and spent less time eating from the novel object compared to C chicks (Table 3).

4 | DISCUSSION

The present experiment was designed to test the hypothesis that PUFA in the maternal diet could engender differential allocation of

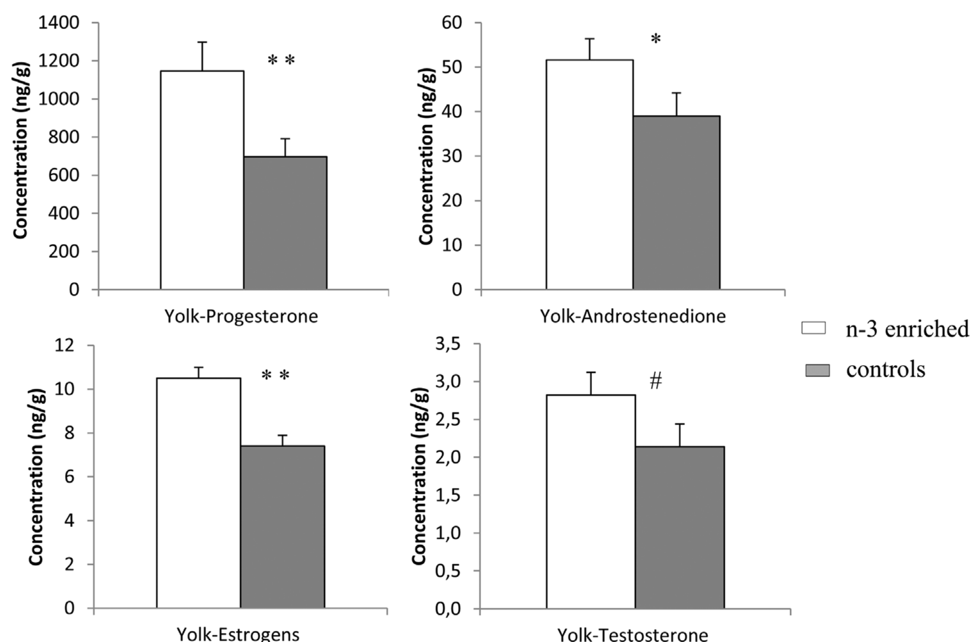

FIGURE 1 Mean (±SE) yolk progesterone (P4), androstenedione (A4), estrogens (E), and testosterone (T) concentrations (ng/g of yolk) in the eggs from n-3 enriched hens (N = 18) and control hens (N = 18). **p < 0.01; *p ≤ 0.05; #0.1 ≤ p ≥ 0.05

TABLE 2 Mean (±SE) percentages of n-3 and n-6 poly-unsaturated fatty acids in the yolk of eggs of hens fed a diet enriched in n-3 (N = 18) or a control diet (N = 18)

	Diets	
Poly-unsaturated fatty acids	n-3 enriched	Control
n-6 fatty acids		
Linoleic acid (LA), C18:2 n-6	13.5% ± 0.23^{a}	18.9% ± 0.47^{b}
Arachidonic acid (AA), C20:4 n-6	0.64% ± 0.05^{a}	1.81% ± 0.18^{b}
Docosatetraenoic acid (DTA), C22:4 n-6	0.03% ± 0.005^{a}	0.22% ± 0.02^{b}
n-3 fatty acids		
α-linoleic acid (ALA), C18:3 n-3	0.47% ± 0.02^{a}	0.71% ± 0.03^{b}
Eicosapentaenoic acid (EPA), C20:5 n-3	0.22% ± 0.02^{a}	0.00% ± 0.0^{b}
Docosapentaenoic acid (DPA),C22:5 n-3	0.15% ± 0.01^{a}	0.07% ± 0.02^{b}
Docosahexaenoic acid (DHA) C22:6 n-3	1.72% ± 0.17^{a}	0.74% ± 0.07^{b}
Ratio n-6/n-3	6.15% ± 0.53^{a}	13.9% ± 0.42^{b}

Means between columns with a different superscript (a, b) differ (p < 0.05)

yolk hormones of maternal origin. We found higher corticosterone metabolites, reflecting heightened HPA-activity, in the fecal droppings of hens fed the diet enriched in n-3 compared to control animals. As expected, yolk n-6:n-3 ratio strongly reflected that of the maternal diet. There were significantly higher concentrations of yolk progesterone, androstenedione, and estrogens, as well as a trend for higher yolk testosterone in eggs of hens fed the n-3 enriched diet compared to controls. Chicks of hens fed the n-3 enriched diet showed a lower body weight at hatching and higher neophobia when exposed to a novel object compared to chicks of hens fed the control diet.

After 6 weeks of consuming a diet enriched in n-3 PUFAs, hens had elevated concentrations of corticosterone metabolites in the feces and elevated levels of yolk hormones compared to hens fed a control diet. Additionally, hens of the n-3 diet laid lighter eggs, with lower yolk/egg mass ratios than controls. Diets were equal in protein and energy content, and were selected to meet the dietary requirements of laying hens (Van Elswyk, 1997). Food intake and body weight followed a similar pattern for n-3 and control hens. Thus, differences between groups cannot be explained by differences in the energy content of the diet or differences in food intake. Rather, they demonstrate an influence of n-6/n3 PUFA ratio in the diet on the metabolism and physiology of hens.

These results suggest a potential action for the n-6:n-3 PUFA ratio of diet on the HPA-axis of hens as reported in other species (Fisher et al., 2014; Lafourcade et al., 2011; Larrieu et al., 2014). First, hens of the n-3 diet had FCM levels more than three-times greater than controls. FCM levels have been shown to reflect HPA activity (Rettenbacher & Palme, 2009). Second, in chickens with elevated corticosterone levels due to a corticosterone implant, egg mass, yolk mass, and hatchling's weight reduction have also been recorded (Henriksen, Groothuis et al., 2011; Henriksen, Rettenbacher, & Groothuis, 2011) which is comparable to our chicks from hens treated with n-3. Moreover, in birds, environmental stress can induce HPA axis activation, causing a decrease in egg and offspring weight (Hayward & Wingfield, 2004; Henriksen, Groothuis, et al., 2011; Hsu, Dijkstra, Darras, de Vries, & Groothuis, 2016). Third, it is known that maternal environmental stress induces similar increases in hens' yolk hormones as we found in the hens fed the n-3 diet (Bertin et al., 2013; Guibert et al., 2010). In mammals, the effects of n-3 PUFAs on HPA axis functioning are generally beneficial, but not always. As in mice, a deficiency of n-3 PUFA can alter corticosterone secretion via modifications in glucocorticoid receptors (GRs) of the prefrontal cortex, but not in the HPA-axis (Larrieu, Hilal, De Smedt-Peyrusse, Sans, & Laye, 2016). In the chicken, GRs are also found in the pituitary

TABLE 3 Mean (±SE) Latency to eat and time spent eating during neophobia tests of n-3 chicks (N = 24 pairs) and control chicks (N = 25 pairs) and Mann–Whitney U-test outcome

Neophobia tests	Parameters measured	n-3 chicks	Control chicks	Z	p-values
Habituation	Latency to eat (s)	40.5 ± 3.3	48.3 ± 6.5	−0.66	0.51
	Time spent eating (s)	48.9 ± 3.8	52.1 ± 5.2	−1.07	0.28
Novel food	Latency to eat (s)	99.1 ± 13.2	85.4 ± 10.6	−0.30	0.76
Test 1	Time spent eating (s)	18.4 ± 3.7	22.8 ± 3.8	−0.90	0.36
Novel food	Latency to eat (s)	84.3 ± 13.9	107.8 ± 10.2	−0.96	0.33
Test 2	Time spent eating (s)	32.0 ± 9.1	24.2 ± 5.9	−0.15	0.88
Novel object	Latency to eat (s)	174.1 ± 5.9	155.2 ± 8.5	−2.11	0.03
	Time spent eating (s)	2.1 ± 2.0	14.11 ± 5.1	−2.27	0.02

(Bossis, Nishimura, Muchow, & Porter, 2004) and frontal brain areas (Bordone, Schrott, & Sparber, 1997). Glucocorticoid receptors can be modified by stressful conditions in a variety of avian species (Japanese quail, Zimmer & Spencer, 2014; starlings, Dickens, Romero, Cyr, Dunn, & Meddle, 2009; house sparrow, Lattin & Romero, 2014) and play a role in the lipid metabolism of the white-crowned sparrow (Landys, Piersma, Ramenofsky, & Wingfield, 2004) (Landys, Ramenofsky, Guglielmo, & Wingfield, 2004). Our results on HPA-functioning are a likely consequence of the surplus of n-3 in the experimental diet. This may have resulted in a lack of arachidonic acid (AA), which is essential for metabolic and hormonal functioning (Huang, Leibovitz, Lee, & Millar, 1990). Based on our results, we hypothesize that the ratio of n-6:n-3 PUFAs in the diet influence HPA activity in birds, but the mechanisms require further investigation. It is important to notice that domestic chickens have been raised for food thousands of years ago and were mainly fed with wheat seeds. As wheat seeds are poor in n-3 and rich in n-6 fatty acids, these animals may have developed specific adaptations and, as a consequence, may cope better with high n-6:n-3 ratios than with low n-6:n-3 ratios in the diet.

Egg mass and yolk proportion was reduced in n-3 hens. These results are in accordance with those obtained by administering diets enriched with 2–4% fish oil (Gonzalez-Esquerra & Leeson, 2000; Huang et al., 1990) in which hens' egg mass was found to decrease linearly with increasing percentages of fish oil (high in n-3). Diets enriched with fish oil can increase albumin production and decrease yolk production compared to control diets (Huang et al., 1990). The n-3 diet we applied contained little linoleic acid (a molecular precursor essential to the production of n-6 PUFA AA). Moreover, AA production appeared to be strongly inhibited by the dominance of docohexaenoic acid (DHA) in the n-3 diet as demonstrated by the amount measured in the yolk. This could severely hamper AA metabolism, resulting in metabolic changes in the hens receiving the n-3 diet, and thereby altering egg composition (Huang et al., 1990). PUFAs in the maternal diet can thus have an important effect on the amount and constitution of the resources available for embryo development.

Moreover, our data showed that fish oil in the diet led to higher concentrations of yolk progesterone, androstenedione, and oestradiol and a trend for higher concentrations of yolk testosterone. These results add support to the hypothesis that the nutritional state of female birds causes variation in yolk hormone levels. However, at the present stage, the interpretation of the mechanisms that mediate variations in yolk hormone levels is bound to be speculative. As mentioned previously, it could be that increased levels of yolk hormones are the result of HPA axis activation in the mother. In accordance with this hypothesis, recent studies showed that maternal plasma corticosterone levels influence reproductive hormone concentrations in the yolk (Henriksen, Groothuis et al., 2011). The underlying physiological mechanisms remain poorly understood and are barely addressed in the literature. As argued before, it might be possible that corticosterone levels affect the steroidogenic activity of follicles. Another hypothesis could be related to the direct influence of PUFAs and their oxygenated derivatives on steroid synthesis. Several factors of the steroidogenic machinery are directly regulated by AA and AA-derived eicosanoids (Wathes, Abayasekara, & Aitken, 2007). The n-3/n-6 PUFA ratio in the diet largely determines the balance between the various types of oxygenated metabolites deriving from AA or from EPA and DHA (Yates, Calder, & Rainger, 2014). Therefore, the decreased AA/DHA + EPA ratio and the resulting eicosanoid pattern in n-3 supplemented hens may have directly contributed to the observed differences in yolk hormone levels. Consistently, dietary fat (such as those derived from fish oil) in the maternal diet is known to influence egg weight and plasma oestrogen levels (Whitehead, 1995), a role for PUFAs in the hormonal metabolism of birds seems, therefore, a plausible explanation.

We found that n-3 chicks were more inhibited (longer latency to eat and less time spent eating) than C chicks in the novel object test. This result indicates a higher level of object neophobia in n-3 chicks than C chicks. On the other hand, n-3 and C chicks did not differ in food neophobia test. Since the n-3 maternal diet modified the embryonic environment in several ways (yolk mass, yolk hormones, and fatty acids), it is difficult to determine which mechanisms and which hormones affected object neophobia. Little is currently known regarding the developmental influence of yolk progesterone and androgens on neophobia. The influence of progesterone is barely investigated. In Northern bobwhite quail (*Colinus virginianus*), a recent study showed that elevated yolk progesterone levels elevate emotional reactivity in neonates (Herrington, Vallin, & Lickliter, 2015). Interestingly, an artificial increase in yolk hormone levels has been found to alter object neophobia but not food neophobia in domestic chicks (Bertin et al., 2015), but the direction of effects was not the same as in the present study. Lower object neophobia was found in chicks exposed in ovo to increased yolk progesterone, androstenedione and estradiol levels compared to controls. Discrepancies in the direction of yolk hormonal effects are also seen in latencies to approach novel objects depending on species and the injected dose (Vergauwen, Eens, & Muller, 2012). Nevertheless, all of these experiments suggest that variation in yolk hormone levels can alter specific aspects of neophobia.

The data concerning the effects of environmental conditions during ontogeny on the development of object or food neophobia are inconclusive. In accordance with our findings, zebra finch chicks (*Taeniopygia guttata*) from eggs with artificially elevated yolk testosterone show no difference in latency to eat novel foods than control animals (Tobler & Sandell, 2007). Similarly, in canaries (*Serinus canaria*), increased levels of yolk testosterone was found to not be the primary source of variation in the expression of food neophobia (Vergauwen, Eens, et al., 2012). Our data highlight the importance of studying the quality of the maternal diet in order to understand more fully the ontogeny of emotional and behavioral traits involved in adapting to new resources. This could be of particular importance when food resources become scarcer under the projected future of climate change. In captive bird populations (for production or conservation purposes), no specific recommendation is made regarding PUFA quantity and quality in the diet. This study highlights the importance of carefully considering food quality in captive bird populations as they might engender transgenerational effects on development and behavior. In our study, chicks were all feeding

around 2/3 days after hatching, therefore, we cannot totally exclude the possibility that the behavior of the chicks was influenced by their respective diet. Two points are of importance when considering this hypothesis. First, even when chicks start feeding, the residual yolk continues to be taken up and thereby provides a source of fatty acids of maternal origin (Romanoff, 1960). Second, in the domestic chicken and king penguin (*Aptenodytes patagonicus*) it was consistently found that the proportion of fatty acids in the brain phospholipids was particularly refractory to the effects of the chicks' diet compared to other tissues (Anderson et al., 1992; Thil, Speake, & Groscolas, 2003). More specifically, high levels of brain n-3 produced by exposure to a maternal diet rich in fish oil are not reversible on a short time period (Anderson et al., 1992). Although caution must be exercised, we argue that maternal effects more likely contributed to the differences observed in hatchlings' mass and behavior than the post-natal diet.

The present data show maternal diet composition as an overlooked pathway by which the environment could engender large variation in yolk hormones levels. This may play a role in the development of offspring behavior and phenotype. In farm birds, as unresolved welfare issues are mediated by unknown cues, this study lends support to perspectives in the field of maternal nutrition. Further investigations to the mechanisms are required but this study suggests common principles regarding the regulative action of n-3 and n-6 PUFA on fear-related behavior across vertebrate taxa. Using precocial birds could yield new insights into the mechanism by which the prenatal resources in PUFA influences behavioral development of individuals in vertebrates.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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