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

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3 **1 Lipids in maternal diet influence yolk hormone levels and post-hatch**
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6 **2 neophobia in the domestic chick.**
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54 25 **Running title:** maternal diet engenders maternal effects
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5 28 We assessed whether the ratio of dietary n-6/n-3 polyunsaturated fatty acids (PUFA)
6
7 29 during egg formation engenders transgenerational maternal effects in domestic chicks. We
8
9
10 30 analysed yolk lipid and hormone concentrations, and HPA-axis activity in hens fed a control
11
12 31 diet (high n-6/n-3 ratio) or a diet enriched in n-3 PUFAs (low n-6/n-3 ratio) for six
13
14 32 consecutive weeks. Their chicks were tested for neophobia during the first week of life. We
15
16 33 found higher corticosterone metabolites in droppings of hens fed the diet enriched in n-3 and
17
18 34 significantly higher concentrations of yolk progesterone, androstenedione and estradiol in
19
20 35 their eggs compared to controls. Chicks of hens fed the n-3 enriched diet showed a lower
21
22 36 body mass at hatch than controls and expressed higher neophobia when exposed to a novel
23
24 37 object. These results add support to the hypothesis that the nutritional state of female birds
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26 38 produces variation in yolk hormone levels and engender maternal effects.
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48 **Keywords:** *Gallus gallus domesticus*, maternal effects, yolk hormones, poly unsaturated fatty
49 acids, neophobia

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3 52 Understanding the reasons for phenotypic variation is one of the cornerstones of
4
5 53 behavioural biology. Non-genetic maternal effects play a key role in generating phenotypic
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7 54 variation in many taxa (Ledón-Rettig, Richards, & Martin, 2013). In birds, prenatal maternal
8
9 55 effects can affect the morphology, behaviour and physiology of offspring (Ruuskanen, 2015).
10
11 56 Such effects are partly mediated via variation in yolk-hormone concentrations of maternal
12
13 57 origin according to different environmental conditions (Groothuis, Muller, von Engelhardt,
14
15 58 Carere, & Eising, 2005). Yolk androgens (mainly testosterone and its precursor
16
17 59 androstenedione) have been widely studied in wild bird populations. Differential allocation of
18
19 60 androgens into eggs is presumed to be a way to adapt offspring to their hatching environment
20
21 61 (Gil, 2008; Groothuis, et al., 2005). Androgen concentrations can vary substantially within
22
23 62 and between egg clutches as a consequence of the maternal environment; factors such as
24
25 63 social conditions (density, aggressive interactions, mate quality) (Kingma et al., 2009; Pilz &
26
27 64 Smith, 2004; Schwabl, 1997), parasitic infection (Müller, Heylen, Eens, Rivera-Gutierrez, &
28
29 65 Groothuis, 2013) and predation risk (Coslovsky, Groothuis, de Vries, & Richner, 2012) can
30
31 66 influence hormone production. Because yolk hormone levels show such a strong context-
32
33 67 dependency within species (Ruuskanen, 2015), the specific environmental cues triggering
34
35 68 maternal effects must be elucidated further.

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41 69 Recently, food resource availability has been identified as an environmental factor
42
43 70 engendering differential maternal hormone allocation. In the lesser black-backed gull (*Larus*
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45 71 *fuscus*), pied flycatcher (*Ficedula hypoleuca*) and black-legged kittiwake (*Rissa tridactyla*)
46
47 72 food supplementation prior to, and during oviposition affected androgen quantity in the yolk
48
49 73 (Verboven et al., 2003; Verboven, Monaghan, Nager, & Evans, 2010). While in great tits
50
51 74 (*Parus major*), no effect of food supplementation on yolk androgens has been recorded (S.
52
53 75 Ruuskanen, Darras, de Vries, Visser, & Groothuis, 2016). Manipulation of food resources
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55 76 also shows to increase the within clutch variation of yolk-hormones in canaries (*Serinus*
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3 77 *canaria*) (Jonas Vergauwen, Goerlich, Groothuis, Eens, & Müller, 2012) and zebra finches
4
5 78 (*Taeniopygia guttata*) (Sandell, Adkins-Regan, & Ketterson, 2007). These studies
6
7 79 demonstrate that the nutritional state of maternal birds during oviposition contributes to
8
9 80 differences in yolk hormone concentrations between or within a clutch. In the reported
10
11 81 studies, enrichment of the maternal diet was achieved by providing additional protein and/or
12
13 82 lipids from different sources (e.g. seeds, boiled eggs). This procedure leaves open the question
14
15 83 on the influence of the nutritional composition of the food provided to the mother. To our
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17 84 knowledge the effect of the quality of maternal diet on egg hormone levels and offspring
18
19 85 phenotype hasn't been investigated so far in birds.
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24 86 This question is of particular importance for factory-farmed birds which are fed an
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26 87 unique diet. The domestic chicken (*Gallus gallus domesticus*) is the most abundant bird
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28 88 species on the earth but, despite this importance, many avenues of research remained to be
29
30 89 explored in order to advance our knowledge on maternal effects and behavioral development
31
32 90 (Dixon, Sparks, & Rutherford, 2016). Housing conditions (Janczak, Torjesen, &
33
34 91 Rettenbacher, 2009), unpredictable access to food (Janczak, Torjesen, Palme, & Bakken,
35
36 92 2007; Nätt et al., 2009), maternal social status (Muller, Eising, Dijkstra, & Groothuis, 2002),
37
38 93 early social stress (Goerlich, Nätt, Elfving, Macdonald, & Jensen, 2012) and thermal
39
40 94 environment (Bertin et al., 2013) cause variation in yolk androgens and progesterone levels in
41
42 95 domestic hens. This caused modifications in growth, feeding behaviors, and emotional
43
44 96 reactivity of their chicks (Goerlich, Nätt, Elfving, Macdonald, & Jensen, 2012; Nätt et al.,
45
46 97 2009; Bertin et al., 2013). In domestic hens, maternal stress during egg formation —mimicked
47
48 98 with subcutaneous corticosterone implantation— increased plasma corticosterone levels and
49
50 99 decreased the synthesis of reproductive hormones which accumulate in the yolk (Henriksen,
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52 100 Groothuis, & Rettenbacher, 2011). In addition, on-farm longitudinal observations showed that
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54 101 parental stress physiology correlate with offspring's anxiety and expression of damaging
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3 102 behavior (de Haas, Bolhuis, Kemp, Groothuis, & Rodenburg, 2014; Rodenburg & de Haas,
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5 103 2016).
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8 104 In domestic hens we previously showed that adding fish oil to the maternal diet
9
10 105 induced food neophobia in the offspring (Aigueperse, Calandreau, & Bertin, 2013). As fish oil
11
12 106 is particularly rich in n-3 poly-unsaturated fatty acids (PUFA), this result suggested that
13
14 107 PUFA in the maternal diet may directly affect the development of behaviour in the offspring.
15
16 108 The aim of the present study was to test this hypothesis and assess whether the ratio between
17
18 109 n-6 and n-3 PUFA in the maternal diet during egg formation could engender maternal effects.
19
20 110 N-3 and n-6 PUFA unambiguously influence the development of the central nervous system
21
22 111 in both mammalian and avian species (Noble & Cocchi, 1989). But these effects are better-
23
24 112 understood for mammals. In mammals, PUFAs and their mediators are known to regulate
25
26 113 many processes within the brain, such as neurotransmission, cell survival and neuro-
27
28 114 inflammation, and also affect hypothalamic–pituitary–adrenal (HPA) axis responses, thereby
29
30 115 possibly playing a role in anxiety-like behavior and cognition (e.g. Bazinet & Layé, 2014;
31
32 116 Lafourcade et al., 2011). In the domestic chicken, PUFA ratios in the maternal diet are
33
34 117 transferred to the egg yolk (Noble & Cocchi, 1990) and subsequently to the tissues of the
35
36 118 developing chick embryo (Maldjian, Farkas, Noble, Cocchi, & Speake, 1995). Numerous
37
38 119 studies show that the PUFA profile of the developing chick brain reflects the PUFA
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40 120 composition in the yolk (Cherian & Sim, 1993; Anderson, Connor, Corliss, & Lin, 1989) but
41
42 121 the consequences on behavioral development remain to be investigated.
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49 122 We analysed yolk lipid and hormone concentrations, and HPA-axis activity in hens
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51 123 fed a control diet (high n-6/n-3 ratio) or a diet enriched in n-3 PUFAs (low n-6/n-3 ratio).
52
53 124 Development and neophobia (i.e. fear of novelty) of the hatchlings were analysed. Neophobia
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55 125 is considered to be a temperament trait which can impair an animal's capacity to adapt to new
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57 126 resources, new habitats and express behavioural innovation (Greenberg & Mettke-hofmann,
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3 127 2001; Webster & Lefebvre, 2001). As a high level of neophobia can reduce fitness (Brown,
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5 128 Ferrari, Elvidge, Ramnarine, & Chivers, 2013), this behavioural trait is a growing topic of
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7 129 interest for behavioural biologists (Camín, Martín-Albarracín, Jefferies, & Marone, 2016;
8
9 130 Greggor, Thornton, & Clayton, 2015). The precocial domestic chick is a particularly
10
11 131 appropriate model in which to analyse developmental influences on neophobia since chicks
12
13 132 start food selection soon after hatching (Bertin et al., 2010). In addition, under commercial
14
15 133 conditions the refusal to accept new foods leads to a major reduction in feed intake and,
16
17 134 subsequently, in growth and animal welfare (Murphy, 1977).
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23 136 **Methods**

26 137 *Ethics statement*

28
29 138 All birds were maintained at the Experimental Unit PEAT of INRA (Nouzilly, France). The
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31 139 Experimental Unit is registered by the ministry of Agriculture with the license number B-37-
32
33 140 175-1 for animal experimentation. All experiments were approved by the Ethic Committee in
34
35 141 Animal Experimentation of Val de Loire, CEEA Vdl (reference number 02153.02). The
36
37 142 CEEA vdl is registered by the National Committee “Comité National de Réflexion Ethique sur
38
39 143 l’Expérimentation Animale” under the number 19. All experiments were performed in
40
41 144 accordance with the European Communities Council Directive 2010/63/UE. All animals were
42
43 145 sold for rehabilitation at the end of the experiment.
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48 146 *Maternal hens and diets*

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51 147 Hens (n=40) and diets were provided by the experimental unit PEAT of INRA (Nouzilly,
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53 148 France). Two groups of 20 one-year old White Leghorn laying hens were matched for body
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55 149 weight, and divided into two dietary treatment groups, a control diet and a diet enriched in n-3
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57 150 FA. Diets were administered for six consecutive weeks. The control diet contained soya-oil
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3 151 (3%) with a n-6:n-3 ratio of 7.99, a value within the range recommended for commercial
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5 152 laying hens (Van Elswyk, 1997). The n-3 diet contained 3% menhaden fish-oil (Sigma Life
6
7 153 Science) having an n-6:n-3 ratio of 0.82. Diets were iso-caloric (2820 kcal ME/kg) and iso-
8
9
10 154 nitrogenous (170 g CP/kg), differing only in the composition of PUFAs (electronic
11
12 155 supplementary material, table S1). Hens were housed individually in wire-mesh cages of 100
13
14 156 x 100 x 50 cm (*l x w x h*), with the possibility of tactile, visual, and vocal contact with
15
16 157 neighbouring hens. Cages contained wood shavings and a wooden perch on the floor, a nest
17
18 158 enclosed by red curtain flaps, a nipple drinker and a feed trough. Water and food were
19
20 159 provided *ad libitum*. Four hens were excluded from the experiment, three due to a lack of
21
22 160 habituation to the experimental diets (food conservatism) and one death unrelated to the diet
23
24
25 161 treatment, resulting in n =18 hens per diet group.

26 27 28 162 *Morpho-physiological measurements of hens*

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30
31 163 All hens were weighed once per week. 24 h-feed intake per hen was measured once a week by
32
33 164 calculating the difference in weight of the feeder before and after 24 h. The number of eggs
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35 165 laid was recorded daily. Laying rate was calculated as a percentage of the number of eggs laid
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37 166 per female per day.

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39
40 167 In order to evaluate HPA activity in chickens, faecal corticosterone metabolite (FCM)
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42 168 concentrations were measured (Rettenbacher, Mostl, Hackl, Ghareeb, & Palme, 2004). In the
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44 169 sixth week of diet treatment, one fresh faecal dropping per hen was collected from the home
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46 170 cage. Urine was removed from the sample as the concentrations of FCM in the liquid portions
47
48 171 of droppings are more likely to represent acute HPA activity caused by handling or disturbing
49
50 172 the animals during collection (Rettenbacher, et al., 2004). Each sample was homogenized and
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52 173 stored at -20°C. From each sample an aliquot (0.5 g) was extracted with 60% methanol
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3 174 (Mostl, Spendier, & Kotrschal, 2001) and analysed by a cortisone enzyme immunoassay
4
5 175 (EIA) validated for chickens and fully described in (Rettenbacher, et al., 2004).
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8 176 *Analysis of egg quality, yolk hormones and yolk lipids*
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10
11 177 As the vitellogenesis has an average length of 8 days (Lacassagne, 1960), we collected eggs
12
13 178 for hormonal and lipid assays during the fifth and sixth weeks of treatment to ensure hens
14
15 179 were fully habituated to the diets, and that differences in egg components were a result of the
16
17 180 maternal diet treatments. One egg per female was weighed and stored at -20°C for hormonal
18
19 181 assay. Eggshells were separated and dried for 24 h and weighed. Frozen yolk was separated
20
21 182 from the albumen and weighed. The weight of albumen was calculated by subtracting the
22
23 183 weight of the eggshell and yolk from that of the whole egg. We then determined the ratio of
24
25 184 each component relative to the egg mass (yolk mass / egg mass; albumen mass / egg mass;
26
27 185 shell mass / egg mass) for each female. A total of 18 eggs were collected per group. The
28
29 186 concentrations of immunoreactive progesterone, testosterone, androstenedione and oestrogens
30
31 187 were analysed by EIAs. Details of the extraction protocol are found in (Guesdon et al., 2011).
32
33 188 For a full description of the assays including specific antibodies, see (Palme, Touma, Arias,
34
35 189 Dominchin, & Lepschy, 2013). Intra- and inter-assay coefficients of variation were less than
36
37 190 10% and 15%, respectively.
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43 191 In order to measure yolk lipid content, three yolks per female were pooled and homogenized.
44
45 192 We then determined the total lipid content of the yolk and the percentages of saturated, mono-
46
47 193 unsaturated and poly-unsaturated FAs within the lipid fraction. Yolk lipid analysis was
48
49 194 conducted by trans-methylation with gas chromatography (Perkin Elmer Autosystem, St.
50
51 195 Quentin en Yvelines, France) following the protocol described in (Chartrin, Berri, Lebihan-
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53 196 Duval, Quentin, & Baéza, 2005).
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3 197 During the fourth and fifth week of treatment, hens were artificially inseminated three times
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5 198 (same mix of sperm for all the hens). Two hundred and thirty nine eggs (mean of 5.6 ± 0.4
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7 199 eggs per female) produced offspring. Eggs were incubated at 37.8°C with 56% relative
8
9 200 humidity for 21 days.

11
12 201 *Chicks and housing conditions*

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14
15 202 Forty-eight chicks from hens fed the n-3 enriched diet (n-3 chicks) and 50 control chicks (C
16
17 203 chicks) were used in this experiment. Chicks were identified with a metal leg-ring. Chicks
18
19 204 were housed in non-sibling pairs but with birds from the same diet treatment. We chose not to
20
21 205 mix treatments within each cage as potential unknown differences in chicks' social or
22
23 206 emotional behaviour between treatments may mask prenatal effects. Chicks were housed in
24
25 207 plastic cages (50 cm x 40 cm x 30 cm) placed in rows with treatments in alternation within a
26
27 208 row (two adjacent cages were from different treatments). All the cages were equally
28
29 209 distributed between two identical rooms. Cages had wood shavings on the floor and a wire-
30
31 210 mesh lid. Food and water were provided *ad libitum*. Chicks were fed a postnatal starter mash
32
33 211 with a PUFA ratio similar to the maternal diet. We used this diet so that the PUFA ratio of the
34
35 212 chick's diet would not counteract the effects induced by the maternal diet on offspring's brain
36
37 213 PUFA levels. This is especially important since precocial birds adapt their feeding behaviour
38
39 214 to their mother's diet choices (Hess, 1964). Moreover, when the chick's brain experiences a
40
41 215 deficiency in PUFAs due to restriction of the maternal diet, it is able to compensate for this
42
43 216 deficiency by altering lipid metabolism and demonstrating preferential uptake of the PUFA
44
45 217 lacking in the diet (Anderson, Van Winkle, & Connor, 1992). As chicks rely on the vitelline
46
47 218 sac reserve for 2/3 days after hatching, any phenotypic differences during the first days of life
48
49 219 between chicks of both maternal diets could therefore be attributed to maternal diet effects.
50
51 220 The n-3 diet administered to experimental chicks had an n-6:n-3 ratio of 1.03, and the control
52
53 221 diet had an n-6:n-3 ratio of 8.15. Both diets were iso-caloric (2900 kcal ME/kg) and iso-
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3 222 nitrogenous (207.2 g CP/kg) differing solely in PUFA composition (electronic supplementary
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5 223 material, table S1). To determine the sex we looked for the development of the comb at 23
6
7 224 days of age. The body weight of each chick was measured at hatching and at seven days of
8
9 225 age.

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11
12 226 *Behavioural characterization of chicks*

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14
15 227 To characterize neophobia, behavioural responses to two novel foods and a novel object were
16
17 228 assessed with a protocol previously described by (Bertin et al., 2015). Each test was executed
18
19 229 at the same age for all chicks. All tests lasted 180 s.

20
21
22
23 230 Because chicks become distressed when socially isolated (Hocking, Haldane, Davidson,
24
25 231 Sandøe, & Kristensen, 2015), we tested cage mates together (n = 24 pairs of n-3 chicks and n
26
27 232 = 25 pairs of C chicks). Testing took place in a separate room within an experimental cage
28
29 233 that had the same features as their home cage. Testing commenced 90 min after the feeder
30
31 234 was removed from their home cage. Pairs were transported to the test room in a 15 cm × 15
32
33 235 cm × 15 cm plastic container, and were gently deposited in an opaque enclosure (20 cm × 6
34
35 236 cm × 20 cm) within the testing cage, opposite the feeding trough. After 30 s, the enclosure
36
37 237 was lifted and removed while chicks remained inside the testing cage. The behaviour of one
38
39 238 marked focal chick of each pair was recorded by an unseen observer. Focal chicks were
40
41 239 chosen randomly at two days of age and were tagged with a blue-coloured mark on the head.
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43 240 The latency (s) to eat (the moment swallowing was observed) and time spent eating (s) were
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45 241 recorded.

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50 242 (i) *Habituation test/familiarization*: at three days of age, chicks were familiarized with the
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52 243 testing cage and handling procedure. As chicks rely on their vitelline sac reserve the first days
53
54 244 of life, this test also measured food motivation. The metallic feeding trough of their home
55
56 245 cage was placed in the test cage, filled with 200 g of their usual diet.

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3 246 (ii) *Food neophobia tests*: we used these tests to assess the immediate reaction to novel foods.
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5 247 Chicks were exposed to changes in sensory properties of the food. At four days of age, the
6
7 248 first test was done with cracked corn-wheat (cereals contained in familiar pellets). Millet
8
9 249 seeds (a novel food) was used at five days of age for the second test.

10
11
12 250 (iii) *Object neophobia test*: chicks were tested at six days of age. The novel object was an
13
14 251 unfamiliar feeder containing the familiar diet of the test pair.

15 16 17 18 252 **Statistical analysis**

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20
21 253 Data were analyzed with Statview (SAS Institute Inc., Cary, NC). The distribution of
22
23 254 variables was tested with a Kolmogorov-Smirnov test. Hen and egg data were normally
24
25 255 distributed. Groups were compared using a t-test (FCM, mass of egg components, yolk-
26
27 256 hormones, yolk-PUFA concentrations) or repeated measures ANOVA (hen body weight, food
28
29 257 intake and laying rate) with the main effect of diet, time, and the interaction between time and
30
31 258 diet. The number of chicks hatched (hatching success) and sex ratio between diet groups were
32
33 259 compared with a Chi-square test. The weight of chicks at hatching and their growth rate
34
35 260 during the first week were analyzed via ANOVA. Offspring behavioural data did not follow a
36
37 261 normal distribution even after transformation, so the non-parametric Mann-Whitney *U*-test
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39 262 was used to compare groups. Data are presented as mean \pm SEM, with significance accepted
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41 263 at $p < 0.05$.

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46 47 48 265 **Results**

49
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51 266 *Dietary effects on laying performance of hens*
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3 267 After five weeks of consuming their respective diets, n-3 enriched hens had significantly
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5 268 higher FCM levels than hens of the C diet (398 ± 57 ng/g vs. 99 ± 19 ng/g, $t = 2.98$, $p =$
6
7 269 0.006).

8
9
10 270 No effect of diet or interaction between diet and time were observed for body weight, 24h-
11
12 271 food intake, or laying rate (see Table 1). For food intake, a time effect was observed (time:
13
14 272 $F_{4,33} = 15.3$, $p < 0.01$). Hen food intake was lower during the first week compared to the
15
16 273 second week for both diets (Table 1).

17
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19
20 274 >>> INSERT Table 1 <<<

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23 275 *Dietary effects on egg components, yolk hormones and yolk lipids*

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25
26 276 The eggs of n-3 hens were significantly lighter than those of C hens (54.9 ± 1.25 g vs. $58.0 \pm$
27
28 277 0.86 g, $t = -2.11$, $p = 0.04$). N-3 eggs had a significantly lower yolk mass/egg mass ratio (0.28
29
30 278 ± 0.04 vs. 0.29 ± 0.06 , $t = 2.47$, $p = 0.02$), and higher albumin mass/egg mass ratio ($0.64 \pm$
31
32 279 0.05 vs. 0.62 ± 0.04 , $t = -2.07$, $p = 0.05$) than C eggs. Eggshell mass/egg mass ratio did not
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34 280 differ between diets (0.09 ± 0.02 for both diets, $t = -0.41$, $p = 0.68$).

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38 281 Compared to C hens, the egg yolk of n-3 hens contained a significantly higher concentration
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40 282 of immunoreactive progesterone ($t = 2.96$, $p = 0.006$), oestrogens ($t = 5.97$, $p < 0.0001$) and
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42 283 androstenedione ($t = 2.01$, $p = 0.05$) with a trend in the same direction for testosterone ($t =$
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44 284 1.68 , $p = 0.10$; Figure 1). If instead of yolk hormone concentrations (ng/g) we analyse
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46 285 hormone contents (ng/yolk), the results remained the same except for androstenedione (n-3
47
48 286 eggs vs. n-6 eggs: progesterone: 17897.2 ± 2241.07 ng vs. 12042.04 ± 1635.8 ng, $t = 2.1$, $p =$
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50 287 0.04 ; oestrogens: 162.5 ± 4.9 ng vs. 134.05 ± 5.4 ng, $t = 3.85$, $p = 0.0005$; androstenedione:
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52 288 785.6 ± 75.06 ng vs. 641.7 ± 94.16 ng, $t = 1.2$, $p = 0.23$; testosterone: 42.7 ± 4.7 ng vs. $30.4 \pm$
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54 289 5.2 ng, $t = 1.77$, $p = 0.08$).

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3 290 The percentage of total lipid content in the yolk did not differ between diets ($34.7 \pm 0.6\%$ vs.
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5 291 $33.1 \pm 1.0\%$, $t = 1.36$, $p = 0.18$). Within the lipid fraction of the yolk, the percentage of
6
7 292 saturated fatty acids did not significantly differ between diets ($33.6 \pm 0.47\%$ vs. 33.7 ± 0.37
8
9 293 $\%$, $t = -0.28$, $p = 0.78$). For n-3 hens, the percentage of mono-unsaturated fatty acids was
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11 294 higher, while the percentage of PUFAs was lower in comparison to eggs from the C hens
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13 295 (mono: $49.5 \pm 0.62\%$ vs. $44.0 \pm 0.65\%$, $t = -6.2$, $p < 0.001$, PUFA: $16.7 \pm 0.36\%$ vs. $22.5 \pm$
14
15 296 0.60% , $t = 8.22$, $p < 0.001$). The yolk of eggs from n-3 hens had significantly higher
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17 297 percentages of n-3 PUFAs with a significantly lower n-6/n-3 ratio, while the yolk of eggs
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19 298 from C hens had significantly higher percentages of n-6 PUFAs and a significantly higher n-
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21 299 6/n-3 ratio (Table 2).

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26 300 >>> INSERT Figure 1 <<<

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29 301 >>> INSERT Table 2 <<<

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32 302 *Dietary effects on hatching success and weight of chicks*

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35 303 Hatching success did not differ between diets (98 hatched out of 111 fertile eggs for the n-3
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37 304 group vs. 125 out of 138 fertile eggs for the n-6 group; χ^2 -test, $p = 0.67$), neither did sex-
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39 305 ratio (25 females and 23 males in the n-3 group and 28 females and 22 males in the C group,
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41 306 χ^2 -test, $p = 0.69$). N-3 chicks were lighter at hatching than C chicks ($39.4 \pm 0.4\text{ g}$ vs. $40.9 \pm$
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43 307 0.5 g , $t = -2.29$, $p = 0.02$) but did not differ significantly in growth rate during the first week
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45 308 of life ($8.60 \pm 0.16\text{ g/week}$ vs. $9.72 \pm 1.86\text{ g/week}$, $F_{3,93} = 0.66$, $p = 0.57$).

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49 309 *Dietary effects on neophobic behaviour in the chicks*

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52 310 Latencies to eat and the time spent eating did not differ significantly between n-3 and C
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54 311 chicks in the habituation/familiarization test and both food neophobia tests (Table 3). In the

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3 312 novel object test, n-3 chicks demonstrated a longer latency to eat and spent less time eating
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5 313 from the novel object compared to C chicks (Table 3).
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8 314 >>> INSERT Table 3 <<<<
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10 11 315 **Discussion** 12

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14 316 The present experiment was designed to test the hypothesis that PUFA in the maternal diet
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16 317 could engender differential allocation of yolk hormones of maternal origin. We found higher
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18 318 corticosterone metabolites, reflecting heightened HPA-activity, in the faecal droppings of
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20 319 hens fed the diet enriched in n-3 compared to control animals. As expected, yolk n-6:n-3 ratio
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22 320 strongly reflected that of the maternal diet. There were significantly higher concentrations of
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24 321 yolk progesterone, androstenedione and oestrogens, as well as a trend for higher yolk
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26 322 testosterone in eggs of hens fed the n-3 enriched diet compared to controls. Chicks of hens fed
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28 323 the n-3 enriched diet showed a lower body weight at hatching and higher neophobia when
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30 324 exposed to a novel object compared to chicks of hens fed the control diet.
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35 325 After six weeks of consuming a diet enriched in n-3 PUFAs, hens had elevated
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37 326 concentrations of corticosterone metabolites in the faeces and elevated levels of yolk
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39 327 hormones compared to hens fed a control diet. Additionally, hens of the n-3 diet laid lighter
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41 328 eggs, with lower yolk/egg mass ratios than controls. Diets were equal in protein and energy
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43 329 content, and were selected to meet the dietary requirements of laying hens (Van Elswyk,
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45 330 1997). Food intake and body weight followed a similar pattern for n-3 and control hens. Thus,
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47 331 differences between groups cannot be explained by differences in the energy content of the
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49 332 diet or differences in food intake. Rather, they demonstrate an influence of n-6/n3 PUFA ratio
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51 333 in the diet on the metabolism and physiology of hens.
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3 334 These results suggest a potential action for the n-6:n-3 PUFA ratio of diet on the HPA-
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5 335 axis of hens as reported in other species (Fisher et al., 2014 ; Larrieu et al., 2014; Lafourcade,
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7 336 et al., 2011). First, hens of the n-3 diet had FCM levels more than 3-times greater than
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9 337 controls. FCM levels have been shown to reflect HPA activity (Rettenbacher & Palme, 2009).
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11 338 Second, in chickens with elevated corticosterone levels due to a corticosterone implant, egg
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13 339 mass, yolk mass and hatchling's weight reduction have also been recorded (Rie Henriksen,
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15 340 Groothuis, & Rettenbacher, 2011; R. Henriksen, Rettenbacher, & Groothuis, 2011) which is
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17 341 comparable to our chicks from hens treated with n-3. Moreover, in birds, environmental stress
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19 342 can induce HPA axis activation, causing a decrease in egg and offspring weight (Hayward &
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21 343 Wingfield, 2004; R. Henriksen, et al., 2011; Hsu, Dijkstra, Darras, de Vries, & Groothuis,
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23 344 2016). Third, it is known that maternal environmental stress induces similar increases in hens'
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25 345 yolk hormones as we found in the hens fed the n-3 diet (Bertin, et al., 2013; Guibert et al.,
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27 346 2010). In mammals, the effects of n-3 PUFAs on HPA axis functioning are generally
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29 347 beneficial, but not always. As in mice, a deficiency of n-3 PUFA can alter corticosterone
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31 348 secretion via modifications in glucocorticoid receptors (GRs) of the prefrontal cortex, but not
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33 349 in the HPA-axis (Larrieu, Hilal, De Smedt-Peyrusse, Sans, & Laye, 2016). In the chicken,
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35 350 GRs are also found in the pituitary (Bossis, Nishimura, Muchow, & Porter, 2004) and frontal
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37 351 brain areas (Bordone, Schrott, & Sparber, 1997). Glucocorticoid receptors can be modified by
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39 352 stressful conditions in a variety of avian species (Japanese quail (Zimmer & Spencer, 2014),
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41 353 starlings (Dickens, Romero, Cyr, Dunn, & Meddle, 2009), house sparrow (Lattin & Romero,
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43 354 2014)) and play a role in the lipid metabolism of the white-crowned sparrow (Landys,
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45 355 Piersma, Ramenofsky, & Wingfield, 2004) (Landys, Ramenofsky, Guglielmo, & Wingfield,
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47 356 2004). Our results on HPA-functioning are a likely consequence of the surplus of n-3 in the
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49 357 experimental diet. This may have resulted in a lack of arachidonic acid (AA), which is
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51 358 essential for metabolic and hormonal functioning (Huang, Leibovitz, Lee, & Millar, 1990).
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3 359 Based on our results, we hypothesize that the ratio of n-6:n-3 PUFAs in the diet influence
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5 360 HPA activity in birds, but the mechanisms require further investigation. It is important to
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7 361 notice that domestic chickens have been raised for food thousands of years ago and were
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9 362 mainly fed with wheat seeds. As wheat seeds are poor in n-3 and rich in n-6 fatty acids, these
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11 363 animals may have developed specific adaptations and, as a consequence, may cope better with
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13 364 high n-6:n-3 ratios than with low n-6:n3 ratios in the diet.
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17 365 Egg mass and yolk proportion was reduced in n-3 hens. These results are in
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19 366 accordance with those obtained by administering diets enriched with 2%, 3% or 4% fish oil
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21 367 (Gonzalez-Esquerria & Leeson, 2000; Huang, et al., 1990) in which hens' egg mass was found
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23 368 to decrease linearly with increasing percentages of fish oil (high in n-3). Diets enriched with
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25 369 fish oil can increase albumin production and decrease yolk production compared to control
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27 370 diets (Huang, et al., 1990). The n-3 diet we applied contained little linoleic acid (a molecular
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29 371 precursor essential to the production of n-6 PUFA AA). Moreover, AA production appeared
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31 372 to be strongly inhibited by the dominance of docoheptaenoic acid (DHA) in the n-3 diet as
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33 373 demonstrated by the amount measured in the yolk. This could severely hamper AA
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35 374 metabolism, resulting in metabolic changes in the hens receiving the n-3 diet, and thereby
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37 375 altering egg composition (Huang, et al., 1990). PUFAs in the maternal diet can thus have an
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39 376 important effect on the amount and constitution of the resources available for embryo
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41 377 development.
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45 378 Moreover, our data showed that fish oil in the diet led to higher concentrations of yolk
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47 379 progesterone, androstenedione and oestradiol and a trend for higher concentrations of yolk
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49 380 testosterone. These results add support to the hypothesis that the nutritional state of female
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51 381 birds causes variation in yolk hormone levels. However, at the present stage, the interpretation
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53 382 of the mechanisms that mediate variations in yolk hormone levels is bound to be speculative.
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55 383 As mentioned previously, it could be that increased levels of yolk hormones are the result of
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3 384 HPA axis activation in the mother. In accordance with this hypothesis; recent studies showed
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5 385 that maternal plasma corticosterone levels influence reproductive hormone concentrations in
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7 386 the yolk (Henriksen, Groothuis, & Rettenbacher, 2011). The underlying physiological
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9 387 mechanisms remain poorly understood and are barely addressed in the literature. As argued
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11 388 before, it might be possible that corticosterone levels affect the steroidogenic activity of
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13 389 follicles. Another hypothesis could be related to the direct influence of PUFAs and their
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15 390 oxygenated derivatives on steroid synthesis. Several factors of the steroidogenic machinery
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17 391 are directly regulated by AA and AA-derived eicosanoids (Wathes, Abayasekara, & Aitken,
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19 392 2007). The n-3/n-6 PUFA ratio in the diet largely determines the balance between the various
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21 393 types of oxygenated metabolites deriving from AA or from EPA and DHA (Yates, Calder, &
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23 394 Rainger, 2014). Therefore, the decreased AA/DHA+EPA ratio and the resulting eicosanoid
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25 395 pattern in n-3 supplemented hens may have directly contributed to the observed differences in
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27 396 yolk hormone levels. Consistently, dietary fat (such as those derived from fish oil) in the
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29 397 maternal diet is known to influence egg weight and plasma oestrogen levels (Whitehead,
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31 398 1995), a role for PUFAs in the hormonal metabolism of birds seems, therefore, a plausible
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33 399 explanation.
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39 400 We found that n-3 chicks were more inhibited (longer latency to eat and less time
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41 401 spent eating) than C chicks in the novel object test. This result indicates a higher level of
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43 402 object neophobia in n-3 chicks than C chicks. On the other hand, n-3 and C chicks did not
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45 403 differ in food neophobia test. Since the n-3 maternal diet modified the embryonic
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47 404 environment in several ways (yolk mass, yolk hormones and fatty acids), it is difficult to
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49 405 determine which mechanisms and which hormones affected object neophobia. Little is
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51 406 currently known regarding the developmental influence of yolk progesterone and androgens
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53 407 on neophobia. The influence of progesterone is barely investigated. In Northern bobwhite
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55 408 quail (*Colinus virginianus*), a recent study showed that elevated yolk progesterone levels
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3 409 elevate emotional reactivity in neonates (Herrington, Vallin, & Lickliter, 2015). Interestingly,
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5 410 an artificial increase in yolk hormone levels has been found to alter object neophobia but not
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7 411 food neophobia in domestic chicks (Bertin, et al., 2015), but the direction of effects was not
8
9 412 the same as in the present study. Lower object neophobia was found in chicks exposed *in ovo*
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11 413 to increased yolk progesterone, androstenedione and estradiol levels compared to controls.
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13 414 Discrepancies in the direction of yolk hormonal effects are also seen in latencies to approach
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15 415 novel objects depending on species and the injected dose (Vergauwen, Eens, & Muller, 2012).
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17 416 Nevertheless, all of these experiments suggest that variation in yolk hormone levels can alter
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19 417 specific aspects of neophobia.
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23 418 The data concerning the effects of environmental conditions during ontogeny on the
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25 419 development of object or food neophobia are inconclusive. In accordance with our findings,
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27 420 zebra finch chicks (*Taeniopygia guttata*) from eggs with artificially elevated yolk testosterone
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29 421 show no difference in latency to eat novel foods than control animals (Tobler & Sandell,
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31 422 2007). Similarly, in canaries (*Serinus canaria*), increased levels of yolk testosterone was
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33 423 found to not be the primary source of variation in the expression of food neophobia (
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35 424 Vergauwen, et al., 2012). Our data highlight the importance of studying the quality of the
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37 425 maternal diet in order to understand more fully the ontogeny of emotional and behavioural
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39 426 traits involved in adapting to new resources. This could be of particular importance when food
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41 427 resources become scarcer under the projected future of climate change. In captive bird
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43 428 populations (for production or conservation purposes), no specific recommendation is made
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45 429 regarding PUFA quantity and quality in the diet. This study highlights the importance of
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47 430 carefully considering food quality in captive bird populations as they might engender
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49 431 transgenerational effects on development and behaviour. In our study, chicks were all feeding
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51 432 around two/three days after hatching, therefore we cannot totally exclude the possibility that
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53 433 the behaviour of the chicks was influenced by their respective diet. Two points are of
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3 434 importance when considering this hypothesis. First, even when chicks start feeding, the
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5 435 residual yolk continues to be taken up and thereby provides a source of fatty acids of maternal
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7 436 origin (Romanoff, 1960). Second, in the domestic chicken and king penguin (*Aptenodytes*
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9 437 *patagonicus*) it was consistently found that the proportion of fatty acids in the brain
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11 438 phospholipids was particularly refractory to the effects of the chicks' diet compared to other
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13 439 tissues (Thil, Speake, & Groscolas, 2003; Anderson, et al., 1992). More specifically, high
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15 440 levels of brain n-3 produced by exposure to a maternal diet rich in fish oil are not reversible
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17 441 on a short time period (Anderson, et al., 1992). Although caution must be exercised, we argue
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19 442 that maternal effects more likely contributed to the differences observed in hatchlings' mass
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21 443 and behaviour than the post-natal diet.
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26 444 The present data show maternal diet composition as an overlooked pathway by which
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28 445 the environment could engender large variation in yolk hormones levels. This may play a role
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30 446 in the development of offspring behaviour and phenotype. In farm birds, as unresolved
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32 447 welfare issues are mediated by unknown cues, this study lends support to perspectives in the
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34 448 field of maternal nutrition. Further investigations to the mechanisms are required but this
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36 449 study suggests common principles regarding the regulative action of n-3 and n-6 PUFA on
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38 450 fear-related behavior across vertebrate taxa. Using precocial birds could yield new insights
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40 451 into the mechanism by which the prenatal resources in PUFA influences behavioral
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42 452 development of individuals in vertebrates
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For Peer Review

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710 Table 1: Mean (\pm SE) body mass, food intake and laying rate of adult n-3 hens ($N = 18$) and control hens ($N = 18$) during dietary treatment.

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Parameters	Hens	Week 1	Week 2	Week 3	Week 4	Week 5
Body mass (g)	n-3 enriched	1958.7 \pm 33.9	1854.5 \pm 40.6	1863.8 \pm 37.3	1835.3 \pm 35.3	1856.6 \pm 36.5
	controls	1966.6 \pm 67.2	1880.9 \pm 60.9	1974.1 \pm 62.5	1865.4 \pm 60.8	1873.5 \pm 62.3
24h feed intake (g)	n-3 enriched	154.7 \pm 23.4	144.7 \pm 7.4	173.7 \pm 16.7	194.6 \pm 22.8	224.4 \pm 28.1
	controls	133.7 \pm 11.8	169.6 \pm 21.1	161.8 \pm 20.5	219.0 \pm 24.5	265.4 \pm 24.3
Laying rate (number per day)	n-3 enriched	0.78 \pm 0.07	0.89 \pm 0.03	0.91 \pm 0.02	0.78 \pm 0.04	0.69 \pm 0.06
	controls	0.71 \pm 0.07	0.82 \pm 0.06	0.77 \pm 0.07	0.69 \pm 0.07	0.65 \pm 0.06

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718 Table 2: Mean (\pm 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
 719 control diet ($N = 18$).

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

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

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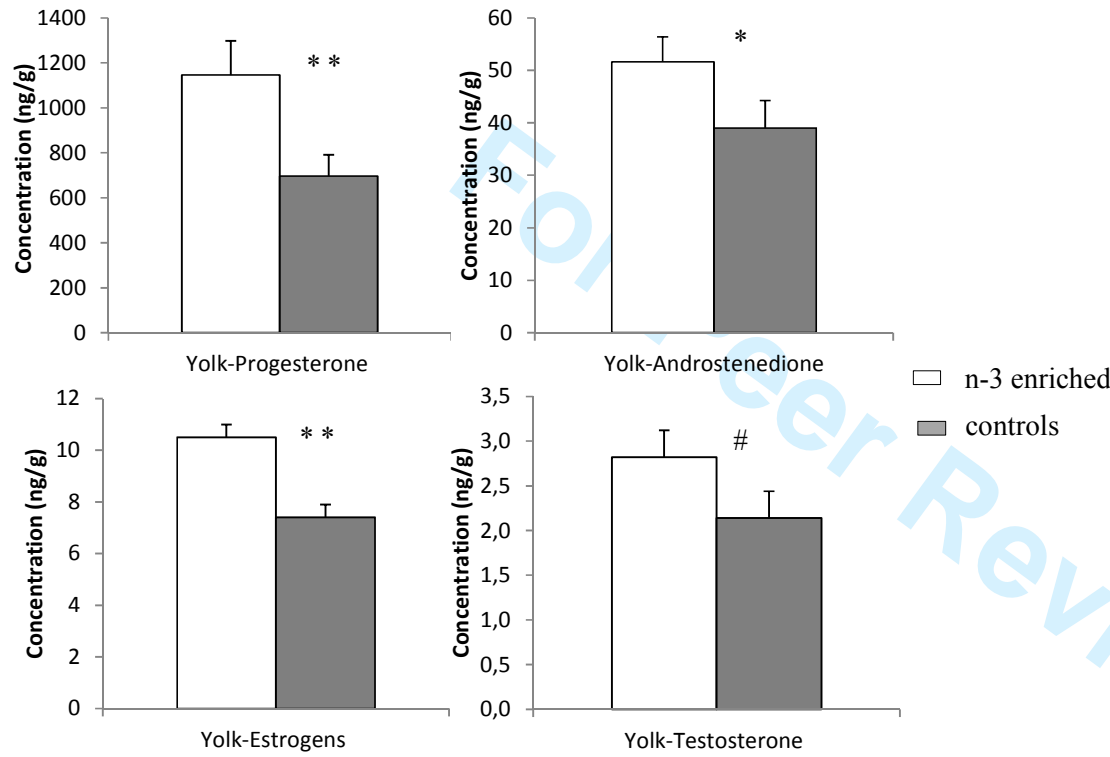
728 Table 3: Mean (\pm SE) Latency to eat and time spent eating during neophobia tests of n-3 chicks ($N = 24$ pairs) and control chicks ($N = 25$ pairs)
729 and Mann-Whitney U -test outcome.

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

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737 FIGURE 1



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3 741 **Figure caption**
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6 742 FIGURE 1: Mean (\pm SE) yolk progesterone (P4), androstenedione (A4), estrogens (E) and
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8 743 testosterone (T) concentrations (ng/g of yolk) in the eggs from n-3 enriched hens ($N = 18$) and
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10 744 control hens ($N = 18$). ** $p < 0.01$; * $p \leq 0.05$; # $0.1 \leq p \leq 0.05$
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