Nutrigenetics of carotenoid metabolism in the chicken: a polymorphism at the β,β-carotene 15,15′-mono-oxygenase 1 (*BCMO1*) locus affects the response to dietary β-carotene

Maamer Jlali[1], Benoit Graulet[2,3], Béatrice Chauveau-Duriot[2,3], Estelle Godet[1], Christophe Praud[1], Carlos Simões Nunes[4], Elisabeth Le Bihan-Duval[1], Cécile Berri[1]*† and Michel J. Duclos[1]†

[1]*INRA, UR83 Recherches Avicoles, F-37380 Nouzilly, France*
[2]*INRA, UMR1213 Herbivores, F-63122 Saint-Genès-Champanelle, France*
[3]*Clermont Université, VetAgroSup, UMR Herbivores, BP 10448, F-63000 Clermont-Ferrand, France*
[4]*DSM Nutritional Products France, NRD/CA, BP 170, F-68305 Saint-Louis Cedex, France*

Abstract

The enzyme β,β-carotene-15,15′-mono-oxygenase 1 (BCMO1) is responsible for the symmetrical cleavage of β-carotene into retinal. We identified a polymorphism in the promoter of the *BCMO1* gene, inducing differences in *BCMO1* mRNA levels (high in adenines (AA) and low in guanines (GG)) and colour in chicken breast muscle. The present study was designed to test whether this polymorphism could affect the response to dietary β-carotene. Dietary β-carotene supplementation did not change the effects of the genotypes on breast muscle properties: *BCMO1* mRNA levels were lower and xanthophyll contents higher in GG than in AA chickens. Lower vitamin E levels in the plasma and duodenum, plasma cholesterol levels and body weight were also observed in GG than in AA chickens. In both genotypes, dietary β-carotene increased vitamin A storage in the liver; however, it reduced numerous parameters such as *SCARB1* (scavenger receptor class B type I) in the duodenum, *BCMO1* in the liver, vitamin E levels in the plasma and tissues, xanthophyll contents in the pectoralis major muscle and carcass adiposity. However, several diet × genotype interactions were observed. In the GG genotype, dietary β-carotene increased *ISX* (intestine-specific homeobox) and decreased *BCMO1* mRNA levels in the duodenum, decreased xanthophyll concentrations in the duodenum, liver and plasma, and decreased colour index and HDL-cholesterol concentration in the plasma. Retinol accumulation following dietary β-carotene supplementation was observed in the duodenum of AA chickens only. Therefore, the negative feedback control on β-carotene conversion through *ISX* appears as functional in the duodenum of GG but not of AA chickens. This could result in a higher availability of β-carotene in the duodenum of GG chickens, reducing the uptake of xanthophylls, liposoluble vitamins and cholesterol.

Key words: Carotenoids: Vitamins: Genetics: Chickens

Carotenoids are lipophilic pigments synthesised by plants, algae and some micro-organisms. They are involved in various biological processes such as embryonic development[1], immunity[2], reproduction[3], and cell growth and differentiation[4], and they are known as antioxidants[3,5]. Epidemiological studies have reported that carotenoids and their metabolites prevent several diseases, including cancers[6], CVD[7] and age-related macular degeneration[8]. Because animals do not synthesise carotenoids, they must obtain them from their diet. Considerable amounts of carotenoids accumulate in organs and tissues in response to feed intake[9–11], and they have an important role in the determination of animal colour[12]. Several genetic polymorphisms have

recently been identified that affect human and animal blood and tissue carotenoid status. In humans, several mutations or SNP associated with variations in carotenoid content affect the activity of β,β-carotene-15,15′-mono-oxygenase 1 (BCMO1), an enzyme responsible for the symmetrical cleavage of β-carotene into retinal[13–15]. Polymorphisms such as SNP have also been identified in the *BCDO2* gene that encodes β,β-carotene-9′, 10′-dioxygenase 2 responsible for the asymmetric cleavage of carotenoids into apocarotenoids[16,17]. They are also present in proteins involved in lipid metabolism, including membrane transporters such as scavenger receptor class B type I (SR-BI or SCARB1) and cluster determinant 36 (CD36), fatty acid-binding proteins and apolipoproteins[18–22].

In the chicken, two fully linked SNP have also been identified within the proximal promoter of the *BCMO1* gene at -678 and -621 upstream from the ATG codon[23]. This polymorphism is associated with differential expression of the *BCMO1* gene in the pectoralis major muscle, and is strongly related to variations in breast meat xanthophyll concentrations (lutein and zeaxanthin). The effects of the mutation have been further characterised by comparing chickens sharing a common genetic background but differing in this specific mutation[24]. Reared on a classical maize-based diet, such birds showed marked differences in colour, lutein and zeaxanthin contents and *BCMO1* mRNA levels in the pectoralis major muscle, but not in other tissues. By contrast, these birds showed no differences in vitamin A and E status and no differences in the activity of other genes involved in carotenoid transport (*SCARB1* and *CD36*) and metabolism (*BCDO2*). The aim of the present study was to evaluate whether the *BCMO1* polymorphism could affect the response to dietary β-carotene, the preferred substrate of the BCMO1 enzyme[25–27], added to a wheat-based diet containing limited amounts of carotenoids. The activities of the *BCMO1* and *BCDO2* genes (involved in carotenoid conversion) and the *SCARB1* and *CD36* genes (involved in carotenoid transport) were determined from the site of uptake (duodenum) to the sites of accumulation (liver and skeletal muscle). The activity of intestine-specific homeobox (ISX), a major sensor of retinoid status involved in the feedback regulation of *BCMO1* gene activity in the intestine, was also measured. The overall physiological consequences were evaluated through the record of the levels of carotenoids, fat-soluble vitamins A and E and cholesterol, and of performance traits (chicken growth, tissue yields and coloration).

Materials and methods

Reagents

Extraction reagent (RNA Now) for total RNA was obtained from Ozyme. DNase treatment was performed using Ambion's DNA-Free 1906 Kit obtained from CliniSciences, Moloney murine leukaemia virus RT (Superscript II) was purchased from Invitrogen and random primers were obtained from Promega. Real-time PCR premix (SYBR® Green I qPCR Master Mix Plus) was obtained from Eurogentec. All-*E*-β-carotene, retinol, lutein, α-tocopherol, δ-tocopherol, γ-tocopherol, tocopheryl acetate, retinyl acetate, retinyl myristate, retinyl palmitate and retinyl stearate were purchased from Sigma. Zeaxanthin, β-cryptoxanthin, echinenone, 9Z-β-carotene and 13Z-β-carotene were purchased from Carotenature. All solvents used were of ultra-performance liquid chromatography grade and obtained from VWR. Ultrapure water was prepared using a Milli-Q system (Millipore).

Animals, housing and diets

In the chicken, two fully linked SNP were identified within the proximal promoter of the *BCMO1* gene at -678 and -621 upstream from the ATG codon[23,28]. They corresponded to the substitution of two adenines (A/A) by two guanines (G/G) and defined two haplotypes, $AN_{57}A$ and $GN_{57}G$, which segregate within chicken lines maintained at INRA. A total of 533 chicks were produced from nineteen males and seventy females whose status at the *BCMO1* locus was known. Genotype was determined by high-resolution melting[23] analysis from genomic DNA extracted, according to Nadaf *et al.*[29], from blood (breeders) or from two wing feathers removed at hatching (offspring). Because the two SNP are fully linked, genotyping took into account only the SNP positioned at -621 upstream from the ATG codon. The following three genotypes were defined: homozygous AA and GG, and heterozygous AG.

From hatching to 3 weeks, all chickens were reared in a conventional poultry house[23] and received the same wheat-based starter diet (Table 1). From 3 to 9 weeks, only homozygous chickens were reared, representing 119 AA chickens, of which sixty-seven were females and fifty-two were males, and ninety-two GG chickens, of which sixty were females and thirty-two were males. Chickens were equally and randomly distributed into two groups within each genotype and sex that received a wheat-based diet with or without supplementation with 10 mg β-carotene/kg food (Table 1). The birds were individually weighed at

Table 1. Ingredients and analysed or calculated compositions of chicken starter (0–3 weeks) and growth (3 and 9 weeks) diets

	Starter	Growth	
		Control	β-Carotene*
Ingredients (g/kg)			
Wheat	491·0	541·7	540·7
Soyabean meal	225·5	162·9	162·9
Pea	80·0	100·0	100·0
Maize starch	50·0	50·0	50·0
Extruded soyabeans	50·0	50·0	50·0
Rapeseed oil	33·9	32·3	32·3
Wheat gluten	30·0	30·0	30·0
Dicalcium phosphate	16·2	13·7	13·7
Calcium carbonate	11·6	8·4	8·4
Salt (NaCl)	3·0	3·0	3·0
L-Lys HCl	2·0	1·3	1·3
DL-Met	1·7	1·7	1·7
Thr	0·1	0·0	0·0
Premix†	5·0	5·0	5·0
β-Carotene	0·0	0·0	0·01
Analysed composition (μg/g DM)			
Lutein	3·10	2·80	3·15
Zeaxanthin	0·62	0·00	0·00
β-Carotene	0·88	0·16	0·42
Vitamin A	4·68	5·49	4·34
α-Tocopherol	17·95	13·13	19·61
γ-Tocopherol	32·17	28·85	40·15
Calculated composition			
Metabolisable energy (MJ/kg)	12·33	12·54	12·54
Crude protein (g/kg)	215	200	200

* β-Carotene is the control wheat-based growth diet supplemented with 10 mg β-carotene/kg food.
† Supplied per kg diet: 5·1 mg retinyl acetate; 0·1 mg cholecalciferol; 100 mg DL-β-tocopherol acetate; 5 mg menadione; 5 mg thiamin; 8 mg riboflavin; 7 mg pyridoxine; 0·02 mg cyanocobalamin; 100 mg niacin; 3 mg folic acid; 0·3 mg biotin; 25 mg calcium pantothenate; 550 mg choline; 80·8 mg manganese oxide; 90·1 mg zinc sulphate; 58·2 mg ferric sulphate; 20·0 mg copper sulphate; 2 mg calcium iodine; 0·2 mg sodium selenium; 0·6 mg cobalt carbonate; 50 mg antioxidant.

hatching, and at 3, 6 and 9 weeks of age. The present study was conducted in accordance with the European Union Guidelines for Animal Care and under authorisation 37-112 delivered to C. B. by the French Ministry of Agriculture.

Sampling and phenotyping

At 9 weeks of age, chickens were weighed and slaughtered, and carcasses were processed as described in Berri et al.[30]. Blood samples were collected on heparin during bleeding and immediately chilled on ice. Blood samples were centrifuged at 3000 g for 10 min at 4°C, and serum was aliquoted and stored at −20°C until analysed. Samples from the duodenal mucosa, liver and pectoralis major muscle were collected 15 min post-mortem from eight males and eight females of each genotype (AA v. GG) and diet (control v. β-carotene), and then immediately frozen in liquid N_2 and stored at −80°C until analysis.

All carcasses were processed 1 d after slaughter. Abdominal fat, breast muscles (pectoralis minor and pectoralis major) and liver were removed and weighed, and their respective yields were calculated and expressed as the percentage of live body weight at slaughter. Lipid contents of the liver and pectoralis major muscle were determined according to the method described by Folch et al.[31]. The colour of the pectoralis major muscle (internal part), duodenum (external face) and abdominal fat was measured by a Miniscan spectrocolorimeter (Hunterlab) according to the CIELAB trichromic system for lightness (L^*), redness (a^*) and yellowness (b^*) values 15 min post-mortem for the duodenum and 24 h post-mortem for the other tissues. The absorbance spectrum of the plasma was measured between the wavelength of 400 and 700 nm by a spectrophotometer (Infinite M200; Tecan). Each absorbance spectrum was translated to make the value at 530 nm equal to zero[32,33]. The colour index (CI), which corresponds to the absolute value of the integral of the translated spectrum between the wavelength of 450 and 530 nm, was measured by calculating the trapezoid area (TA) between the 450 and 530 nm wavelengths as follows:

$$CI_{450-530\,nm} = TA_{450-460} + TA_{460-470} + TA_{470-480} + TA_{480-490}$$
$$+ TA_{490-500} + TA_{500-510} + TA_{510-520}$$
$$+ TA_{520-530}.$$

The values were only taken into account when the CV between three repetitions was less than 5%.

Quantitative PCR analysis

Total RNA was extracted from the duodenum, liver and pectoralis major muscle of eight females and eight males from each treatment group (genotype × diet), and quantitative PCR was performed using complementary DNA synthesised as described previously[34]. Primers were designed (Table 2) and purchased from Eurogentec, quantitative PCR assays were run on a Roche LightCycler® 480 II using the LightCycler® 480 SYBR Green I Master (Roche Applied Science), according to the manufacturer's recommendations. Quantitative PCR conditions were set at 95°C for 5 min, followed by forty-five cycles of 10 s at 95°C, 20 s at 60°C or 62°C and 10 s at 72°C. The specificity of quantitative PCR was assessed by analysing the melting curves of the products and by verifying the size and sequence of the amplicons. Each PCR included negative control and unknown samples that were run in triplicate. The samples were normalised internally by using simultaneously the average cycle quantification of three reference genes (18S ribosomal RNA (18S), β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) whose stability in all the selected tissues was investigated using the geNorm application[35]. As recommended, the threshold for eliminating a gene was $M \geq 1$[36]. Expression levels are expressed as normalised values[35].

Biochemical analyses

Carotenoids and fat-soluble vitamins in plasma and tissues (duodenum, liver and pectoralis major muscle) were quantified by ultra-performance liquid chromatography (Waters), as described previously[37]. After precipitation of proteins by ethanol, carotenoids and fat-soluble vitamins (A and E) were extracted from plasma and tissues with n-hexane and separated by ultra-performance liquid chromatography. The analytical conditions for ultra-performance liquid chromatography analysis were those recommended by Chauveau-Duriot et al.[37]. Under these conditions, carotenoids, and the entire vitamin A (retinol and retinyl esters) and vitamin E (α- and γ-tocopherol) forms were quantified at wavelengths of 450, 325 and 292 nm, respectively. Quantification of the compounds was

Table 2. Primers used for real-time PCR analysis

	Primer sequences (5′–3′)			
Genes	Forward	Reverse	Accession no.	Product size (bp)
18S rRNA	TCCAGCTAGGAATAATGGAATAGGA	CCG GCCGTCCCTCTTAAT	AF173612	83
ACTB	CTGGCACCTAGCACAATGAA	CTGCTTGCTGATCCACATCT	NM_205518	103
GAPDH	AGGCGAGATGGTGAAAGTCGGAGT	TGCCCTTGAAGTGTCCGTGTGT	NM_204305	176
BCMO1	AACAAAGAAGAGCATCCAGAGCC	GCCAAGCCATCAAACCAGTG	NM_204635	145
BCDO2	GGTTTCTTTCTGAGCCTGACCTTG	CCAGCTAGGAATAATGGAATAGGA	XM_417929	156
SCARB1	ACCTCTTTGAAGTGCAGAACCC	ATGACAACGACACCGTCTCCT	XM_415106	135
CD36	CTGTTTCTCTTTGTGGCCTTTG	CGTGAGAGAAGCTGTATGGAGG	NM_001030731	136
ISX	AACCAGAGAGCCAAGTGGAGGA	TGCCCTTGCAGTGGTGAGTAGT	XM_416296.3	179

18S rRNA, 18S ribosomal RNA; ACTB, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BCMO1, β,β-carotene 15,15′-mono-oxygenase 1; BCDO2, β,β-carotene 9′,10′-dioxygenase 2; SCARB1, scavenger receptor class B type 1; CD36, cluster determinant 36. ISX, intestine-specific homeobox.

performed using Empower Pro software (Waters), and their corresponding concentrations were calculated by using an external standard curve. The concentrations obtained were adjusted according to the percentage of the internal standard recovered.

Concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG, glucose and albumin were determined in plasma by enzymatic methods (Roche Diagnostics) using an automated assay (Hitachi Cobas C501; Roche Diagnostics). Plasma minerals (Ca, Fe, Mg and P) were determined according to the Association of Official Analytical Chemists method[38] using a Vista-MPX ICP OES spectrophotometer (Varian Australia Private Limited).

Statistical analyses

Data were analysed using SAS 8.1 (SAS Institute, Inc.). Type I error was accepted as 5%. The effects of BCMO1 genotype (AA *v.* GG), diet (control *v.* β-carotene) and their possible interactions were analysed using a two-way ANOVA (generalised linear model procedure) with type III sum of squares. Comparisons of means for each significant effect were made by Tukey's test using the least-squares mean statement of the generalised linear model procedure. Data are presented as least-squares means with their standard errors.

Results

Gene expression

The expression of BCMO1, BCDO2, SCARB1 and CD36 was detected in all the tissues tested (Tables 3 and 4). The expression of ISX was detected at much higher levels in the duodenum (about 60-fold) than in the liver, but was not detected in the pectoralis major muscle. The normalised expression of BCMO1 was affected by genotype and diet in a tissue-specific manner. In both diet conditions, there was a genotype effect in the pectoralis major muscle (AA ≫ GG, $P < 0.0001$). An opposite effect of the genotype was observed in the duodenum of chickens receiving the control diet (AA < GG, $P = 0.0001$) that was no longer observed when they were supplemented with the β-carotene diet. The addition of β-carotene to the diet lowered ($P = 0.004$) the mRNA levels of BCMO1 in the liver in both genotypes. The expression of SCARB1 was not affected by genotype, but was significantly decreased ($P < 0.0001$) when supplemented with the β-carotene diet, but only in the duodenum. The expression of ISX was also affected by diet but only in the GG genotype (β-carotene > control, $P = 0.04$). By contrast, mRNA levels of BCDO2 and CD36 were invariable between the genotypes and diets.

Carotenoid and vitamin status

As for mRNA, lutein and zeaxanthin concentrations were affected by genotype and diet in a tissue-specific manner (Fig. 1). Concentrations of lutein and zeaxanthin were affected by genotype (AA < GG, $P < 0.001$) and diet in the pectoralis major muscle, but without any interaction: lutein and zeaxanthin concentrations were reduced ($P = 0.02$ and $P < 0.0001$, respectively) by supplementation of the β-carotene diet. Plasma lutein and zeaxanthin concentrations were lowered ($P < 0.05$) by supplementation of the β-carotene diet only in the GG genotype. The concentration of lutein was reduced by β-carotene supplementation in the duodenum ($P = 0.02$)

Table 3. Normalised mRNA levels of β,β-carotene 15,15′-mono-oxygenase 1 (BCMO1), β,β-carotene 9′,10′-dioxygenase 2 (BCDO2) and scavenger receptor class B type 1 (SCARB1) genes* in relation to genotype (G) and dietary β-carotene (D) in various tissues of 63-d-old chickens

(Least-squares mean values with their standard errors; *n* 16)

	Genotype				Diet				P		
	AA		GG		Control		β-Carotene				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	G	D	G×D
Duodenum											
BCDO2	0·28	0·02	0·25	0·02	0·27	0·02	0·26	0·02	NS	NS	NS
SCARB1	0·23	0·03	0·29	0·03	0·36	0·03	0·16	0·03	NS	<0·0001	NS
CD36	0·22	0·03	0·22	0·03	0·20	0·03	0·24	0·03	NS	NS	NS
Liver											
BCMO1	0·44	0·03	0·51	0·03	0·55	0·03	0·41	0·03	NS	0·004	NS
BCDO2	0·38	0·03	0·40	0·03	0·38	0·03	0·39	0·03	NS	NS	NS
SCARB1	0·40	0·03	0·40	0·03	0·43	0·03	0·36	0·03	NS	NS	NS
CD36	0·55	0·02	0·59	0·02	0·55	0·02	0·59	0·02	NS	NS	NS
ISX	0·36	0·04	0·44	0·04	0·40	0·04	0·40	0·04	NS	NS	NS
Pectoralis major muscle											
BCMO1	0·57	0·02	0·25	0·02	0·40	0·02	0·42	0·02	<0·0001	NS	NS
BCDO2	0·37	0·03	0·45	0·03	0·40	0·03	0·43	0·03	NS	NS	NS
SCARB1	0·30	0·03	0·34	0·03	0·30	0·03	0·34	0·03	NS	NS	NS
CD36	0·33	0·03	0·27	0·03	0·28	0·03	0·31	0·03	NS	NS	NS
ISX	ND		ND		ND		ND		–	–	–

CD36, cluster determinant 36; ISX, intestine-specific homeobox; ND, not detected.

* Data were normalised internally using three reference genes simultaneously (18S ribosomal RNA, β-actin and glyceraldehyde-3-phosphate dehydrogenase) whose stability in all the selected tissues was investigated using the geNorm application[32].

Table 4. Intestine-specific homeobox (ISX) mRNA levels and retinol concentration in the duodenum and HDL-cholesterol concentration and colour index in the plasma of AA or GG chickens fed the control or the β-carotene-supplemented diet

(Least-squares mean values with their standard errors; n 16)

| | AA | | | | GG | | | | P |
| | Control | | β-Carotene | | Control | | β-Carotene | | G×D |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Duodenum									
BCMO1	0·23[b]	0·03	0·16[b]	0·03	0·38[a]	0·03	0·19[b]	0·03	0·056
ISX	0·40[a,b]	0·05	0·36[a,b]	0·05	0·31[b]	0·05	0·51[a]	0·05	0·03
Retinol	0·25[b]	0·03	0·38[a]	0·03	0·31[a,b]	0·03	0·27[a,b]	0·03	0·015
Plasma									
HDL-cholesterol	119·1[a]	1·6	124·0[a]	1·6	117·1[a,b]	1·6	108·6[b]	1·6	0·048
Colour index (arbitrary units)	10·35[a]	0·46	10·23[a]	0·44	10·31[a]	0·44	7·46[b]	0·43	0·003

G, genotype; D, dietary β-carotene; BCMO1, β,β-carotene 15,15′-mono-oxygenase 1.
[a,b] Least-squares mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

and liver ($P=0.03$) of GG chickens. Zeaxanthin concentration was absent in the liver and not regulated in the duodenum.

Vitamin A and vitamin E status were affected by genotype or the incorporation of β-carotene in the diet, but the effects varied according to the tissue (Table 5). Lower concentrations of α- and γ-tocopherol were observed in the plasma ($P=0.001$ and $P=0.003$ for α and γ, respectively) of GG chickens. The concentration of α-tocopherol was also lower ($P=0.02$) in the duodenum of GG chickens. The concentration of α-tocopherol was lowered in the plasma ($P=0.001$) and in the tissues ($P=0.07$ in the duodenum, $P=0.01$ in the liver and $P=0.001$ in the pectoralis major muscle) of chickens fed the β-carotene diet. By contrast, the concentration of vitamin A metabolites increased in response to the β-carotene diet.
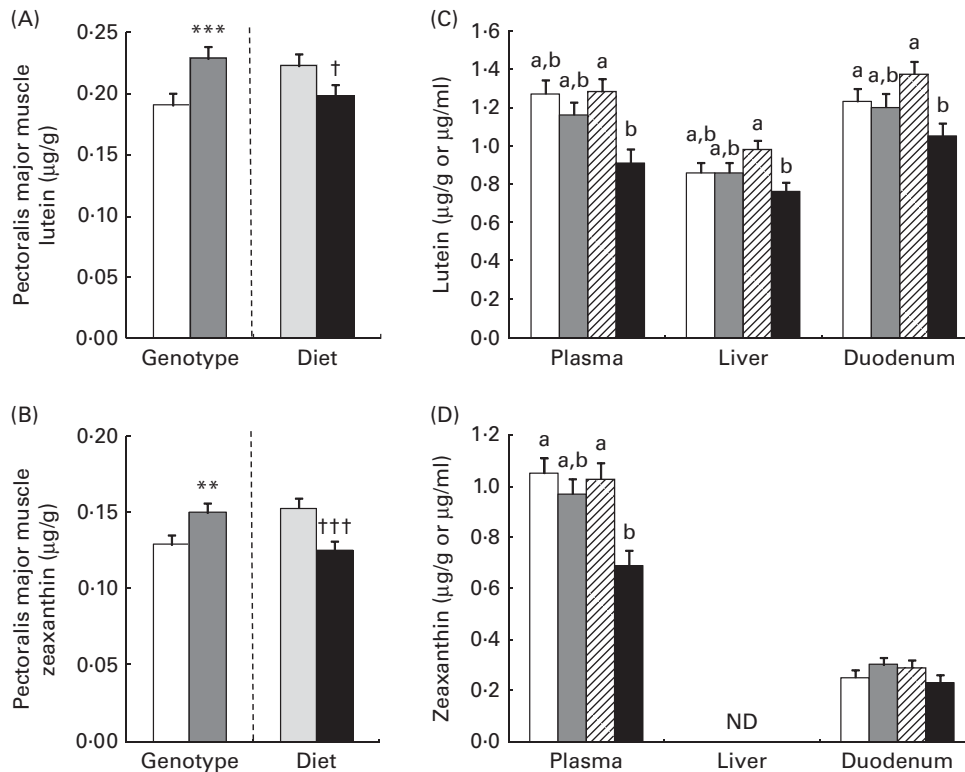

Fig. 1. Effects of genotype (G) or diet (D) on lutein (A) and zeaxanthin (B) concentrations in the pectoralis major muscle (□, AA; ■, GG; □, control; ■, β-carotene). Lutein (C) and zeaxanthin (D) concentrations in the plasma, liver and duodenum of AA or GG chickens fed the control or β-carotene-supplemented diet (□, AA control; ■, AA β-carotene; ▨, GG control; ■, GG β-carotene). Values are least-squares means (n 16 chickens per treatment), with their pooled standard errors represented by vertical bars. [a,b] Least-squares mean values with unlike letters were significantly different within a tissue or plasma ($P<0.05$). Least-squares mean value was significantly different from that of the AA genotype: ** $P<0.01$, *** $P<0.001$. Least-squares mean value was significantly different from that of the control diet: † $P < 0.05$, ††† $P < 0.001$. For lutein concentrations, there was a significant effect for diet ($P=0.002$ (plasma), $P=0.03$ (liver) and $P=0.02$ (duodenum)) and the genotype × diet interaction was significant ($P=0.004$ (plasma), $P=0.02$ (liver) and $P=0.04$ (duodenum)). For plasma zeaxanthin concentrations, there were significant genotype ($P=0.02$) and diet ($P=0.001$) effects, and the genotype × diet interaction was significant ($P=0.03$). ND, not determined.

Table 5. Concentrations* of vitamin A and E metabolites in relation to genotype (G) and dietary β-carotene (D) in several tissues and plasma of 63-d-old chickens

(Least-squares mean values with their standard errors; *n* 8)

| | Genotype | | | | Diet | | | | P | | |
| | AA | | GG | | Control | | β-Carotene | | | | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	G	D	G×D
Plasma											
Retinol	1·80	0·04	1·75	0·05	1·75	0·05	1·81	0·05	NS	NS	NS
α-Tocopherol	29·9	1·63	21·9	1·74	30·08	1·72	21·73	1·66	0·001	0·001	NS
γ-Tocopherol	1·29	0·06	1·00	0·07	1·21	0·07	1·08	0·07	0·003	NS	NS
Duodenum											
α-Tocopherol	6·85	0·75	4·40	0·70	6·59	0·72	4·66	0·72	0·02	0·07	NS
Liver											
Retinol	20·5	3·7	26·2	3·6	23·2	3·7	23·5	3·6	NS	NS	NS
Retinyl myristate	22·5	2·6	25·6	2·5	20·0	2·6	28·1	2·5	NS	0·03	NS
Retinyl palmitate	247·7	12·3	237·3	11·8	209·8	12·3	275·2	11·8	NS	0·001	NS
Retinyl stearate	220·3	16·4	222·7	15·8	203·0	16·4	240·0	15·9	NS	NS	NS
Total vitamin A	511·0	31·1	511·8	30·0	456·0	31·1	566·9	30·0	NS	0·02	NS
α-Tocopherol	9·29	1·29	7·82	1·33	11·20	1·33	5·92	1·29	NS	0·01	NS
γ-Tocopherol	1·79	0·53	1·87	0·31	1·83	0·52	1·83	0·32	NS	NS	NS
Pectoralis major muscle											
Retinol	0·26	0·01	0·26	0·01	0·26	0·01	0·26	0·01	NS	NS	NS
α-Tocopherol	7·32	0·31	6·85	0·31	7·87	0·31	6·29	0·31	NS	0·001	NS
γ-Tocopherol	0·66	0·02	0·68	0·02	0·69	0·02	0·66	0·02	NS	NS	NS

* Data are expressed in μg/g for tissues or μg/ml for plasma.

Several forms of retinyl esters (the storage forms of vitamin A) were detected only in the liver. Concentrations of total vitamin A were increased ($P=0.02$) following β-carotene supplementation, essentially as a result of increased myristate and palmitate ester contents ($P=0.03$ and $P=0.001$, respectively). Retinol concentration was also increased (0·38 (SE 0·03) *v.* 0·25 (SE 0·03) μg/g; $P<0.05$) in response to dietary β-carotene supplementation in the duodenum of AA chickens (Table 4).

Plasma metabolites and tissue lipid contents

Of all the plasma metabolites measured, only cholesterol differed between the genotypes (Table 6). Total cholesterol concentration was higher ($P=0.01$) in AA than in GG chickens. For HDL-cholesterol concentration, a significant effect of genotype was observed ($P=0.01$) in a genotype × diet interaction manner ($P=0.05$): HDL-cholesterol concentration was higher in AA than in GG chickens when fed the β-carotene diet (Table 4). A slight decrease ($P<0.05$) in Ca concentration was also observed following dietary β-carotene supplementation. The lipid content of the liver and pectoralis major muscle was unaffected by genotype or diet.

Growth, body composition and colour parameters

Body weight at slaughter was higher ($P=0.02$) in AA than in GG chickens, but not affected by diet (Table 7). Body composition was affected by both genotype and diet. Breast muscle yields were lower ($P=0.01$) in AA than in GG chickens, and a trend towards higher values was observed following dietary β-carotene supplementation ($P=0.06$). Dietary β-carotene increased leg yield ($P=0.002$) and decreased abdominal fat yield ($P=0.03$). The pectoralis major muscle was more yellow ($P=0.03$) in GG than in AA chickens. The plasma CI was decreased by β-carotene supplementation in GG chickens (Table 4).

Table 6. Concentrations of plasma metabolites and tissue lipid contents of 63-d-old chickens in relation to genotype (G) and dietary β-carotene (D)

(Least-squares mean values with their standard errors; *n* 16)

| | Genotype | | | | Diet | | | | P | | |
| | AA | | GG | | Control | | β-Carotene | | | | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	G	D	G×D
Plasma metabolites (mg/l)											
Ca	126	1·6	124	1·6	127	1·6	123	1·6	NS	0·05	NS
Total cholesterol	1536	30	1421	30	1508	30	1449	30	0·01	NS	NS
LDL-cholesterol	233	11·6	221	11·6	236	11·6	218	11·6	NS	NS	NS
Lipid content (%)											
Liver	4·8	0·18	4·5	0·18	4·8	0·18	4·6	0·18	NS	NS	NS
Pectoralis major muscle	0·92	0·02	0·90	0·02	0·91	0·02	0·91	0·02	NS	NS	NS

Table 7. Body weight and yields and colour traits of 63-d-old chickens in relation to genotype (G) and dietary β-carotene (D)

(Least-squares mean values with their standard errors; n 92–120 per genotype and n 105–106 per diet except for plasma colour index)

	Genotype				Diet				P		
	AA		GG		Control		β-Carotene				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	G	D	G×D
Body weight (g)	1825	24	1742	27	1799	25	1768	25	0·02	NS	NS
Yields (% of body weight)											
Abdominal fat	3·06	0·08	3·08	0·10	3·20	0·09	2·93	0·09	NS	0·03	NS
Breast	12·16	0·09	12·52	0·10	12·21	0·10	12·47	0·10	0·01	0·06	NS
Leg	22·83	0·10	22·74	0·12	22·54	0·11	23·04	0·11	NS	0·002	NS
Liver	1·71	0·02	1·66	0·02	1·70	0·02	1·67	0·02	NS	NS	NS
Pectoralis major muscle colour traits											
L*	47·87	0·25	47·80	0·29	47·92	0·27	47·75	0·27	NS	NS	NS
a*	−0·71	0·07	−0·64	0·08	−0·67	0·08	−0·69	0·08	NS	NS	NS
b*	6·89	0·08	7·14	0·09	7·08	0·08	6·95	0·08	0·03	NS	NS

L*, lightness; a*, redness; b*, yellowness.

Discussion

By selecting chicken breeders of a pure line for their status at the BCMO1 locus, we were able to produce offspring homozygous (AA or GG) for this specific mutation, but sharing a common genetic background. This model is particularly pertinent to evaluate the consequences of variations in BCMO1 expression on chicken growth and metabolism in different nutritional conditions (presence or absence of β-carotene). The addition of β-carotene in the diet significantly altered the vitamin A status as evidenced by the increase in liver retinol accumulation, which is probably the result of BCMO1 gene activity. Numerous parameters were consistently affected in both genotypes. In the liver, two forms of retinyl ester (but not retinol) were increased in response to β-carotene supplementation in the diet, indicating an efficient conversion of dietary β-carotene in both genotypes. By contrast, concentrations of α-tocopherol in the plasma and all tissues, as well as concentrations of lutein (0·20 v. 0·22 μg/g) and zeaxanthin (0·12 v. 0·15 μg/g) in the pectoralis major muscle, were decreased by β-carotene supplementation. Similarly, Wang et al.[11] observed a significant reduction of lutein concentration in the plasma and several tissues including heart in leghorn chickens fed high supplements of dietary β-carotene. However, a discrepancy was found by these authors for zeaxanthin concentrations that were not different in the heart but in other tissues and plasma. Dietary β-carotene also reduced abdominal fat and slightly increased leg and breast yields without affecting overall chicken growth. The effects of β-carotene, a pro-vitamin A carotenoid, on body fat are consistent with previous results showing that dietary β-carotene reduces the lipid storage capacity of adipocytes in a BCMO1-dependent manner[39,40], suggesting a vitamin A-dependent mechanism. Interestingly, a reduction in Ca concentration was also observed in the plasma of chickens supplemented with the β-carotene diet. It has already been shown that vitamin A antagonises the Ca response to vitamin D in human subjects[41]. The activity of the BCMO1 gene was negatively regulated by dietary β-carotene in the liver, but not

in the pectoralis major muscle. However, the negative effect of β-carotene on BCMO1 in the duodenum was only significant in the GG genotype. A decline in the mRNA levels of SCARB1 in the duodenum was also observed in response to dietary β-carotene supplementation. Several studies[42–44] have reported a negative control of BCMO1 and SCARB1 gene expression or enzyme activity by pro-vitamin A carotenoids, and/or vitamin A or its derivatives, such as retinoic acid, especially in the intestinal tract. This negative feedback is mediated by retinoid receptors (retinoic acid receptor and retinoid X receptor)[42,45], and a common regulatory pathway for these two genes has previously been described in the intestine, involving the ISX response element[44,46]. In the present study, the expression of ISX was up-regulated in response to dietary β-carotene supplementation in the duodenum of GG but not AA chickens. The concomitant increase in the expression of ISX and a decrease in the expression of BCMO1 in GG chickens suggest an efficient negative feedback that would allow maintaining stable duodenal retinol content. By contrast, this negative feedback appears as non-functional in the duodenum of AA chickens, which could explain the increased retinol accumulation following dietary β-carotene supplementation.

Significant interactions between genotype and diet were also observed for lutein concentrations in the plasma, liver and duodenum and for zeaxanthin concentrations in the plasma. These concentrations were similar between the genotypes in the control conditions, and significantly decreased by β-carotene supplementation only in the GG genotype. Competition between carotenoids has been reported, in which increased supply of β-carotene resulted in lower absorption of xanthophylls[11]. As vitamin E derivatives and cholesterol share the same transporters[47,48], we make the hypothesis that a similar mechanism could induce lower levels of all those metabolites in the plasma or tissues of chickens supplemented with β-carotene. Moreover, the increased β-carotene conversion may lower its competitive effect in AA chickens, while it may be maximal in GG chickens, and explain the decrease in plasma lutein and zeaxanthin concentrations, duodenum and

liver lutein contents and circulating HDL-cholesterol levels following dietary β-carotene supplementation.

The effect of the genotypes on breast meat yellow colour (AA < GG), lutein and zeaxanthin contents (AA < GG) and *BCMO1* mRNA levels (AA > GG) in the pectoralis major muscle, previously observed with chickens fed a maize-based diet[23,24], was confirmed in the present study using a wheat-based diet, without any interaction with the dietary supplementation of β-carotene. In this tissue, lutein and zeaxanthin contents were lowered following β-carotene supplementation without any alteration in the gene expression of *BCMO1*. Thus, the differential activity of the *BCMO1* gene between the AA and GG genotypes remains tightly linked with xanthophylls levels in this tissue. Although xanthophylls are not pro-vitamin A substrates for the BCMO1 enzyme[25,27], the observations of the present study in chickens and the recent discovery of human genetic variants in the *BCMO1* gene that are associated with variations in circulating or tissue levels of zeaxanthin and lutein[14,15] argue in favour of a possible role of the BCMO1 enzyme in the metabolism of xanthophylls, which deserves further studies.

A significant effect of genotype, without interaction with diet, was recorded for a number of traits that did not differ in the previous study[24]. We observed a significant effect of genotype on overall growth (AA > GG) and breast muscle yield (AA < GG). Plasma concentrations of the vitamin E isoforms α- and γ-tocopherol and duodenal content of α-tocopherol were significantly higher in AA than in GG chickens. The differences between the results of that study[24] and the present study could be explained by the increased statistical power due to the greater number of animals studied in the present study or might be the consequences of the reduced zeaxanthin content of the wheat-based diet used in the present study. As discussed above, competition phenomena are known to occur between carotenoids[11,49] and between carotenoids and other nutrients[50]. In particular, β-carotene, vitamin E and cholesterol share similar transport mechanisms[47,48]. The lower availability of β-carotene in the duodenum of AA chickens due to higher conversion by the BCMO1 enzyme may therefore result in the increased absorption of vitamin E and cholesterol.

By comparing genetic variants at the *BCMO1* locus in chickens fed with or without dietary β-carotene supplementation, we identified a defect in the feedback regulation of *BCMO1* gene expression by retinoids in the duodenum of AA chickens. The ISX-mediated negative feedback was observed in response to dietary β-carotene supplementation in GG chickens only. We make the hypothesis that the resulting decrease in β-carotene conversion by the BCMO1 enzyme could result in a lower uptake of xanthophylls, liposoluble vitamins and cholesterol due to competition in the duodenum of GG chickens. In the breast muscle, where ISX was not detectable, the gene expression of *BCMO1* was insensitive to dietary β-carotene, while storage of xanthophylls was decreased. The relationship between *BCMO1* mRNA levels and lutein and zeaxanthin contents in the pectoralis major muscle deserves further research for a complete explanation. From a basic perspective, the present data show that a SNP in the promoter of a metabolic gene (*BCMO1*) can lead to tissue-specific alterations of its expression and to diet × genotype interactions for a number of physiological parameters. Some are directly linked with the activity of the corresponding enzyme, such as retinol concentration in the duodenum, while others may rely on indirect effects such as the impact on lutein concentrations in the plasma, liver and duodenum. This illustrates the complexity of nutrigenetics. From an applied perspective, the present data show that the polymorphism at the *BCMO1* locus is important to consider whether breast meat yellow colour and lutein content are traits of high added value, which could be the case for the production of yellow chickens. The present study also shows that dietary β-carotene is efficiently converted by broiler chickens into vitamin A, with favourable effects on carcass quality, such as decreased adiposity and increased breast muscle yields.

References

1. Mora O, Kuri-Melo L, González-Gallardo A, *et al.* (2004) A potential role of β-carotene in avian embryonic development. *Int J Vitam Nutr Res* **74**, 116–122.
2. Koutsos EA, Clifford AJ, Calvert CC, *et al.* (2003) Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). *J Nutr* **133**, 1132–1138.
3. Surai PF (2002) *Natural Antioxidants in Avian Nutrition and Reproduction.* Nottingham: Nottingham University Press.
4. Lampert JM, Holzchuh J, Hessel S, *et al.* (2003) Provitamin A conversion to retinal via the β,β-carotene-15,15′-oxygenase (bcox) is essential for pattern formation and differentiation during zebrafish embryogenesis. *Development* **130**, 2173–2186.

5. Müller L, Fröhlich K & Böh V (2011) Comparative anti-oxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem* **129**, 139–148.

6. Wang Y, Ausman LM, Greenberg AS, *et al.* (2010) Dietary lycopene and tomato extract supplementations inhibit non-alcoholic steatohepatitis-promoted hepatocarcinogenesis in rats. *Int J Cancer* **126**, 1788–1796.

7. Voutilainen S, Nurmi T, Mursu J, *et al.* (2006) Carotenoids and cardiovascular health. *Am J Clin Nutr* **83**, 1265–1271.

8. Krinsky NI, Landrum JT & Bone RA (2003) Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annu Rev Nutr* **23**, 171–201.

9. Na JC, Song JY, Lee BD, *et al.* (2004) Effect of polarity on absorption and accumulation of carotenoids by laying hens. *Anim Feed Sci Technol* **117**, 305–315.

10. Chung HY, Ferreira AL, Epstein S, *et al.* (2009) Site-specific concentrations of carotenoids in adipose tissue: relations with dietary and serum carotenoid concentrations in healthy adults. *Am J Clin Nutr* **90**, 533–539.

11. Wang Y, Illingworth DR, Connor SL, *et al.* (2010) Competitive inhibition of carotenoid transport and tissue concentrations by high dose supplements of lutein, zeaxanthin and beta-carotene. *Eur J Nutr* **49**, 327–336.

12. Bhosale P & Bernstein PS (2007) Vertebrate and invertebrate carotenoid-binding proteins. *Arch Biochem Biophys* **458**, 121–127.

13. Lindqvist A, Sharvill J, Sharvill DE, *et al.* (2007) Loss-of-function mutation in carotenoid 15,15′-monooxygenase identified in a patient with hypercarotenemia and hypovitaminosis A. *J Nutr* **137**, 2346–2350.

14. Ferrucci L, Perry JRB, Matteini A, *et al.* (2009) Common variation in the β-carotene 15,15′-monooxygenase 1 gene affects circulating levels of carotenoids: a genome-wide association study. *Am J Human Genet* **84**, 123–133.

15. Borel P, De Edelenyi FS, Vincent-Baudry S, *et al.* (2011) Genetic variants in BCMO1 and CD36 are associated with plasma lutein concentrations and macular pigment optical density in humans. *Ann Med* **43**, 47–59.

16. Tian R, Pitchford WS, Morris CA, *et al.* (2009) Genetic variation in the β,β-carotene-9′,10′-dioxygenase gene and association with fat colour in bovine adipose tissue and milk. *Anim Genet* **41**, 253–259.

17. Våge DI & Boman IA (2010) A nonsense mutation in the beta-carotene oxygenase 2 (BCO2) gene is tightly associated with accumulation of carotenoids in adipose tissue in sheep (*Ovis aries*). *BMC Genet* **11**, 10.

18. Ortega H, Castilla P, Gómez-Coronado D, *et al.* (2005) Influence of apolipoprotein E genotype on fat-soluble plasma antioxidants in Spanish children. *Am J Clin Nutr* **81**, 624–632.

19. Herron KL, McGrane MM, Waters D, *et al.* (2006) The ABCG5 polymorphism contributes to individual responses to dietary cholesterol and carotenoids in Eggs. *J Nutr* **136**, 1161–1165.

20. Borel P, Moussa M, Reboul E, *et al.* (2007) Human plasma levels of vitamin E and carotenoids are associated with genetic polymorphism in genes involved in lipid metabolism. *J Nutr* **137**, 2653–2659.

21. Borel P, Moussa M, Reboul E, *et al.* (2009) Human fasting plasma concentrations of vitamin E and carotenoids, and their association with genetic variants in apo C-III cholesteryl ester transfer protein, hepatic lipase, intestinal fatty acid binding protein and microsomal triacylglycerol transfer protein. *Br J Nutr* **101**, 680–687.

22. Borel P (2012) Genetic variations involved in interindividual variability in carotenoid status. *Mol Nutr Food Res* **56**, 228–240.

23. Le Bihan-Duval E, Nadaf J, Berri C, *et al.* (2011) Detection of a Cis [corrected] eQTL controlling BCMO1 gene expression leads to the identification of a QTG for chicken breast meat color. *PLoS One* **6**, e14825.

24. Jlali M, Graulet B, Chauveau-Duriot B, *et al.* (2012) A mutation in the promoter of the chicken β,β-carotene-15,15′-monooxygenase 1 gene alters xanthophyll metabolism through a selective effect on its mRNA abundance in the breast muscle. *J Anim Sci* **90**, 4280–4288.

25. Lindqvist A & Andersson S (2002) Biochemical properties of purified recombinant human beta-carotene 15,15′-monooxygenase. *J Biol Chem* **277**, 23942–23948.

26. Lakshman MR (2004) Alpha and omega of carotenoid cleavage. *J Nutr* **134**, 241S–245S.

27. Kim YS & Oh DK (2009) Substrate specificity of a recombinant chicken beta-carotene 15,15′-monooxygenase that converts beta-carotene into retinal. *Biotechnol Lett* **31**, 403–408.

28. Le Bihan-Duval E, Nadaf J, Berri C, *et al.* (2010) >Marqueurs génétiques pour la coloration de la viande (Genetic markers for coloring meat). License deposit 25 August 2008, no. EP2161345A1.

29. Nadaf J, Gilbert H, Pitel F, *et al.* (2007) Identification of QTL controlling meat quality traits in an F$_2$ cross between two chicken lines selected for either low or high growth rate. *BMC Genomics* **8**, 155.

30. Berri C, Le Bihan-Duval E, Debut M, *et al.* (2007) Consequence of muscle hypertrophy on Pectoralis major characteristics and breast meat quality of broiler chickens. *J Anim Sci* **85**, 2005–2011.

31. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.

32. Nozière P, Grolier P, Durand D, *et al.* (2006) Variations in carotenoids, fat-soluble micronutrients, and color in cows' plasma and milk following changes in forage and feeding level. *J Dairy Sci* **89**, 2634–2648.

33. Calderón F, Chauveau-Duriot B, Pradel P, *et al.* (2007) Variations in carotenoids, vitamins A and E, and color in cow's plasma and milk following a shift from hay diet to diets containing increasing levels of carotenoids and vitamin E. *J Dairy Sci* **90**, 5651–5664.

34. Sibut V, Le Bihan-Duval E, Tesseraud S, *et al.* (2008) AMP-activated protein kinase involved in the variations of muscle glycogen and breast meat quality between lean and fat chickens. *J Anim Sci* **86**, 2888–2896.

35. Vandesompele J, De Preter K, Pattyn F, *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

36. Hellemans J, Mortier G, De Paepe A, *et al.* (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**, R19.

37. Chauveau-Duriot B, Doreau M, Nozière P, *et al.* (2010) Simultaneous quantification of carotenoids, retinol, and tocopherols in forages, bovine plasma, and milk: validation of a novel UPLC method. *Anal Bioanal Chem* **397**, 777–790.

38. AOAC (1990) *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed. Washington, DC: Association of Official Analytical Chemists.

39. Lobo GP, Amengual J, Li HN, *et al.* (2010) Beta, beta-carotene decreases peroxisome proliferator receptor gamma

activity and reduces lipid storage capacity of adipocytes in a beta, beta-carotene oxygenase 1-dependent manner. *J Biol Chem* **285**, 27891–27899.

40. Amengual J, Gouranton E, van Helden YG, *et al.* (2011) Beta-carotene reduces body adiposity of mice via *BCMO1*. *PLoS One* **6**, e20644.

41. Johansson S & Melhus H (2001) Vitamin A antagonizes calcium response to vitamin D in man. *J Bone Mineral Res* **16**, 1899–1905.

42. Bachmann H, Desbarats A, Pattison P, *et al.* (2002) Feedback regulation of beta, beta-carotene 15, 15′-mono-oxygenase by retinoic acid in rats and chickens. *J Nutr* **132**, 3616–3622.

43. Wyss A (2004) Carotene oxygenases: a new family of double bond cleavage enzymes. *J Nutr* **134**, 246S–250S.

44. Lobo GP, Hessel S, Eichinger A, *et al.* (2010) ISX is a retinoic acid-sensitive gatekeeper that controls intestinal β,β-carotene absorption and vitamin A production. *FASEB J* **24**, 1656–1666.

45. Takitani K, Zhu CL, Inoue A, *et al.* (2006) Molecular cloning of the rat β-carotene 15,15′-monooxygenase gene and its regulation by retinoic acid. *Eur J Nutr* **45**, 320–326.

46. Lietz G, Lange J & Rimbach G (2010) Molecular and dietary regulation of β,β-carotene 15,15′-monooxygenase 1 (BCMO1). *Arch Biochem Biophys* **502**, 8–16.

47. van Bennekum A, Werder M, Thuahnai ST, *et al.* (2005) Class B scavenger receptor-mediated intestinal absorption of dietary β-carotene and cholesterol. *Biochemistry* **44**, 4517–4525.

48. Reboul E, Klein A, Bietrix F, *et al.* (2006) Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte. *J Biol Chem* **281**, 4739–4745.

49. van den Berg H & van Vliet T (1998) Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl ester responses in the triacylglycerol-rich lipoprotein fraction of men. *Am J Clin Nutr* **68**, 82–89.

50. Mamatha BS & Baskaran V (2011) Effect of micellar lipids, dietary fiber and β-carotene on lutein bioavailability in aged rats with lutein deficiency. *Nutrition* **27**, 960–966.