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Supplementation of xanthophylls decreased proinflammatory and increased anti-inflammatory cytokines in hens and chicks

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Abstract

The present study investigated the effects of xanthophylls (containing 40 % of lutein and 60 % of zeaxanthin) on proinflammatory cytokine (IL-1β, IL-6, interferon (IFN)-γ and lipopolysaccharide-induced TNF-α factor (LITAF)) and anti-inflammatory cytokine (IL-4 and IL-10) expression of breeding hens and chicks. In Expt 1, a total of 432 hens were fed diets supplemented with 0 (as the control group), 20 or 40 mg/kg xanthophylls (six replicates per treatment). The liver, duodenum, jejunum and ileum were sampled at 35 d of the trial. The results showed that both levels of xanthophyll addition decreased IL-1β mRNA in the liver and jejunum, IL-6 mRNA in the liver, IFN-γ mRNA in the jejunum and LITAF mRNA in the liver compared to the control group. Expt 2 was a 2 × 2 factorial design. Male chicks hatched from 0 or 40 mg/kg xanthophyll diet of hens were fed a diet containing either 0 or 40 mg/kg xanthophylls. The liver, duodenum, jejunum and ileum were collected at 0, 7, 14 and 21 d after hatching. The results showed that *in ovo* xanthophylls decreased proinflammatory cytokine expression (IL-1β, IL-6, IFN-γ and LITAF) in the liver, duodenum, jejunum and ileum and increased anti-inflammatory cytokine expression (IL-4 and IL-10) in the liver, jejunum and ileum mainly at 0–7 d after hatching. *In ovo* effects gradually vanished and dietary effects began to work during 1–2 weeks after hatching. Dietary xanthophylls modulated proinflammatory cytokines (IL-1β, IL-6 and IFN-γ) in the liver, duodenum, jejunum and ileum and anti-inflammatory cytokine (IL-10) in the liver and jejunum mainly from 2 weeks onwards. In conclusion, xanthophylls could regulate proinflammatory and anti-inflammatory cytokine expression in different tissues of hens and chicks.

Key words: Xanthophylls: Proinflammatory cytokines: Anti-inflammatory cytokines: Hens: Chicks

The health benefits of carotenoid pigment were first elucidated in mammals more than 80 years ago[1], and the following decades of research have helped us to raise our interest and understanding of the functions of carotenoids in animals such as mammals, birds and fishes. A large body of experimental evidence has revealed that carotenoids modulate the immune system. For example, β-carotene supplementation increased $CD4^+$, natural killer and IL-2 receptor cells[2] and the ratio of $CD4^+$-to-$CD8^+$ cells[3] in human peripheral blood, and inhibited the growth of mammary tumours in mice[4], suggesting that β-carotene might be useful in immune regulation. Lutein could blunt systemic indices of inflammatory response in chicks[5], change lymphocyte subsets and proliferation in dogs[6] and cats[7], and inhibit mammary tumour growth and enhance phytohaemagglutinin-induced lymphocyte proliferation in mice[8]. Recent studies reported that proinflammatory mediators and cytokines could be regulated by lutein[9], β-carotene[10,11], β-cryptoxanthin[11], astaxanthin[12,13] and lycopene[14,15]. Therefore, modulation of proinflammatory and anti-inflammatory cytokines and other immune mediators' expression may be an important and significant mechanism for the immunomodulatory effects of carotenoids.

The intestine has a highly specialised immune system and constitutes one of the largest immunological organs. The intestine not only permits absorption of nutrients, but also maintains the ability to respond appropriately to a diverse milieu of dietary and microbial antigenic components[16,17]. The intestinal mucosa is challenged by diet-derived pro-oxidants (including Fe, Cu, H_2O_2, haem, lipid peroxides, aldehydes

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; LITAF, lipopolysaccharide-induced TNF-α factor.

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and nitrite), mutagens, carcinogens and endogenously generated reactive oxygen species, leading to oxidative stress in the gastrointestinal tract[18,19]. Carotenoids do not seem to be as well absorbed as vitamins C and E[19], and hence their concentrations could be much higher in the lumen of the gastrointestinal tract than are ever achieved in plasma or other body tissues, making a functional action in the gastrointestinal tract more likely[19]. Many of the functions of T cells in the gastrointestinal immune system are mediated by secreted cytokines[16]. Hence, investigating the effects of carotenoids on cytokine expression of the gut is meaningful for our understanding of how carotenoids work in the gut.

A maize–soyabean basal diet is used in a wide geographical area (such as the USA and China) and lutein is added to pigment skin and eggs to satisfy consumer acceptance, and so both lutein and zeaxanthin exist in the diet. In the present study, we used a rice–soyabean basal diet and applied lutein and zeaxanthin as additives not only because lutein and zeaxanthin are the main carotenoids of the chicken yolk, but also because they have little or no provitamin A activity. There have been few studies pertaining to carotenoids on proinflammatory and anti-inflammatory cytokines of hens and chicks, especially in the intestine. Therefore, our objectives were to investigate the effects of xanthophylls on proinflammatory and anti-inflammatory cytokine expression in the liver and small intestine (duodenum, jejunum and ileum) of hens and chicks.

Materials and methods

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Committee of Animal Experiments of South China Agricultural University (approval ID 201004152). All efforts were made to minimise suffering.

Expt 1

To examine the effects of dietary xanthophylls (containing 40 % of lutein and 60 % of zeaxanthin, as we determined; Juyuan Biochemical Company Limited) on proinflammatory cytokine (IL-1β, IL-6, interferon (IFN)-γ and lipopolysaccharide-induced TNF-α factor (LITAF)) and anti-inflammatory cytokine (IL-4 and IL-10) expression of breeding hens, 432 hens at 34 weeks of age with similar weight and genetic background were randomly assigned to three treatments. Each treatment was replicated six times and there were twenty-four breeding hens per replicate. The hens were fed either a carotenoid-depleted diet supplemented with 0 mg/kg xanthophylls (as the control group; containing 0·05 mg/kg xanthophylls, as we determined) or a carotenoid-replete diet supplemented with 20 or 40 mg/kg xanthophylls (containing 20·07 and 30·94 mg/kg xanthophylls, as we determined, respectively). The diets were formulated according to Chinese Feeding Standard of Chicken (2004) and NRC (1994). Details of ingredient composition and calculated nutrient content of diets for hens are provided in Table 1. The experiment lasted for 35 d, and water and diet were provided *ad libitum*.

Table 1. Basal diet composition and calculated nutrient content for meat-type breeding hens* and chicks†, on an as-fed basis

Item	Breeding hens	Chicks
Ingredient (%)		
Wheat bran	1·585	
Rice	66·00	58·69
Soyabean meal	20·18	32·93
Fish meal	2·00	2·00
Soyabean oil		2·10
Limestone powder	7·35	1·23
Calcium monohydrogen phosphate	1·54	1·50
Premix compound	1·00	1·00
DL-Met	0·095	0·25
Salt	0·25	0·30
Total	100·00	100·00
Calculated composition		
ME (MJ/kg)	11·72	12·56
CP (%)	17·00	21·50
Lys (%)	0·91	1·19
Met (%)	0·39	0·60
Met + Cys (%)	0·64	0·91
Non-phytate P (%)	0·45	0·45
Ca (%)	3·30	1·00

ME, metabolisable energy; CP, crude protein.

* Premix compound (mineral and vitamin mix) provided per kg of diet: vitamin A, 3·6 mg; cholecalciferol, 0·06 mg; vitamin E, 30 mg; menadione, 1·5 mg; riboflavin, 9·0 mg; niacin, 35 mg; D-pantothenic acid, 12 mg; vitamin B_{12}, 0·012 mg; biotin, 0·2 mg; folacin, 1·2 mg; vitamin B_1, 2·0 mg; vitamin B_6, 4·5 mg; 8·0 mg of Cu from $CuSO_4 \cdot 5H_2O$; 80 mg of Zn from $ZnSO_4 \cdot H_2O$; 100 mg of Mn from $MnSO_4 \cdot H_2O$; 80 mg of Fe from $FeSO_4 \cdot H_2O$; 1·0 mg of I from KI; 0·3 mg of Se from Na_2SeO_3.

† Premix compound (mineral and vitamin mix) provided per kg of diet: vitamin A, 2·4 mg; cholecalciferol, 0·025 mg; vitamin E, 20 mg; menadione, 0·5 mg; riboflavin, 8·0 mg; niacin, 35 mg; D-pantothenic acid, 10 mg; vitamin B_{12}, 0·01 mg; biotin, 0·18 mg; folacin, 0·55 mg; vitamin B_1, 2·0 mg; vitamin B_6, 3·5 mg; 8·0 mg of Cu from $CuSO_4 \cdot 5H_2O$; 100 mg of Zn from $ZnSO_4 \cdot H_2O$; 120 mg of Mn from $MnSO_4 \cdot H_2O$; 100 mg of Fe from $FeSO_4 \cdot H_2O$; 0·7 mg of I from KI; 0·3 mg of Se from Na_2SeO_3.

Production performance (egg number, total egg weight, feed intake, broken eggs, qualified eggs and hen mortality) of each replicate was recorded daily. Blood of hens (two hens for each replicate) was sampled at 7, 14, 21, 28 and 35 d after xanthophyll supplementation to determine the carotenoid content in plasma according to Koutsos *et al.*[20], which indicated that serum carotenoids reach a new steady state after 21 d of xanthophyll supplementation (Fig. S1, supplementary material for this article can be found at http://www.journals.cambridge.org/bjn). From 29 to 35 d of the trial, 510 eggs (eighty-five eggs from each replicate) were collected from the control group or the 40 mg/kg xanthophyll group, and hatched artificially to determine the fertilisation rate, hatchability of fertilised eggs, chick birth weight and healthy chick rate. The eggs were collected from 29 d of the trial because yolk carotenoid concentration had reached a new steady state after about 3 weeks of supplementation in the hens' diet, as we (Fig. S2, supplementary material for this article can be found at http://www.journals.cambridge.org/bjn) and other researchers have determined[21,22]. On the 35th day of the trial, two hens from each replicate (twelve hens for each treatment) were weighed and slaughtered. Liver, duodenum, jejunum and ileum samples were collected immediately after slaughter and flash-frozen in liquid N_2 for the determination of proinflammatory and anti-inflammatory cytokines.

Expt 2

To examine the effects of *in ovo* and dietary xanthophylls (containing 40 % of lutein and 60 % of zeaxanthin) on proinflammatory cytokine (IL-1β, IL-6, IFN-γ and LITAF) and anti-inflammatory cytokine (IL-4 and IL-10) expression of chicks, a 2 × 2 factorial arrangement of treatments consisting of two *in ovo* xanthophyll levels and two dietary xanthophyll levels was designed. A total of 510 eggs were collected from hens fed a carotenoid-depleted diet or a 40 mg/kg xanthophyll diet in Expt 1. On the day of hatching, fertilisation rate, hatchability of fertilised eggs, chick birth weight and healthy chick rate were calculated, and 180 healthy male chicks from each *in ovo* xanthophyll treatment were chosen randomly and assigned to one of two dietary xanthophyll levels: a basal diet supplemented with 0 or 40 mg/kg xanthophylls (containing 0·07 and 40·02 mg/kg xanthophylls, as we determined, respectively). A total of twelve chicks (two chicks for each replicate) without feeding from each *in ovo* xanthophyll treatment were weighed and slaughtered within 12 h after hatching (0 d of chicks), following which the liver, duodenum, jejunum and ileum samples were removed immediately after slaughter and flash-frozen in liquid N_2 for further analysis. There were four groups of progeny designed as follows: parents and chicks fed the carotenoid-replete diets, parents fed the carotenoid-replete and chicks fed the carotenoid-depleted diets, parents fed the carotenoid-depleted and chicks fed the carotenoid-replete diets, parents and chicks fed the carotenoid-depleted diets. Each of the four progeny groups contained six replicate pens with fifteen male chicks in each. The diets were formulated according to Chinese Feeding Standard of Chicken (2004) and NRC (1994). Details of ingredient composition and calculated nutrient content of diets for chicks are provided in Table 1. Dietary xanthophyll levels were chosen to be similar to those fed to commercial poultry. Chicks were housed in battery cages, and water and diet were provided *ad libitum*. The experiment lasted for 21 d and the growth performance (average daily feed intake, average daily gain and gain-to-feed ratio) of chicks was analysed after the experiment. A total of six chicks from each of the four groups (one chick for each replicate) were weighed and slaughtered at 7, 14 and 21 d after hatching. Liver, duodenum, jejunum and ileum samples were collected immediately after slaughter and flash-frozen in liquid N_2 for the determination of proinflammatory and anti-inflammatory cytokines.

Determination of proinflammatory and anti-inflammatory cytokines by real-time quantitative PCR

Total RNA was isolated from the frozen tissue (liver, duodenum, jejunum and ileum) of hens and chicks using a High Pure RNA Tissue Kit (Roche) following the manufacturer's recommendations. Total RNA was quantified using a spectrophotometer at an optical density of 260 nm (OD_{260}), and the purity was assessed by determining the ratio of OD_{260} to OD_{280}. This ratio ranged from 1·8 to 2·0 for all samples. Each total RNA sample was reverse-transcribed to complementary DNA using the ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer's instructions.

Quantitative real-time PCR analysis of IL-1β, IL-6, IFN-γ, LITAF, IL-4 and IL-10 mRNA was performed using the ABI 7500 Real-time PCR System (Applied Biosystems). Primer pairs were designed for IL-1β, IL-6, IFN-γ, LITAF, IL-4, IL-10 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) based on the published sequences with the following respective Genbank accession nos.: NM_204524, NM_204628, NM_205149, AY765397, NM_001007079, NM_001004414 and NM_204305, respectively. The forward and reverse primers for IL-1β were AGAAGAAGCCTCGCCTGGAT and CCGCAGCAGTTTGGTCAT; for IL-6: ATAAATCCCGATGAAGTGG and CTCACGGTCTTCTCCATAAA; for IFN-γ: TGAGCCAGATTGTTTCGA and ACGCCATCAGGAAGGTTG; for LITAF: TTCTATGACCGCCCAGTT and CAGAGCATCAACGCAAAA; for IL-4: GAGAGGTTTCCTGCGTCAAG and TGACGCATGTTGAGGAAGAG; for IL-10: CAATCCAGGGACGATGAAC and GCAGGTGAAGAAGCGGTGA; for GAPDH: ACTGTCAAGGCTGAGAACGG and CATTTGATGTTGCTGGGGTC, respectively.

Selection of a reference gene is of crucial importance for gene expression studies. *GAPDH* was selected as the reference gene because we found that many studies have used *GAPDH* as a housekeeping gene in the intestine and liver and used only one reference gene[23–27], although recent research has showed that housekeeping gene expression may vary in different tissues[28,29] and use of a single gene for normalisation may lead to relatively large errors[30]. A validation experiment was performed for each set of primers to confirm efficiency, amplification of a single gene and to optimise primer concentrations. PCR products from each gene were visualised by gel electrophoresis on 1 % agarose stained with ethidium bromide to ensure that a single product was produced of the predicted size. Single-band PCR products for each gene were sequenced and the products had 99 % homology to their respective gene transcripts. The 20 μl final PCR volume contained 1 μl RT product, 300 nmol/l forward and reverse primers and 10 μl 2 × concentrated SYBR Green Master mix (Roche). The PCR cycle was set at 95°C for 10 min followed by forty cycles of denaturing, annealing and extension at 95°C for 15 s and 58°C for 1 min (except at 56°C for LITAF). After the forty cycles were completed, a melting-curve analysis was performed to confirm that a single gene product was amplified. PCR data obtained from the ABI 7500 Real-time PCR System were automatically analysed by Applied Biosystems Software. The relative standard-curve method was used to quantify the mRNA concentrations of each gene in relation to the reference gene (*GAPDH*). Our approach was based on the calibrator-normalised relative quantification including correction for PCR efficiency. All samples were analysed in triplicate.

Statistical analysis

Statistical analysis of the data was performed using SAS 8.1 (SAS Institute Inc.). Fertilisation rate, hatchability of fertilised eggs, chick birth weight, healthy chick rate, and proinflammatory and anti-inflammatory cytokines at 0 d of chicks were analysed by *t* test between the two treatments. For analysing production performance, proinflammatory and anti-inflammatory cytokines of breeding hens, one-way ANOVA was performed. The dependent

variable was examined for the main effect of dietary xanthophylls in hens. For the growth performance at 21 d of chicks, and proinflammatory and anti-inflammatory cytokines at 7, 14 and 21 d of chicks, data were analysed by using two-way ANOVA. The model included the main effects of *in ovo*, diet, and their interaction. Replicate was used as the experimental unit. Data are presented as means with their pooled standard errors. When the main effect(s) or interaction was significant, differences among means were determined using Tukey's honestly significant difference test. Differences among means were considered significant at $P \leq 0{\cdot}05$.

Results

Effects of xanthophylls on proinflammatory cytokine expression of hens

Supplementation of 20 or 40 mg/kg xanthophylls had no effect on fertilisation rate, hatchability of fertilised eggs, chick birth weight, healthy chick rate and production performance of hens (data not shown). Compared to the control group, addition of 20 or 40 mg/kg xanthophylls decreased IL-1β mRNA level in the liver ($P=0{\cdot}028$ and 0·008, respectively; Fig. 1) and jejunum ($P=0{\cdot}032$ and 0·037, respectively), IL-6 mRNA level in the liver ($P=0{\cdot}007$ and 0·004, respectively; Fig. 2), IFN-γ mRNA level in the jejunum ($P=0{\cdot}040$ and 0·015, respectively; Fig. 3) and LITAF mRNA level in the liver ($P=0{\cdot}026$ and 0·038, respectively; Fig. 4). In addition, the 40 mg/kg xanthophyll group also had reduced liver IFN-γ and duodenum LITAF mRNA level in contrast to the control group ($P=0{\cdot}003$ and 0·012, respectively).

Effects of xanthophylls on anti-inflammatory cytokine expression of hens

Supplementation of 40 mg/kg xanthophylls increased liver IL-10 mRNA level compared to the 20 mg/kg xanthophyll and control groups ($P=0{\cdot}018$ and 0·014, respectively; Fig. 5). There was no difference in IL-4 mRNA level among treatments (data not shown).

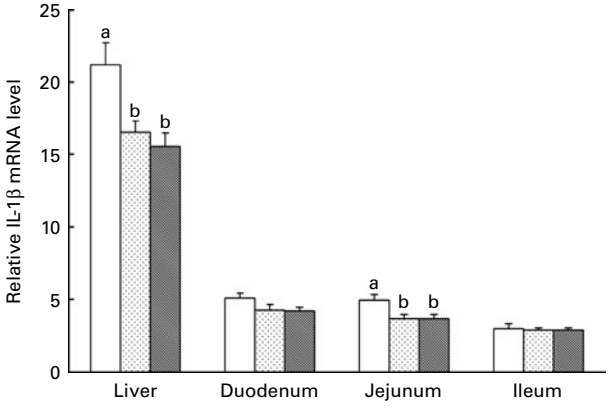

Fig. 1. Effects of xanthophylls on relative IL-1β mRNA level in the liver, duodenum, jejunum and ileum of hens. Values are means, with their standard errors represented by vertical bars, n 6. [a,b]Mean values with unlike letters were significantly different ($P<0{\cdot}05$). P value (0·007, 0·139, 0·019 and 0·931) and pooled SEM (1·13, 0·33, 0·33 and 0·23) from one-way ANOVA for liver, duodenum, jejunum and ileum, respectively. ⊏, Control; ⊏, 20 mg/kg xanthophylls; ■, 40 mg/kg xanthophylls.

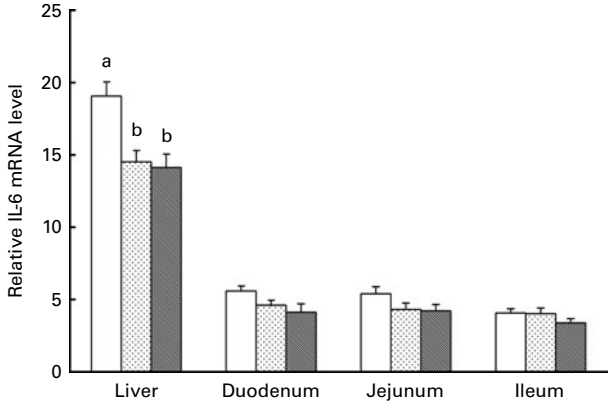

Fig. 2. Effects of xanthophylls on relative IL-6 mRNA level in the liver, duodenum, jejunum and ileum of hens. Values are means, with their standard errors represented by vertical bars, n 6. [a,b]Mean values with unlike letters were significantly different ($P<0{\cdot}05$). P value (0·002, 0·096, 0·157 and 0·327) and pooled SEM (0·90, 0·45, 0·46 and 0·35) from one-way ANOVA for liver, duodenum, jejunum and ileum, respectively. ⊏, Control; ⊏, 20 mg/kg xanthophylls; ■, 40 mg/kg xanthophylls.

Effects of xanthophylls on proinflammatory cytokine expression of chicks

Xanthophyll supplementation from *in ovo*, diet, or their interaction had no effect on the growth performance of chicks (data not shown). *In ovo* xanthophylls decreased liver IL-1β and IL-6 mRNA at 0 and 7 d, duodenum IL-6 and IFN-γ mRNA at 7 d and LITAF mRNA at 0 and 7 d, jejunum IL-1β mRNA at 0, 7 and 14 d and IL-6 mRNA at 0 d, and ileum IL-1β mRNA at 0 d and IFN-γ mRNA at 0 and 7 d (Tables 2–5). Dietary xanthophyll supplementation decreased liver IL-6 mRNA at 14 d and IFN-γ mRNA at 14 and 21 d, duodenum IL-6 and IFN-γ mRNA at 14 d, jejunum IL-1β mRNA at 14 and 21 d, and ileum IFN-γ mRNA at 21 d. LITAF mRNA in the liver, duodenum, jejunum and ileum were not affected by dietary xanthophylls.

Effects of xanthophylls on anti-inflammatory cytokine expression of chicks

Xanthophylls from *in ovo* increased liver IL-4 mRNA at 7 d and IL-10 mRNA at 0, 7 and 14 d, jejunum IL-4 and IL-10 mRNA at 0 and 7 d, and ileum IL-10 mRNA at 0 and 7 d (Tables 6 and 7). *In ovo* xanthophyll supplementation did not affect IL-4 and IL-10 mRNA in the duodenum. Dietary xanthophylls also enhanced liver IL-10 mRNA at 14 and 21 d, and jejunum IL-10 mRNA at 14 d. Addition of dietary xanthophylls had no effect on IL-4 mRNA in the liver, duodenum, jejunum and ileum.

Discussion

Measuring cytokine production is an integral part of measuring the immune response[31]. Cytokines can be generally divided into two types: proinflammatory (IL-1β, IL-6, IFN-γ, LITAF) and anti-inflammatory cytokines (IL-4, IL-10, TGF-β)[32]. Proinflammatory cytokines are essential for the development and functioning of both the innate and adaptive immune response, while their over-expression is associated with pathological

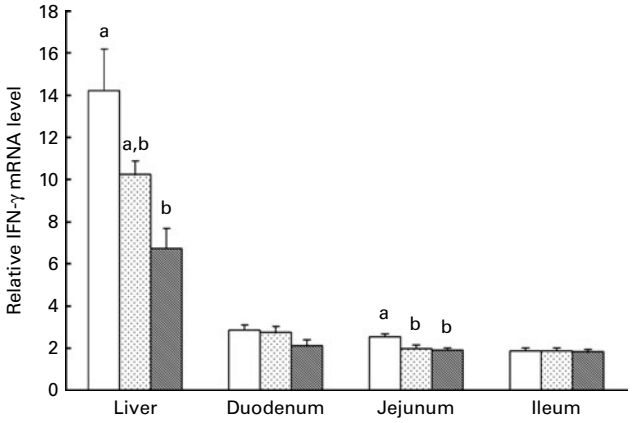

Fig. 3. Effects of xanthophylls on relative interferon (IFN)-γ mRNA level in the liver, duodenum, jejunum and ileum of hens. Values are means, with their standard errors represented by vertical bars, *n* 6. [a,b]Mean values with unlike letters were significantly different ($P<0·05$). *P* value (0·004, 0·150, 0·012 and 0·962) and pooled SEM (1·32, 0·28, 0·14 and 0·14) from one-way ANOVA for liver, duodenum, jejunum and ileum, respectively. ⊏, Control; ⊏, 20 mg/kg xanthophylls; ■, 40 mg/kg xanthophylls.

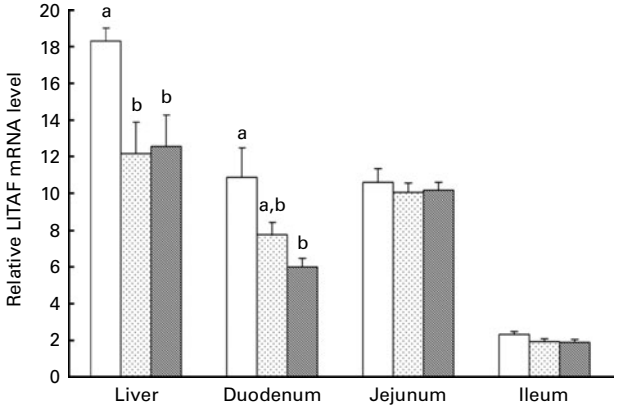

Fig. 4. Effects of xanthophylls on relative lipopolysaccharide-induced TNF-α factor (LITAF) mRNA level in the liver, duodenum, jejunum and ileum of hens. Values are means, with their standard errors represented by vertical bars, *n* 6. [a,b]Mean values with unlike letters were significantly different ($P<0·05$). *P* value (0·017, 0·014, 0·779 and 0·148) and pooled SEM (1·48, 1·04, 0·58 and 0·16) from one-way ANOVA for liver, duodenum, jejunum and ileum, respectively. ⊏, Control; ⊏, 20 mg/kg xanthophylls; ■, 40 mg/kg xanthophylls.

conditions of the immune system[33]. Recently, researchers[34–37] found that high levels of TNF-α, IFN-γ, IL-1β and IL-6 could induce intestinal epithelial permeability. Research has shown that LITAF played an important role in the activation of TNF-α gene expression in mice[38] and humans[39]. TNF has not been identified in chickens, whereas LITAF could induce the expression of TNFSF 15 (a member of the TNF ligand super family) and may play an important role in the regulation of TNF-α gene expression[40]. In Expt 1, a novel finding of the present study was that xanthophyll addition could decrease proinflammatory cytokine (IL-1β, IL-6, IFN-γ, LITAF) expression in the liver, duodenum and jejunum of hens, but not in the ileum. The same *in vivo* results were reported by research which showed that lutein supplementation reduced the concentrations of NO, TNF-α, IL-6, PGE2 and macrophage inflammatory protein-2 in the aqueous humor of endotoxin-induced uveitis in rats[9] and liver IL-1β mRNA in turkeys following lipopolysaccharide injection[41]. The inhibition of proinflammatory cytokines was also observed following the supplementation of other carotenoids in animals or cell lines, such as β-carotene in rats[10], astaxanthin in mice and rats[13,42], β-cryptoxanthin in murine macrophages[11] and lycopene in human macrophages[14,15].

Anti-inflammatory cytokines (IL-4, IL-10 and TGF-β) could prevent the over-activation of immune responses and the further production of proinflammatory cytokines and other mediators, thus mediating the balance between proinflammatory and anti-inflammatory responses[32]. Liver IL-10 mRNA of hens was increased by 40 mg/kg xanthophyll supplementation, indicating that xanthophylls could modulate anti-inflammatory cytokine expression. The effects of supplementing 40 mg/kg xanthophylls are better than 20 mg/kg xanthophylls based on our results that the 40 mg/kg xanthophyll group not only had reduced liver IFN-γ and duodenum LITAF mRNA but also elevated liver IL-10 mRNA level, which did not happen in the 20 mg/kg xanthophyll group. Xanthophylls did not affect proinflammatory and anti-inflammatory cytokine expression in the hen ileum as we measured, suggesting that the ileum is not the action site for xanthophylls. This may lie in the finding that xanthophylls, which are highly susceptible to oxidation, could be depleted before reaching the ileum. The main action site for xanthophylls may be in the liver because not only proinflammatory cytokine (IL-1β, IL-6, IFN-γ and LITAF) but also anti-inflammatory cytokine (IL-10) expression was modulated in the liver as compared to changed cytokine expression in the duodenum (LITAF) and the jejunum (IL-1β and IFN-γ).

Most yolk-derived carotenoids are deposited into the embryonic liver[43], but dietary carotenoids are more broadly distributed in the post-hatch chick[44]. In Expt 2, we demonstrated that *in ovo* or dietary xanthophylls decreased proinflammatory cytokines in the liver, duodenum, jejunum and ileum. Furthermore, *in ovo* xanthophylls increased anti-inflammatory cytokines in the liver, jejunum and ileum, but

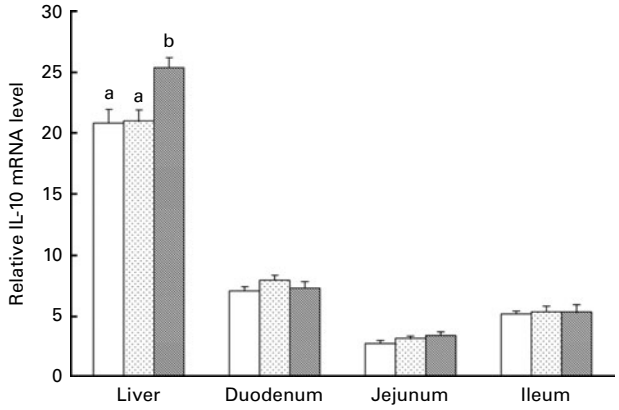

Fig. 5. Effects of xanthophylls on relative IL-10 mRNA level in the liver, duodenum, jejunum and ileum of hens. Values are means, with their standard errors represented by vertical bars, *n* 6. [a,b]Mean values with unlike letters were significantly different ($P<0·05$). *P* value (0·008, 0·416, 0·219 and 0·974) and pooled SEM (0·99, 0·46, 0·25 and 0·48) from one-way ANOVA for liver, duodenum, jejunum and ileum, respectively. ⊏, Control; ⊏, 20 mg/kg xanthophylls; ■, 40 mg/kg xanthophylls.

Table 2. Effects of *in ovo* and dietary xanthophylls on relative IL-1β mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	1·10[a]	1·10[a]	1·68[b]	1·68[b]	0·13	0·012		
7 d	1·34[a]	1·55[a,b]	1·70[a,b]	1·90[b]	0·13	0·013	0·142	0·991
14 d	1·19	1·02	1·12	1·19	0·11	0·654	0·663	0·297
21 d	1·44	1·48	1·41	1·39	0·10	0·569	0·926	0·720
Duodenum								
0 d	1·23	1·23	1·25	1·25	0·08	0·873		
7 d	2·43	2·29	2·58	2·94	0·27	0·149	0·689	0·364
14 d	2·64	2·86	2·65	3·01	0·29	0·779	0·328	0·809
21 d	2·15	2·34	2·93	2·41	0·24	0·101	0·506	0·163
Jejunum								
0 d	1·53[a]	1·53[a]	2·72[b]	2·72[b]	0·19	0·002		
7 d	1·51[a]	1·57[a]	2·33[a,b]	2·52[b]	0·23	0·001	0·607	0·779
14 d	1·46[a]	1·93[a,b]	1·96[a,b]	2·45[b]	0·22	0·032	0·041	0·973
21 d	1·58[a]	2·04[a,b]	1·61[a]	2·50[b]	0·21	0·248	0·004	0·317
Ileum								
0 d	1·35[a]	1·35[a]	2·07[b]	2·07[b]	0·19	0·021		
7 d	1·97	2·14	2·31	2·99	0·35	0·108	0·251	0·482
14 d	1·93	2·35	1·87	2·66	0·31	0·690	0·062	0·550
21 d	1·97	2·41	2·24	2·90	0·32	0·237	0·099	0·738

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

not in the duodenum. Dietary xanthophylls also increased anti-inflammatory cytokines in the liver and jejunum, but not in the duodenum and ileum. Generally speaking, maternal xanthophylls modulated proinflammatory and anti-inflammatory cytokine expression mainly at 0–7 d after hatching. During 7–14 d after hatching, the maternal effects gradually disappeared and the progeny's diet began to take over. Dietary xanthophylls modulated cytokine expression mainly from 2 weeks onwards. The cytokine results were consistent with the liver carotenoid change of chicks as we (Table S1, supplementary material for this article can be found at http://www.journals.cambridge.org/bjn) and other researchers have determined[21]. However, some studies revealed that lutein did not affect liver IL-6[45] and the liver and spleen IL-1 mRNA of chicks following lipopolysaccharide challenge[46]. The difference may be due to methodological diversity, such as environment (housing condition and density), dosage and type of carotenoids used, the interaction with other antioxidants

Table 3. Effects of *in ovo* and dietary xanthophylls on relative IL-6 mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	1·76[a]	1·76[a]	3·16[b]	3·16[b]	0·38	0·028		
7 d	1·58[a]	1·62[a]	2·64[a,b]	2·83[b]	0·30	0·001	0·705	0·794
14 d	2·98[a]	4·03[a,b]	3·44[a,b]	4·52[b]	0·38	0·230	0·012	0·970
21 d	4·18	4·07	4·22	3·72	0·41	0·715	0·471	0·639
Duodenum								
0 d	1·89	1·89	1·59	1·59	0·14	0·157		
7 d	2·43[a]	3·06[a,b]	4·35[b]	4·22[b]	0·41	0·001	0·556	0·365
14 d	3·14	3·85	3·05	4·77	0·45	0·361	0·013	0·273
21 d	3·35	3·07	3·45	2·99	0·21	0·965	0·092	0·667
Jejunum								
0 d	5·39[a]	5·39[a]	8·72[b]	8·72[b]	0·94	0·031		
7 d	3·38	3·47	4·44	4·64	0·58	0·068	0·794	0·927
14 d	7·17	6·25	6·65	7·61	0·85	0·629	0·983	0·284
21 d	6·13	6·45	7·07	5·84	0·73	0·823	0·542	0·303
Ileum								
0 d	2·90	2·90	3·18	3·18	0·51	0·706		
7 d	4·65	5·56	4·92	5·63	0·60	0·780	0·193	0·866
14 d	6·98	6·01	3·99	5·02	1·29	0·140	0·981	0·449
21 d	6·00	6·32	8·14	6·22	1·11	0·367	0·478	0·322

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 4. Effects of *in ovo* and dietary xanthophylls on relative interferon-γ mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	1·72	1·72	1·71	1·71	0·24	0·985		
7 d	1·86	1·92	2·05	1·85	0·23	0·799	0·757	0·584
14 d	2·27[a]	2·96[a,b]	2·37[a]	4·22[b]	0·43	0·132	0·008	0·195
21 d	3·21	5·01	3·96	4·70	0·54	0·687	0·030	0·343
Duodenum								
0 d	1·41	1·41	2·31	2·31	0·50	0·236		
7 d	4·85[a,b]	3·66[a]	7·72[b]	6·66[a,b]	1·02	0·009	0·283	0·948
14 d	24·34	32·47	23·32	33·99	3·46	0·942	0·013	0·717
21 d	22·50	24·99	24·44	27·52	3·11	0·480	0·381	0·926
Jejunum								
0 d	1·24	1·24	1·15	1·15	0·35	0·861		
7 d	3·03	3·23	3·94	2·78	0·67	0·737	0·487	0·320
14 d	25·32	32·96	29·23	33·71	5·12	0·654	0·251	0·760
21 d	26·76	29·74	33·63	24·65	5·13	0·864	0·565	0·257
Ileum								
0 d	1·49[a]	1·49[a]	2·63[b]	2·63[b]	0·27	0·013		
7 d	3·27	3·34	4·26	4·37	0·46	0·040	0·856	0·967
14 d	31·78	38·29	37·55	39·95	7·56	0·629	0·563	0·789
21 d	32·25[a]	56·18[b]	36·82[a,b]	47·47[a,b]	5·39	0·705	0·004	0·232

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

(vitamin C, vitamin E) and immune challenge (our experiment did not include lipopolysaccharide challenge).

These changes of proinflammatory and anti-inflammatory cytokine expression may lie in the fact that immune cells are particularly sensitive to oxidative stress because their plasma membranes contain a high percentage of PUFA and they generally produce more reactive oxygen species[47]; carotenoids could help protect immune cells from oxidative damage[48]. For a precocial species like chicken, modulation of proinflammatory and anti-inflammatory cytokine expression may be helpful because the hatching process incurs sudden exposure to atmospheric concentrations of oxygen, and then there is a dramatic increase in the metabolic rate with the onset of pulmonary respiration and post-hatching growth[49]. We also noted that the relative average value of IFN-γ mRNA in the duodenum, jejunum and ileum increased rapidly from 0 to 14 d after

Table 5. Effects of *in ovo* and dietary xanthophylls on relative lipopolysaccharide-induced TNF-α factor mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	2·29	2·29	3·93	3·93	0·58	0·073		
7 d	38·84	34·87	44·56	37·69	7·59	0·580	0·483	0·851
14 d	39·34	43·98	39·02	39·63	7·59	0·762	0·734	0·793
21 d	34·43	52·58	33·62	49·41	9·50	0·836	0·089	0·902
Duodenum								
0 d	2·53[a]	2·53[a]	4·62[b]	4·62[b]	0·61	0·035		
7 d	9·04[a]	10·07[a,b]	16·36[c]	15·94[b,c]	1·52	0·000	0·843	0·638
14 d	11·01	12·14	11·66	10·98	1·34	0·849	0·869	0·510
21 d	8·42	9·80	9·41	9·79	1·01	0·634	0·395	0·623
Jejunum								
0 d	5·16	5·16	7·46	7·46	1·19	0·202		
7 d	4·78	6·43	5·83	6·89	1·21	0·539	0·277	0·812
14 d	8·76	8·36	6·39	6·24	1·19	0·073	0·818	0·918
21 d	5·72	6·40	4·77	5·71	1·09	0·459	0·465	0·907
Ileum								
0 d	3·20	3·20	3·20	3·20	0·60	0·996		
7 d	5·81	5·73	5·57	6·07	0·79	0·948	0·795	0·714
14 d	5·04	6·31	4·87	5·55	0·83	0·582	0·253	0·722
21 d	6·22	7·50	5·97	6·19	1·04	0·461	0·482	0·616

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 6. Effects of *in ovo* and dietary xanthophylls on relative IL-4 mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	9·03	9·03	10·70	10·70	1·45	0·433		
7 d	13·59^{a,b}	13·95^{a}	9·62^{a,b}	7·39^{b}	1·58	0·003	0·560	0·420
14 d	12·60	9·34	9·62	10·24	1·71	0·550	0·450	0·272
21 d	13·08	10·86	12·34	10·63	1·05	0·650	0·075	0·815
Duodenum								
0 d	12·20	12·20	9·87	9·87	2·25	0·480		
7 d	6·02	7·17	7·07	6·22	1·10	0·964	0·888	0·376
14 d	3·74	5·10	3·81	3·74	0·66	0·341	0·343	0·296
21 d	3·57	4·16	4·03	4·11	0·54	0·715	0·542	0·639
Jejunum								
0 d	7·21^{a}	7·21^{a}	4·20^{b}	4·20^{b}	0·86	0·033		
7 d	2·22	3·15	1·90	1·16	0·52	0·038	0·854	0·126
14 d	2·30	2·70	1·91	2·06	0·33	0·141	0·421	0·708
21 d	1·43	1·42	2·25	1·35	0·30	0·219	0·141	0·154
Ileum								
0 d	2·05	2·05	2·54	2·54	0·37	0·379		
7 d	2·02	1·65	2·01	1·99	0·25	0·508	0·445	0·494
14 d	2·31	2·08	1·62	2·02	0·35	0·287	0·807	0·372
21 d	2·34	3·33	2·81	2·30	0·51	0·598	0·642	0·159

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

hatching, and reached a relatively steady level, onwards. The same change occurred to LITAF mRNA in the liver and duodenum from 0 to 7 d after hatching, and reached a relatively steady level, onwards. However, the average value of IL-4 mRNA decreased in the duodenum and jejunum after hatching. All of these changes may relate to a rapid increase of feed intake and microbial colonisation of the intestine after hatching. These data showed important developmental physiology of the chick by revealing that different parts of organs may have specific cytokine change as their sensitive indicators during chick development (LITAF for the liver; IFN-γ, LITAF and IL-4 for the duodenum; IFN-γ and IL-4 for the jejunum; IFN-γ for the ileum).

In conclusion, dietary xanthophyll supplementation decreased proinflammatory cytokine expression in the liver, duodenum and jejunum and increased anti-inflammatory cytokine expression in the liver of breeding hens. *In ovo* xanthophylls regulated proinflammatory cytokine (in the liver, duodenum, jejunum and ileum) and anti-inflammatory cytokine expression (in the liver,

Table 7. Effects of *in ovo* and dietary xanthophylls on relative IL-10 mRNA expression in the liver, duodenum, jejunum and ileum of chicks

(Mean values with their pooled standard errors, *n* 6)

	+ *In ovo*		− *In ovo*			*P*		
Items	+ Diet	− Diet	+ Diet	− Diet	Pooled SEM	*In ovo*	Diet	*In ovo* × diet
Liver								
0 d	1·07^{a}	1·07^{a}	0·59^{b}	0·59^{b}	0·12	0·019		
7 d	3·88^{a}	4·04^{a}	2·17^{b}	2·57^{a,b}	0·43	0·001	0·525	0·776
14 d	3·39^{a}	2·89^{a,b}	2·89^{a,b}	1·83^{b}	0·32	0·025	0·026	0·392
21 d	3·64	2·77	3·56	2·27	0·43	0·518	0·021	0·637
Duodenum								
0 d	3·31	3·31	3·40	3·40	0·59	0·917		
7 d	3·44	3·85	2·85	2·99	0·41	0·092	0·512	0·743
14 d	5·38	4·38	3·76	4·08	0·60	0·123	0·582	0·281
21 d	2·45	2·04	3·03	2·52	0·38	0·176	0·239	0·892
Jejunum								
0 d	1·76^{a}	1·76^{a}	1·03^{b}	1·03^{b}	0·17	0·011		
7 d	2·48^{a}	2·00^{a,b}	1·40^{b}	1·48^{b}	0·22	0·002	0·364	0·222
14 d	2·69	2·06	2·54	1·87	0·29	0·553	0·034	0·936
21 d	2·55	1·89	2·28	2·30	0·34	0·838	0·349	0·330
Ileum								
0 d	3·02^{a}	3·02^{a}	1·96^{b}	1·96^{b}	0·21	0·005		
7 d	3·93^{a,b}	4·12^{b}	2·81^{a,c}	2·71^{c}	0·29	0·000	0·877	0·617
14 d	3·67	3·99	3·75	3·92	0·49	0·998	0·616	0·873
21 d	4·07	4·58	4·66	3·35	0·73	0·666	0·591	0·225

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

jejunum and ileum) mainly for at least the first week after hatching in chicks, whereas dietary xanthophylls played an important modulated role in proinflammatory cytokine (in the liver, duodenum, jejunum and ileum) and anti-inflammatory cytokine expression (in the liver and jejunum) mainly from 2 weeks onwards. Moreover, the determination of proinflammatory and anti-inflammatory cytokine expression in different organs (liver, duodenum, jejunum and ileum of chicks) at different times (0, 7, 14 and 21 d) may have special significance for deliberating the developmental physiology of chicks by indicating that different organs may have specific cytokine change during chick growth.

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