Food restriction but not fish oil increases fertility in hens: role of RARRES2?

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Abstract

Overfed hens selected for their rapid growth become fatter and develop reproductive disorders. Herein, we aimed to demonstrate that food restriction leading to a weight reduction and/or a supplementation with fish oil may be effective in preventing reproductive disorders through the regulation of adipokine expression in broiler hens. This study included four groups of food restricted (Rt) or *ad libitum* hens (Ad, feeding at a rate 1.7 times greater than Rt hens) supplemented or unsupplemented with fish oil (1%). The Rt diet significantly increased plasma chemerin (RARRES2) levels during the laying period, delayed sexual maturity by one week and improved egg quality and fertility. These effects were associated with higher progesterone production in response to IGF1 (or LH) in cultured granulosa cells and *in vivo* egg yolk, as compared with Ad hens. Fish oil supplementation had similar effects to the Rt diet on progesterone ($P < 0.05$), but without any effect on fertility. Using RT-PCR, we found that RARRES2 levels were lower in theca cells of Rt hens and NAMPT levels were increased by the fish oil supplementation. A significant positive correlation between RARRES2 expression in granulosa cells and the weight of F1 preovulatory follicle was observed, as well as a negative correlation of plasma RARRES2 levels with hatchability. Thus, food restriction but not fish oil supplementation improved fertility, and this was associated with variations in RARRES2 plasma and ovarian expression in hens.

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Introduction

In domestic animals including poultry, nutrition has profound effects on reproductive functions including folliculogenesis, fertilisation and early embryonic development (Sirotkin & Grossmann 2015, van Emous *et al.* 2015). Reproduction is an energetically costly process. There is a strong association between metabolic disorders and infertility, but some of the mechanisms mediating the influence of metabolism and nutrition on fertility are currently unclear. In chicken, standard broiler breeders have been submitted to high selection pressure for growth and feed efficiency, resulting in metabolic disorders and reproductive dysfunction (Chen *et al.* 2006, Decuypere *et al.* 2010). Overfeeding during reproductive development is associated with disruptions in steroid production, leading to the formation of excessive numbers of ovarian yellow follicles arranged in multiple hierarchies and, in turn, increased production of unshetable eggs (Hocking *et al.* 1989, Heck *et al.* 2004, Decuypere *et al.* 2010). Hocking

et al. (1993) also showed a relationship between hen weight and sexual maturity and with the number of large ovarian follicles. The maintenance of reproductive performance (laying and fertility) can only be ensured if a strict food restriction is applied at a very early age (2–3 weeks after hatching). However, it appears that the application of this restriction has side effects on animal welfare manifested in changes in social and feeding behaviour (Savory & Maros 1993). Moreover, the functional mechanism of the crosstalk between nutrition and reproductive regulation is still not fully explained.

Metabolites, nutrients and metabolic hormones are considered as potential mediators affecting reproduction. Among the metabolic hormones, the adipokine family are produced mainly by white adipose tissue. They include leptin (LEP), visfatin (NAMPT), chemerin (RARRES2) and adiponectin (ADIPOQ) and have been associated with fattening in mammals (Haider *et al.* 2006, Chang *et al.* 2016, de Luis *et al.* 2017). Furthermore, it is well known that normal

levels of adipokines are fundamental to maintaining the integrity of the hypothalamus gonadotrope axis, regular ovulatory processes, successful embryo implantation and physiological pregnancy (Malik *et al.* 2001, Kasher-Meron *et al.* 2014, Maillard *et al.* 2017). They are also particularly important in interactions between metabolism and reproduction in mammals (Farshchian *et al.* 2014, Chen *et al.* 2015, Kort *et al.* 2015). In chicken, the presence of LEP is controversial (Taouis *et al.* 1998, Pitel *et al.* 2010). However, it has been clearly demonstrated that two of these three adipokines (ADIPOQ and NAMPT) play a critical role in the regulation of carbohydrate and lipid metabolism (Yan *et al.* 2013, Li *et al.* 2017), as well as in reproductive functions in poultry (Diot *et al.* 2015a,b). The plasma concentration of ADIPOQ is inversely correlated with adiposity and blood glucose levels in chickens (Hendricks *et al.* 2009) and decreases during the laying period in turkeys (Diot *et al.* 2015b). In addition, ADIPOQ and its receptors, as well as NAMPT, are expressed in ovarian cells (Chabrolle *et al.* 2007, Diot *et al.* 2015a). In contrast to mammals, NAMPT is more of a myokine than an adipokine (Krzysik-Walker *et al.* 2008). Furthermore, we have recently shown that NAMPT is capable of inhibiting the production of progesterone in hen primary granulosa cells (Diot *et al.* 2015a). While NAMPT and ADIPOQ, along with its two main receptors ADIPOR1 and ADIPOR2 have largely been described, very few data about chicken RARRES2 and RARRES2 receptors (CMKLR1, CCRL2 and GPR1) are available.

In mammals, adipokine plasma levels are upregulated by fish oil, which could potentially improve fertility in addition to its beneficial effects on metabolic disorders (Flachs *et al.* 2006, Wathes *et al.* 2007). Thus, fish oil could be a good nutritional supplement for broiler breeder hens with reproductive impairment. However, to our knowledge, the influence of adipokine levels in plasma and ovarian cells on reproductive parameters (laying and fertility) in hens has not been investigated in different nutritional states. In this present study, we hypothesise that circulating and/or ovarian expression levels of adipokines could be modulated by food restriction and/or fish oil supplementation and could be associated with improved egg production and quality, and consequently fertility parameters. Thus, we investigated the effects of food restriction and fish oil supplementation on egg production and quality, ovarian steroidogenesis and fertility parameters in broiler hen breeders. We also aimed to characterise the relationship of these different parameters with circulating and ovarian expression levels of adipokines.

Materials and methods

Ethical issues

An ethics committee 'Comité d'Ethique en Expérimentation Animale Val de Loire' (CEEA VdL N°19) protocol registered

under ref. n° 01607.02 approved all experimental studies, which were consistent with the guidelines provided by the French Council for Animal Care.

Animals

Three hundred and twenty female broiler breeder chicks (Cobb 500) from Hendrix Genetics (Saint Laurent de la Plaine, France) were studied from hatching to 39 weeks old. On the day of hatching, animals were distributed into homogeneous groups of 10, and kept in 32 pens of 3 m² with thermostatically controlled air inlets and a dynamic cross-ventilation system. Each pen was equipped with a hanging feeder, drip nipples and 5 kg of fresh wood shavings as litter. Animals were reared at 'Pôle Expérimental Avicole de Tours' (INRA, Nouzilly, France) under conventional breeding conditions (Cobb-Vantress 2008): 24 h of light on arrival, day length being reduced to approximately 8 h at two days of age, then kept constant until the age of photostimulation (21st week). From 21 weeks of age, there was a gradual increase in exposure to light up to 15 h per day at 25 weeks. The animals were maintained under this light regime until the end of the experiment.

Diets

From day one to four weeks of age, female breeder chicks received an *ad libitum* diet (free access to food) named starting diet. At week 4, animals were divided into two groups. The first group ($n=160$) received a growth restriction diet according to Hendrix Genetics recommendation, while the second group ($n=160$) named '*ad libitum*' daily received the same diet but in an amount 1.7 times greater than that given to the restricted animals. From nine to 39 weeks of age, these two groups were subdivided into two groups, supplemented or unsupplemented with fish oil (group RNS: restricted unsupplemented, group ANS: *ad libitum* unsupplemented, group RS: restricted supplemented, group AS: *ad libitum* supplemented). During this period, the four groups of animals received three different diets (growing, pre-lay and laying). The supplement was a protected encapsulated fish oil OMG750 provided by Kemin (Nantes, France). It is composed of 77% of refined fish oil and 23% gelatin (the capsule). The supplement was manually mixed into the diet at 1% of the total diet. The composition and the nutrient contents of the diet and the fatty acids composition of the supplement (mg/kg) are shown in [Supplementary Table 1A](#) and [B](#), respectively (see section on [supplementary data](#) given at the end of this article). The fatty acid concentration (mg/kg) in the different diets and in eggs yolk are represented in [Supplementary Table 1C](#) and [D](#) respectively. The weight and the fattening of animals were measured every three weeks using an automated balance and ultrasound, respectively ([Supplementary Table 2A](#)). The feed conversion by the animals was calculated as the ratio between the weight gain (6–9, 9–18, 18–21 weeks) or number of eggs laid (23–39 weeks) and the feed intake during the period ([Supplementary Table 2B](#)). Plasma triglyceride, phospholipid, cholesterol, glucose and insulin concentrations were obtained using enzymatic assays at 21, 27, 32 and 39 weeks, as previously described by Diot and coworkers (2015b) ([Supplementary Table 2C](#)).

Measurement of egg production and quality

From the 23rd week the eggs from each pen were collected, counted and weighed using a balance (Ohaus, Pionner) twice a day. The weight of the albumen, the egg yolk and the dehydrated shell were also measured separately using a balance (Ohaus, Pionner). The numbers of normal, soft, double (eggs with two egg yolks) and broken eggs were determined. At the 25th week, the length and the width of the eggs laid by the four groups of animals were measured using a digital calliper (Mitutoyo, CD-20DCX), and their static stiffness (Sd) and their tensile strength (F) were evaluated using an Instron instrument (Instron, UK527). The elastic modulus (Young's modulus expressed in N/mm^2) and toughness ($N/mm^3 = 2$) of the shell were estimated according to the formulae developed by Bain (Sauveur 1988, Bain 1990, Guesdon *et al.* 2006). Micro-cracked eggs were detected using an Acoustic Egg Tester (Coucke *et al.* 1999, Dunn *et al.* 2005).

Determination of number and weight of follicles

At 39 weeks old, 13 hens per group were selected randomly and their preovulatory follicles were collected, hierarchically defined and weighed.

Fertility parameters

The semen of 65 cocks (Cobb500) was collected and pooled to form a single sample. The hens were artificially inseminated with 2×10^8 spermatozoa from the pool at the 27th and 32nd week and eggs were collected and counted daily for two weeks following the artificial insemination and incubated every seven days (Supplementary Table 3A). The number of unfertilised eggs, and early (EEM) and late (LEM) embryonic mortality was evaluated by breaking eggs and candling on the 7th (EEM) and 14th day of incubation (LEM) (Supplementary Table 3B and C). The different percentages (EEM, LEM, hatchability of eggs set, fertility and hatchability of fertile eggs) were measured using the following formulae:

$$\% \text{ EEM} = \text{number of EEM} / \left(\text{number of incubated eggs} - \text{unfertilised eggs} \right) * 100,$$

$$\% \text{ LEM} = \text{number of LEM} / \left(\text{number of incubated eggs} - (\text{unfertilised eggs} + \text{number of EEM}) \right) * 100,$$

$$\% \text{ Hatchability of incubated eggs} = \left(\text{number of hatched chicks} / \text{number of incubated eggs} \right) * 100,$$

$$\% \text{ Fertility} = \left(\frac{\text{number of fertile eggs after 14 days of incubation}}{\text{number of incubated eggs}} \right) * 100,$$

$$\% \text{ Hatchability of incubated eggs} = \left(\frac{\text{number of hatched chicks} / \text{number of fertile eggs after 14 days of incubation}}{\text{number of incubated eggs}} \right) * 100.$$

Measurement of progesterone, oestradiol, androstenedione and testosterone deposition in egg yolk

Steroids from six egg yolks per group were extracted with diethyl ether after intense agitation and centrifugation. The steroid-containing diethyl ether phase was decanted after freezing the tubes in nitrogen for 10 s. The organic solvents were then evaporated and the extracts taken up in phosphate buffer. Steroid hormones (progesterone, oestradiol, testosterone and androstenedione) were then measured in the extracts using ELISA assays. For progesterone, the ELISA assay was performed as described by Canepa *et al.* (2008). The sensitivity of the assay was 0.4 ng/mL. Oestradiol and testosterone concentrations were determined using commercial ELISA assays from Cayman Chemicals and the sensitivity of these assays was 0.01 ng/mL. Androstenedione levels were analysed using an ELISA assay from Abcam and the sensitivity of the assay was 0.01 ng/mL. The intra-assay and inter-assay coefficients of variation (CV) for each assay averaged <10%.

In vitro culture of hen granulosa cells

Granulosa cells from preovulatory F1 follicles of three hens per group (25–26th week, selected randomly) were collected and dispersed in 0.3% collagenase type A (Roche) in F12 medium containing 5% foetal bovine serum (FBS), at 37°C. Cells were pelleted by centrifugation, washed twice with fresh medium and counted in a haemocytometer. The viability of F1 granulosa cells estimated by Trypan Blue exclusion was about 95%. Cells were cultured in a medium composed of DMEM supplemented with 100 U/mL penicillin, 100 mg/L streptomycin, 3 mmol/L L-glutamine and 5% FBS. The cells were initially cultured for 24 h with no treatment. After overnight serum deprivation, cells were stimulated with IGF1 (10^{-8} M) or LH (10^{-8} M) or left untreated for 48 h. All cultures were maintained under a water-saturated atmosphere of 95% air/5% CO₂ at 37°C (Chabrolle *et al.* 2007, Diot *et al.* 2015a).

In vitro measurement of progesterone secretion by granulosa cells

The concentration of progesterone secreted into the medium by granulosa cells under the various conditions was determined according to an ELISA protocol described by Canepa *et al.* (2008). The sensitivity of the kits was 0.4 ng/mL. The intra- and inter-assay coefficients of variation were <10% and <4.3% respectively. This experiment was carried out using four replicates of three hens for all groups studied, and the results are presented according to the average of these twelve experiments.

Adipokine assays

Plasma concentrations of adipokines were obtained using chicken-specific kits (E12V0003: sensitivity 1 ng/mL, E12A0125: sensitivity 0.1 ng/mL, and E12C0104: sensitivity 1 ng/mL, respectively for NAMPT, ADIPOQ and RARRES2; Holzel Diagnostika, Koln, Germany). The measurements were

carried out according to the manufacturer's protocol with an intra-assay coefficient of variation <6%. The absorbance was measured at 450nm and concentrations were estimated referring to a standard reference range.

Measurement of the expression of adipokines and their receptors in granulosa and theca cells

Total RNA from granulosa and theca cells of preovulatory follicle 1 (F1) and 3 (F3) of eight animals from each group was extracted by homogenisation in TRIzol reagent using an ULTRA-TURRAX instrument and purified using a DNA-free kit according to the manufacturer's recommendations (Invitrogen by Life Technologies). cDNA was generated by reverse transcription (RT) of total RNA (1 µg), incubating for one hour at 37°C in a mixture containing: 0.5 mM each deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, dTTP) 2 M of RT buffer, 15 µg/µL of oligodT, 0.125 U of ribonuclease inhibitor and 0.05 U MMLV (Moloney murine leukaemia virus reverse transcriptase). Real-time PCR was performed using the MyiQ Cycle device (Bio-Rad), in a mixture of SYBR Green Supermix 1X reagent (Bio-Rad), 250 nM specific primers (Invitrogen by Life Technologies) (Supplementary Table 4) and 5 µL of cDNA diluted 1:5 in water for a total volume of 20 µL. The samples were set up in duplicate on the same plate according to the following procedure: after an incubation of 2 min at 50°C and a denaturation step of 10 min at 95°C, samples were subjected to 40 PCR cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C). The mRNA expression levels were standardised using three reference genes (*ACTB*, *EEF1A1* and *GAPDH*). These three housekeeping genes showed expression changes among the different conditions studied. Therefore, the data were normalised to the geometric mean of *ACTB*, *EEF1A1* and *GAPDH* (the most stable combination) following a report that suggests the geometric mean of multiple housekeeping genes is an accurate normalisation factor (Vandesompele *et al.* 2002). For each gene, the relative abundance of transcripts was determined by calculating $e^{-\Delta\Delta C_t}$. The relative expression of the gene of interest was then related to the geometric mean of the relative expression of the reference genes.

Statistical analysis

The results are represented as mean $\pm$ S.E.M., with a level of significance less than 0.05 ($*P < 0.05$). An analysis of variance using repeated measurements (Proc.Mix procedure) was used to compare the average numbers of normal, soft, broken and double eggs among the different chicken groups over time. An analysis of variance (Proc.GLM procedure) was used to compare the average concentrations of secreted progesterone and levels of expression of adipokines and their receptors among the different groups. A chi-square test was used for analysis of percentage fertility between the different parameters. A Pearson test was used to analyse correlations between ovarian or plasma adipokine expression and reproductive parameters. The correlation was noted '*r*' and the *P* value was considered significant if $P < 0.05$. SAS software (version 9.3, Cary, USA) was used for all analyses. Different letters indicate significant differences ($P < 0.05$).

Results

Effect of restricted diet and fish oil supplementation on the egg production curve and on the amount and quality of eggs laid

As shown in Fig. 1A, a delay by one week of entry into sexual maturity was observed in the Rt hens (24th week) compared with the Ad hens (23rd week). Similarly, we noted an earlier laying peak in the Ad hens (27th week) compared with the Rt hens (29th week). This can be explained by a delay in ovarian follicle maturation as shown in Fig. 1B. Fish oil supplementation postponed the laying peak of Rt hens (30th week) by one week but had no effect on the beginning of laying. Overall, Rt hens laid significantly fewer eggs than Ad hens (Table 1). This difference was notable from the 23rd to 27th week; thereafter, the number of laying eggs was unaffected by the diet except in the 31st week in which we observed the opposite trend (Fig. 1A). Although the number of eggs laid was fewer in Rt hens, their quality was better. Among the eggs laid by Rt hens, fewer were broken and soft and there were fewer double egg yolks (Table 1). In addition, we noted that the weight of the egg yolk, albumen and consequently the total weight, as well as the length and width of the eggs were reduced in Rt hens (Table 1). On the other hand, the proportion of shell and the static rigidity were increased and the dynamic rigidity decreased in Rt hens compared with Ad hens.

Figure 1 Laying curve (profile) (A) and representation of ovary (B) of broiler hens fed *ad libitum* or with a restricted diet either with (FO) or without (control) fish oil supplementation. (A) Eggs were collected daily for all hens ($n = 80$ for each group). (B) Representation of ovary of 21-week-old restricted (left side) or *ad libitum* (right side) hens. Results are presented as $\pm$ S.E.M. $*P < 0.05$ (diet effect) and $^{\circ}P < 0.05$ (fish oil supplementation effect).

Table 1 Number (23–39th week) and quality (25th week) by pen of laying eggs of broiler hens fed with *ad libitum* or restricted diet either with (FO) or without (control) fish oil supplementation.

Egg quality	Ad libitum diet		Restricted diet		P value		
	Control (n=80)	FO (n=80)	Control (n=80)	FO (n=80)	Diet	Supp	Diet*Supp
Total laying eggs	765 ± 37	744 ± 27	705 ± 38	664 ± 36	0.04	0.35	0.76
Normal laying eggs	727 ± 38	709 ± 27	692 ± 37	651 ± 36	0.16	0.36	0.72
Broken laying eggs	14 ± 3	12 ± 2	6 ± 1	8 ± 1	0.001	0.96	0.20
Soft laying eggs	4 ± 0	4 ± 1	2 ± 0	2 ± 1	<0.0001	0.97	0.97
Double laying eggs	19 ± 2	19 ± 1	6 ± 2 ^b	6 ± 2	0.03	0.96	0.84
Weight total egg (g)	71.9 ± 0.7	69.4 ± 0.8	67.3 ± 0.5	67.0 ± 0.6	<0.0001	0.03	0.10
Weight of egg yolk (g)	14.7 ± 0.2	14.6 ± 0.2	14.0 ± 0.2	13.4 ± 0.3	<0.0001	0.14	0.27
Weight of albumen (g)	35.9 ± 0.6	35.9 ± 0.6	33.1 ± 0.5	32.9 ± 0.5	<0.0001	0.80	0.85
Length (mm)	60.3 ± 0.3	59.3 ± 0.3	58.3 ± 0.3	58.2 ± 0.3	<0.0001	0.07	0.17
Width (mm)	45.9 ± 0.2	45.2 ± 0.2	44.7 ± 0.1	44.6 ± 0.2	<0.0001	0.004	0.09
% of shell	9.2 ± 0.1	9.2 ± 0.1	9.6 ± 0.1	9.5 ± 0.1	0.002	0.83	0.79
Static rigidity of shell	145.7 ± 4.0	151.8 ± 3.9	156.3 ± 4.0	166.6 ± 3.6	0.001	0.04	0.59
Dynamic rigidity of shell	143943 ± 411	141089 ± 346	127839 ± 264	132797 ± 337	0.0004	0.76	0.25

Results are presented as $\text{lsmeans} \pm \text{s.e.m.}$ P values of the effects of diet, supplementation and the interaction between diet and supplementation were considered as significant if $P \leq 0.05$ (bold). Supp, supplementation.

The fish oil supplementation decreased the total weight of the eggs and their width, while improving their static rigidity (Table 1).

Effect of restricted diet and fish oil supplementation on number and weight of follicles

The Rt diet significantly decreased the mean number of preovulatory follicles (Fig. 2A) as well as the weight of the preovulatory follicle 1 (F1) (Fig. 2B) and 2 (F2) (Fig. 2C), but did not affect the weight of the preovulatory follicle 3 (F3) (Fig. 2D) at the 39th week. The fish oil supplementation had no effect on these parameters.

Effect of restricted diet and fish oil supplementation on fertility

After the first artificial insemination (AI) at 27 weeks the Rt diet significantly decreased the percentage of unfertilised eggs (Table 2) and consequently improved the percentage fertility and hatchability of incubated eggs (Table 2). On the other hand, we noticed that the fish oil supplementation decreased the number of unfertilised eggs without altering the percentage fertility and hatchability (Table 2). Neither the Rt diet nor fish oil supplementation affected the percentage EEM, LEM and hatchability of fertile eggs (Table 2). We found similar results for the second AI (32 weeks) except that the percentage EEM decreased in Rt hens (data not shown).

Effect of restricted diet and fish oil supplementation on in vitro steroidogenesis in hen primary granulosa cells

As shown in Fig. 3, the production of progesterone (Fig. 3A), oestradiol (Fig. 3B), androstenedione (Fig. 3C) and testosterone (Fig. 3D) was significantly greater in the egg yolks of Rt hens than in Ad hens. In addition,

the fish oil supplementation increased the production of progesterone (Fig. 3A) but had no effect on the production of oestradiol (Fig. 3B), androstenedione (Fig. 3C) or testosterone (Fig. 3D) in the egg yolks. As was the case for egg yolks, the Rt diet significantly increased the production of progesterone by granulosa cells stimulated or unstimulated with IGF1 (10^{-8} M) or LH (10^{-8} M). Additionally, the fish oil supplementation increased progesterone production by Rt and Ad hen granulosa cells (Fig. 3E).

Figure 2 Mean number of follicles (A) and weight of preovulatory follicles 1 (B), 2 (C) and 3 (D) of broiler hens fed *ad libitum* or with a restricted diet either with (FO) or without (control) fish oil supplementation. Follicular hierarchy of 13 animals selected randomly from each group was analysed at the 39th week and each preovulatory follicle from each animal was weighed. Results are presented as $\text{lsmeans} \pm \text{s.e.m.}$ Different letters indicate significant differences $P < 0.05$. Capital letters indicate a significant effect of the diet, and lower-case letters indicate a significant effect of fish oil supplementation.

Table 2 Percentage of unfertilised eggs, early (EEM) and late (LEM) embryonic mortality and fertility after first artificial insemination in broiler hens fed with *ad libitum* or restricted diet either with (FO) or without (control) ω 3 PUFA supplementation.

	<i>Ad libitum</i> diet		Restricted diet		<i>P</i> value	
	Control (<i>n</i> =80)	FO (<i>n</i> =80)	Control (<i>n</i> =80)	FO (<i>n</i> =80)	Diet	Supp
% Unfertilised	17.60±1.01	14.55±1.2	10.49±1.65	7.84±1.71	<0.0001	0.05
% EEM	8.63±1.09	6.69±1.54	6.75±1.52	6.68±0.95	0.24	0.35
% LEM	0.82±0.33	1.49±0.51	1.55±0.57	2.22±0.66	0.22	0.11
% Fertility	74.15±0.95	77.65±1.45	83.88±2.29	85.53±0.82	0.05	0.63
% Hatchability of incubated eggs	67.82±1.45	73.08±1.27	78.56±2.85	80.78±1.84	0.02	0.46
% Hatchability of fertile eggs	90.92±1.83	93.15±0.83	95.43±0.99	96.08±0.74	0.5	0.84

Results are presented as $\bar{x}$ ±s.e.m. *P* values of the effects of diet, supplementation and the interaction between diet and supplementation were considered as significant if $P \leq 0.05$ (bold).

EEM, early embryonic mortality; LEM, late embryonic mortality; Supp: supplementation.

Effect of restricted diet and fish oil supplementation on plasma adipokine concentrations (RARRES2, ADIPOQ and NAMPT) during laying and association with fertility parameters

From 21 to 39 weeks, we observed a weak effect on plasma ADIPOQ, RARRES2 and NAMPT concentrations (Table 3). Plasma ADIPOQ and NAMPT concentrations increased gradually, whereas plasma RARRES2 concentrations decreased progressively. We also noted a significant effect of diet only on plasma RARRES2 concentrations. More precisely, these effects appeared at the 21st week with greater concentrations in Rt hens than in Ad hens (Table 3). No effect of fish oil supplementation was observed on plasma adipokine concentrations. On the other hand, we showed that plasma RARRES2 levels were negatively correlated with the percentage hatchability of fertile eggs (number of hatched chicks/number of fertile eggs after 14 days of incubation) after the first AI and that plasma NAMPT levels were positively correlated with the mean number of follicles (Table 4).

Effect of restricted diet and fish oil supplementation on the expression of adipokines in granulosa and theca cells, and association with the weight of ovarian follicles and in vitro progesterone production

In granulosa and theca cells from F1 and F3, we detected the presence of the messenger RNA of all of the adipokines (ADIPOQ, RARRES2, NAMPT) and their receptors (ADIPOR1, ADIPOR2, CMKLR1 and CCRL2), as well as free fatty acid receptors (FFAR4). In theca cells from F1 (Table 5), the Rt diet decreased the expression of RARRES2 and increased the expression of ADIPOR2. In addition, fish oil supplementation increased the expression of NAMPT, CMKLR1 and FFAR4, whereas it decreased expression of ADIPOR1 and ADIPOR2. In theca cells from F3 (Table 5), the Rt diet decreased the expression of RARRES2, NAMPT and ADIPOR1; however, fish oil supplementation decreased the expression of RARRES2, ADIPOR1 and ADIPOR2. In granulosa cells of F1 (data not shown), the Rt diet increased the expression of CCRL2, whereas the fish oil supplementation had no effect. Furthermore,

we showed that in F1, the weight of the follicle was positively correlated with the expression of RARRES2 in granulosa cells and that the production of progesterone by primary F1 granulosa cells was negatively correlated with the expression of RARRES2 in theca cells (Table 6). Similarly, the weight of the follicle in F3 was positively

Figure 3 Levels of progesterone (A), oestradiol (B), androstenedione (C) and testosterone (D) in egg yolk and production of progesterone by granulosa cells (E) of broiler hens fed *ad libitum* or with a restricted diet either with (FO) or without (control) fish oil supplementation. Granulosa cells from preovulatory follicles 1 (F1) were seeded for 24 h and after overnight serum starvation, granulosa cells were incubated with IGF1 (10^{-8} M), or LH (10^{-8} M) for 48 h. The culture medium was then collected and progesterone levels were determined. Hormone levels were assessed by ELISA at 39 weeks. Results are presented as $\bar{x}$ ±s.e.m. Different letters indicate significant differences $P < 0.05$. Capital letters indicate a significant effect of the diet and lower-case letters indicate a significant effect of fish oil supplementation.

Table 3 Plasma ADIPOQ, RARRES2 and NAMPT concentrations of broiler hens fed with *ad libitum* or restricted diet either with (FO) or without (control) fish oil supplementation.

	Diet	Supp	Weeks				
			21–39	21	27	32	39
ADIPOQ (µg/mL)	<i>Ad libitum</i>	Control (<i>n</i> =8)	2.76±0.15	2.7±0.1	1.9±0.2	3.0±0.3	3.4±0.4
		FO (<i>n</i> =8)	2.70±0.10	2.7±0.1	2.2±0.2	2.8±0.2	3.2±0.2
	Restricted	Control (<i>n</i> =8)	2.80±0.12	2.8±0.1	2.3±0.3	2.9±0.2	3.3±0.2
		FO (<i>n</i> =8)	2.70±0.09	2.7±0.1	2.6±0.2	2.5±0.2	3.1±0.2
	<i>P</i> value (week)		<0.0001				
	NAMPT (ng/mL)	<i>Ad libitum</i>	Control (<i>n</i> =8)	158.63±6.54	155.7±10.9	127.8±6.1	159.5±8.7
FO (<i>n</i> =8)			156.84±7.05	136.7±8.0	140.0±12.6	160.9±12.6	189.8±18.2
Restricted		Control (<i>n</i> =8)	167.90±5.85	148.5±7.7	152.2±10.0	180.5±11.9	190.4±13.5
		FO (<i>n</i> =8)	162.28±6.57	145.0±10.2	138.2±4.3	152.6±9.5	213.3±8.9
<i>P</i> value (week)		<0.0001					
RARRES2 (ng/mL)		<i>Ad libitum</i>	Control (<i>n</i> =8)	218.66±5.95 ^a	184±8 ^b	251±14	224±6
	FO (<i>n</i> =8)		239.47±6.93 ^{a,b}	264±11 ^a	239±9	249±12	206±17
	Restricted	Control (<i>n</i> =8)	268.53±11.89 ^c	286±6 ^a	275±27	283±37	230±20
		FO (<i>n</i> =8)	244.20±6.49 ^a	282±6 ^a	252±17	248±12	205±6
	<i>P</i> value						
	Week	0.002					
Diet	0.001	<.0001	ns	ns	ns		
Supp	ns	<.0001	ns	ns	ns		
Diet*Supp	0.007	<.0001	ns	ns	ns		

Results are presented as $\text{lsmeans} \pm \text{s.e.m.}$ *P* values of the effects of week, diet, supplementation and the interaction between diet and supplementation were considered as significant if $P \leq 0.05$ (bold). Different letters indicate significant differences when there is a significant interaction between diet and supplementation effect. ns, non-significant; Supp, supplementation.

correlated with the expression of *ADIPOQ* in granulosa cells (Table 6).

Discussion

In the present study, we showed that feed restriction improved the egg quality, fertility and hatchability of incubated eggs in broiler breeder hens. These effects were associated with higher steroid levels in egg yolk *in vivo* and with greater progesterone secretion by cultured granulosa cells. In addition, we showed for the first time that the plasma concentrations of the three adipokines, *ADIPOQ*, *NAMPT* and *RARRES2* were variable during the laying period but only plasma *RARRES2* concentration was increased by the feed restriction, being negatively correlated with the hatchability of fertile eggs. However, the effects of fish oil supplementation were less pronounced, since they were significant only on the eggshell rigidity, the percentage of unfertilised

eggs and the amount of progesterone produced *in vivo* and *in vitro*.

In the present study, we found that the plasma *RARRES2* concentration was increased, whereas plasma *ADIPOQ* and *NAMPT* concentrations were decreased during the laying period. For *RARRES2*, but not for *ADIPOQ* and *NAMPT*, these results are in good agreement with those already described in turkeys (Diot 2015), suggesting a species-dependent regulation of plasma *ADIPOQ* and *NAMPT* concentrations. In addition, we found that the plasma *ADIPOQ* and *NAMPT* profiles were negatively correlated with the number of eggs laid ($r = -0.5$, $P < 0.0001$ and $r = -0.28$, $P < 0.03$, respectively) between 27 and 39 weeks, whereas the opposite was observed for plasma *RARRES2* ($r = 0.29$, $P < 0.03$, data not shown). We also showed that adipokine amounts in plasma and/or in ovarian cells were correlated with some fertility parameters. Indeed, we showed that plasma *RARRES2* was negatively associated with the ratio of hatched

Table 4 Pearson correlation coefficient (*r*) calculated between plasma adipokines (*RARRES2*, *NAMPT*) concentration and hatchability of fertile eggs after first artificial insemination (AI1) or number of follicles (39 weeks) of broiler hens fed with *ad libitum* or restricted diet either with or without fish oil supplementation.

	Hatchability of fertile eggs AI1 (n=80 per group)	Number of follicles (n=13 per group)
<i>RARRES2</i> (n=8 per group)		
<i>r</i>	-0.47	
<i>P</i> value	0.04	
<i>NAMPT</i> (n=8 per group)		
<i>r</i>		0.35
<i>P</i> value		0.05

Values of *r* and significance of the correlations are indicated. Correlations were considered as significant if $P \leq 0.05$ (bold).

Table 5 mRNA expression of adipokines (ADIPOQ, RARRES2, NAMPT) and adipokine receptors (ADIPO1, ADIPOR2, CMKLR1, CCRL2) and fatty acid receptor (FFAR4) in theca cells from follicle 1 (T1) or 3 (T3).

Tissue	Adipokines	Ad libitum		Restricted		P value	
		Control (n=8)	ω 3 PUFA (n=8)	Control (n=8)	ω 3 PUFA (n=8)	Diet	Supp
T1	ADIPOQ	0.004 $\pm$ 6.76.10 ⁻⁴	0.006 $\pm$ 0.001	0.005 $\pm$ 3.80.10 ⁻⁴	0.006 $\pm$ 9.22.10 ⁻⁴	0.85	0.31
	RARRES2	0.05 $\pm$ 0.004	0.05 $\pm$ 0.002	0.04 $\pm$ 0.003	0.03 $\pm$ 0.002	0.002	0.58
	NAMPT	0.002 $\pm$ 2.77.10 ⁻⁴	0.005 $\pm$ 0.001	0.003 $\pm$ 3.92.10 ⁻⁴	0.005 $\pm$ 8.83.10 ⁻⁴	0.75	0.003
	ADIPO1	0.02 $\pm$ 0.002	0.009 $\pm$ 0.001	0.02 $\pm$ 0.002	0.01 $\pm$ 8.58.10 ⁻⁴	0.61	<0.0001
	ADIPOR2	0.02 $\pm$ 0.002 ^a	0.02 $\pm$ 0.002 ^a	0.05 $\pm$ 0.006 ^b	0.02 $\pm$ 7.99.10 ^{-4a}	<0.0001	<0.0001
	CMKLR1	6.73.10 ⁻⁴ $\pm$ 8.1.10 ⁻⁵	0.001 $\pm$ 4.08.10 ⁻⁴	8.77.10 ⁻⁴ $\pm$ 1.65.10 ⁻⁴	0.003 $\pm$ 7.75.10 ⁻⁴	0.15	0.31
	CCRL2	0.001 $\pm$ 2.46.10 ⁻⁴	0.001 $\pm$ 4.14.10 ⁻⁴	0.001 $\pm$ 2.46.10 ⁻⁴	0.001 $\pm$ 2.55.10 ⁻⁴	0.42	0.17
T3	FFAR4	1.47.10 ⁻⁵ $\pm$ 2.26.10 ⁻⁶	8.55.10 ⁻⁵ $\pm$ 2.45.10 ⁻⁵	1.46.10 ⁻⁵ $\pm$ 2.29.10 ⁻⁶	1.14.10 ⁻⁴ $\pm$ 3.04.10 ⁻⁵	0.46	0.0001
	ADIPOQ	0.003 $\pm$ 7.39.10 ⁻⁴	0.003 $\pm$ 6.46.10 ⁻⁴	0.002 $\pm$ 4.71.10 ⁻⁴	0.002 $\pm$ 6.19.10 ⁻⁴	0.19	0.96
	RARRES2	0.06 $\pm$ 0.006	0.04 $\pm$ 0.005	0.04 $\pm$ 0.005	0.03 $\pm$ 0.003	0.02	0.03
	NAMPT	0.003 $\pm$ 3.48.10 ⁻⁴	0.002 $\pm$ 4.19.10 ⁻⁴	0.002 $\pm$ 8.32.10 ⁻⁵	0.002 $\pm$ 2.72.10 ⁻⁴	0.01	0.27
	ADIPOR1	0.03 $\pm$ 0.002	0.02 $\pm$ 0.001	0.02 $\pm$ 8.24.10 ⁻⁴	0.01 $\pm$ 0.001	0.002	0.0002
	ADIPOR2	0.03 $\pm$ 0.003	0.01 $\pm$ 0.001	0.03 $\pm$ 0.004	0.01 $\pm$ 0.001	0.24	<0.0001
	CMKLR1	0.006 $\pm$ 9.94.10 ^{-4a,b}	0.01 $\pm$ 0.003 ^b	0.01 $\pm$ 0.002 ^b	0.002 $\pm$ 6.19.10 ^{-4a}	0.48	0.12
	CCRL2	0.001 $\pm$ 1.95.10 ^{-4b}	0.001 $\pm$ 2.23.10 ^{-4b}	0.001 $\pm$ 2.49.10 ^{-4b}	4.56.10 ⁻⁴ $\pm$ 7.57.10 ^{-5a}	0.06	0.15
	FFAR4	2.33.10 ⁻⁵ $\pm$ 3.48.10 ⁻⁶	2.38.10 ⁻⁵ $\pm$ 4.25.10 ⁻⁶	2.06.10 ⁻⁵ $\pm$ 4.45.10 ⁻⁶	1.86.10 ⁻⁵ $\pm$ 4.06.10 ⁻⁶	0.34	0.86

Results are presented as $\bar{x}$ means $\pm$ S.E.M. P values of the effects of diet, supplementation and the interaction between diet and supplementation were considered as significant if $P \leq 0.05$ (bold). Different letters indicate significant differences when there is an interaction between diet and supplementation effect. Supp, supplemented; ω 3 PUFA, ω 3 PUFA supplementation.

chicks/fertile eggs after 14 days of incubation after the first AI. Regarding to this data, RARRES2 could be used as a biomarker by breeding companies to select feed-restricted breeder hens with better fertility and hatchability but it might be confirmed using more animals. RARRES2 expression in theca cells was also negatively correlated with progesterone secretion by *in vitro* granulosa cells from F1. In avian species, only one paper has described the presence of RARRES2 in ovarian cells of turkey (Diot *et al.* 2015b), whereas ADIPOQ and NAMPT expression in the female reproductive tract has been already reported in the literature (Maddineni *et al.* 2005, Chabrolle *et al.* 2007, Ons *et al.* 2010, Diot *et al.* 2015a). In mammals, a negative association between RARRES2 and fertility has already been described. For example, treatment with recombinant human RARRES2 decreases *in vitro* granulosa cell steroidogenesis in human and bovine species (Reverchon *et al.* 2012, 2014) and inhibits *in vitro* bovine oocyte maturation (Reverchon *et al.* 2014). We observed that the plasma RARRES2 concentration is significantly higher in Rt hens that are leaner than Ad hens. This regulation is opposite to that described in obese humans. Current data show that plasma RARRES2 levels are reduced after prolonged fasting (van Herpen *et al.* 2013) and they are higher in obese patients compared with lean controls (Chang *et al.* 2016). Furthermore, in contrast to both animal models (Flachs *et al.* 2006, Neschen *et al.* 2006) and humans (Guebre-Egziabher *et al.* 2008), a ω 3 PUFA-enriched diet did not affect plasma ADIPOQ levels in hens, suggesting different metabolic regulation in avian species.

In a good agreement with our data, previous studies have shown that egg performance (amount and quality) and fertility can be altered in overweight broiler breeder hens (Hocking *et al.* 1989, Fattori *et al.* 1991, Heck *et al.* 2004, Decuyper *et al.* 2010). We also observed that feed restriction reduced egg weight, more specifically yolk weight, probably due to a reduction in the F1 and F2 weights. Heck *et al.* (2004) also reported that egg abnormalities and weight were significantly reduced in restricted broiler breeder animals. Fertility parameters were also affected by the nutritional status. Our findings showed that the feed restriction decreased the percentage of unfertilised (assumed infertile) eggs, but it did not affect the embryo mortality (early or late) after the first AI. Hens fed excessively have previously been found to have lower percentages of fertile eggs, hatchability and embryonic viability (Yu *et al.* 1992). Herein, we have shown that the steroid concentrations in egg yolk (progesterone, oestradiol, androstenedione and testosterone) were higher in Rt hens. In hens and other bird species, considerably higher amounts of progesterone than of testosterone or androstenedione have been found around the germinal disc (the outer layer of the yolk) (Hackl *et al.* 2003). Progesterone is the precursor of androgens and oestrogens and is produced by the granulosa cells of the preovulatory follicles

Table 6 Pearson correlation coefficient (*r*) calculated between granulosa (from follicle 1, G1 or 3, G3) or theca (from follicle 1, T1) cells adipokines (*ADIPOQ*, *RARRES2* and *NAMPT*) mRNA expression and weight of follicle (1 or 3) or granulosa from follicle 1 progesterone production (G1) of broiler hens fed with *ad libitum* or restricted diet either with or without fish oil supplementation.

Tissue		Weight of follicle 1 (n = 13 per group)	Progesterone (G1) (n = 3 per group)	Weight of follicle 3 (n = 13 per group)
G1	RARRES2, (n = 8 per group)			
	<i>r</i>	0.50		
	<i>P</i> -value	0.007		
T1	RARRES2, (n = 8 per group)			
	<i>r</i>		−0.55	
	<i>P</i> -value		0.001	
G3	ADIPOQ, (n = 8 per group)			
	<i>r</i>			0.47
	<i>P</i> -value			0.02

Values of *r* and significance of the correlations are indicated. Correlations were considered as significant if $P \leq 0.05$.

(Huang & Nalbandov 1979). We have also showed that granulosa cells from F1 produced more progesterone in Rt hens, probably leading to higher progesterone levels in egg yolks. In birds, progesterone produced by the F1 follicle is capable of triggering the LH preovulatory surge and ovulation (Scanes *et al.* 1977, Johnson & van Tienhoven 1980). Thus, progesterone plays a key role in the control of fertility in hens. In our study, the percentage of unfertilised eggs was lower in Rt hens compared with Ad hens. This improved fertility can probably be explained by higher levels of progesterone in the egg yolks of restricted hens.

PUFAs have a great impact on reproduction, affecting prostaglandin (PG) synthesis, steroidogenesis, transcription factors and membrane properties in mammals and in chicken (Wathes *et al.* 2007). They act via cell surface and intracellular receptors/sensors that control cell signalling and gene expression patterns. Some effects of PUFAs appear to be mediated by, or at least associated with, changes in the fatty acid composition of cell membranes. In birds, many studies have shown that diets containing fish oil components such as EPA and DHA that are especially abundant in fish and can also be derived from alpha linolenic acid of linseed oil (Nettleton 1991) significantly increase sperm mobility (Kelso *et al.* 1997, Zanini *et al.* 2003). In hens, the main energy source for the developing chick embryo is supplied by fats in the egg yolk. Alteration of the egg yolk fatty acid profile could therefore be detrimental to the growth and development of the embryo during the incubation period (Leone *et al.* 2009). However, some nutritional studies performed in males (Cerolini *et al.* 2006) have reported that fish oil supplementation may be beneficial in reducing embryonic losses during incubation. In our study, we showed that a fish oil-enriched diet in broiler breeder hens improved the percentage fertilisation but did not affect the percentage fertility or hatchability, or the level of embryo mortality (early or late). The supplementation level in fish oil (1% of the total diet) used in our study was probably

not sufficient to detect a significant effect on fertility. In our experiment, although the lipids in the egg yolk were enriched with fish oil (data not shown), we observed no effect on the number of eggs. Although many authors have not reported any effect of fish oil supplementation on egg production (Baucells *et al.* 2000, Novak & Scheideler 2001), others (Scheideler & Froning 1996, Aziza *et al.* 2013) have observed increased egg production after supplementation with fish oil, but also with linseed or camelina meal. However, in the present study, we noted a reduction in the weight of the eggs and of chicks at hatching (data not shown) in both Rt and Ad hens, as previously reported by van Elswyk *et al.* Aigueperse *et al.* and Koppenol *et al.* in experiments with diets containing 1.5–2% fish oil (Van Elswyk 1997, Aigueperse *et al.* 2013, Koppenol *et al.* 2015). Furthermore, Scheideler and Froning (1996) showed that inclusion of linseed also led to a reduction in egg weight (Scheideler & Froning 1996). Since egg yolk is primarily derived from hepatically synthesised VLDL and vitellogenin (Burley 1993), one hypothesis is that omega 3 PUFA decrease the secretion of VLDL from the liver resulting in fewer eggs laid and possibly smaller egg yolks. Notably, the expression of the main receptor of EPA and DHA (*FFAR4*) was demonstrated for the first time in chicken ovarian cells in our study, suggesting that the effect of fish oil supplementation on fertility parameters could be mediated by *FFAR4*.

In conclusion, feed restriction but not fish oil at 1% of the total diet improved fertility in broiler breeder hens, and this improvement was associated with an increase in progesterone production *in vivo* in egg yolk and *in vitro* by ovarian cells (granulosa). Moreover, we found that the three adipokines, *ADIPOQ*, *NAMPT* and *RARRES2* are present in plasma and ovarian cells. However, only the plasma concentrations of *RARRES2* were affected by diet throughout the laying period. Furthermore, plasma *RARRES2* levels were negatively correlated with the percentage hatchability of fertile eggs. Further experiments are necessary to better understand the role

of RARRES2 in the interactions between nutrition and reproduction in avian species.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0678>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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