Lutein exposure, *in ovo* or in the diet, reduces parameters of inflammation in the liver and spleen laying-type chicks (*Gallus gallus domesticus*)

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Summary

These trials examined whether the demonstrated effects of embryonic and dietary carotenoid exposure on the inflammatory immune response in fast growing chickens also occur in slow growing chickens. The systemic and local inflammatory responses of chicks were examined in two experiments with two *in ovo* lutein levels (C+, carotenoid replete; or C—, carotenoid-deplete), two dietary lutein levels (0 or 40 mg lutein/kg diet), and two inflammatory challenges [no exposure or lipopolysaccharide (LPS)-vaccinated]. At 24 h after LPS vaccination, spleen weight was not affected by diet or in ovo lutein, but liver weight increased from C+ eggs ($p < 0.01$), and in LPS-vaccinated chicks fed 0 mg lutein ($p < 0.05$), but not in chicks fed 40 mg lutein. Plasma carotenoids and liver carotenoids were reduced post-LPS ($p < 0.05$). Splenic IL-6 mRNA abundance was the greatest post-LPS in C— chicks fed 40 mg lutein vs. C+ chicks fed 40 mg lutein ($p < 0.05$). Hepatic IL-6, iNOS and TGFβ and splenic iNOS and TGFβ were not affected by *in ovo* or dietary lutein. The systemic and local inflammatory results are similar to those observed in fast growing chickens, and support that lutein-depleted birds have greater inflammatory responses.

Introduction

Carotenoids are lipid-soluble organic compounds that pigment the integument of many organisms by absorbing light, and in some situations provide antioxidant protection and immunomodulation (reviewed by Goodwin, 1986). These properties may beneficially affect breeding potential and mate selection (Stradi, 1995; Gray, 1996), progeny development (Lyon et al., 1994; Saino et al., 2000), pigmentation of meat and eggs for consumer acceptance (Koutsos et al., 2003a) and immune function. For example, in zebra finches dietary carotenoid exposure affects phytohemagglutinin (PHA)-induced wing web swelling and antibody responses to sheep red blood cells (McGraw and Ardia, 2003), while embryonic carotenoid exposure affects PHA-induced swelling in barn swallow nestlings (Saino et al., 2003). In domesticated birds, carotenoid exposure also affects parameters of the immune system. In broiler chicks, embryonic or dietary carotenoid exposure affects PHA-induced wing web swelling including skin thickness changes as well as changes in responding leucocyte populations (Koutsos et al., 2007), lymphocyte proliferation and macrophage nitrite production (Selvaraj et al., 2005), and parameters of the systemic inflammatory response to lipopolysaccharide (LPS; Koutsos et al., 2006).

One mechanism by which carotenoids affect immune responses are through retinoid receptor (RXR) binding and heterodimerisation with peroxisome proliferator-activated receptor gamma (PPARγ) bound to fatty acids, eicosanoids, as well as many synthetic ligands (Straus and Glass,

2007). Peroxisome proliferator-activated receptor gamma-RXR dimers bind coactivators and response elements in DNA to affect transcription events. In particular, these dimers suppress the activity of the transcription factor nuclear factor (NF) -JB, and thus regulate the expression of inflammatory immune genes including inducible nitric oxide synthase (iNOS; Selvaraj and Klasing, 2006).

Given the striking differences in growth rates, efficiency of feed utilisation, and immunity between meat-type chickens (i.e. those selected for fast and efficient skeletal muscle growth) and laying-type chickens (i.e. those selected for egg production) (Leshchinsky and Klasing, 2001), we examined whether the effects of embryonic and dietary carotenoid exposure seen in meat-type chickens also occur in layer-type chickens. Additionally, the effects of carotenoid exposure on mRNA abundance for genes related to the inflammatory immune response to LPS in slow growing (layer-type) chickens were examined.

Materials and methods

Experiment 1

To examine the effects of *in ovo* and dietary lutein on systemic inflammatory responses of layer-type chicks, a 2 x 2 x 2 factorial arrangement of treatments with 2 *in ovo* lutein levels, 2 dietary lutein levels and 2 inflammatory challenge levels was designed. To establish the in ovo lutein treatments, Hyline white leghorn hens (UC Davis, Davis, CA, USA) were fed either a lutein-free diet or a diet containing 40 mg lutein/kg diet for 30 days, as previously described (Koutsos et al., 2003a). Following insemination, fertile eggs (resulting in carotenoid-replete (C+) eggs containing ≅ 125 nmol lutein + zeaxanthin/egg or carotenoid-deplete (C—) eggs containing no detectable carotenoids) were collected and transported to Cal Poly, San Luis Obispo to be set for hatch. The UC Davis and the Cal Poly Animal Care and Use Committees approved all procedures for animal care and use at their respective facilities.

After hatch, chicks were housed in a brooder battery cage (Petersime, Gettysburg, OH, USA) under 24 h light and 40.6 °C directly below the brooder, and with *ad libitum* access to water. Chicks from each *in ovo* group (n = 51 from C- eggs, n = 58 from C+ eggs, based on egg availability) were randomly assigned to one of two diets (n = 6 replicates per treatment; 8–10 chicks/replicate). Each pen of chicks was allowed free access to either a basal diet (Table 1) containing 0 mg lutein/kg diet, or the basal diet plus 40 mg lutein/kg diet (Oroglo Dry, Kemin Industries, Des Moines, IA, USA), such that each dietary treatment was replicated three times within in ovo treatment. This dietary lutein level was chosen to be consistent with lutein levels fed to commercial poultry.

At 12 days post-hatch, chicks were randomly assigned to inflammatory treatments: half of the chicks in each pen (n = 4–5/pen) had no exposure to LPS (not vaccinated, control) and half the chicks in each pen (n = 4–5/pen) were vaccinated with LPS (from *Salmonella typhimurium*; Sigma L7261, St Louis, MO, USA) at 100 μg/kg body weight (BW) intravenously (i.v., wing vein). The control group was not vaccinated, as saline injections do not induce an acute-phase

response (Laurin and Klasing, 1987) and we were interested in the effect of no inflammation vs. inflammation and not the specific effect of LPS relative to saline. At 2 h post-vaccination, 1 chick/ inflammatory treatment group/pen (n = 3 per inflammatory group) was killed via cervical dislocation, and the spleen and whole liver were removed, weighed, snap frozen and stored at — 80 °C. At 24 h post-vaccination, a blood sample (cardiac venipuncture) was taken from 1 chick/inflammatory treatment group x pen (n = 3 per inflammatory group). Whole blood was placed in heparinised tubes for plasma isolation after centrifugation. Chicks were then killed by cervical dislocation and the spleens and whole livers were removed, weighed and snap frozen. The dependent variables measured included body weight, liver, and spleen weights at 2 and 24 h post-vaccination, splenic IL-6 mRNA abundance at 2 h post-vaccination, and plasma haptoglobin-like activity at 24 h post-vaccination (Wicher and Fries, 2006).

Splenic IL-6 mRNA abundance was examined to confirm a splenic inflammatory response. Total RNA was isolated and extracted from the spleens collected at 2 h post-vaccination using the RNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Optical density at 260 nm was used to determine RNA concentrations. Each total RNA sample (1.07 μg) was reverse transcribed using the Reverse Transcription System, (Promega, Madison, WI, USA) according to the manufacturer's protocol. Traditional PCR was performed using PUReTaq^{TM} Ready-to-Go PCR beads (GE Healthcare, Piscataway, NJ, USA) to detect splenic IL-6 mRNA (Table 2; melting-95 °C for 2 min, denaturing-94 °C, annealing-58 °C, extension-72 °C, 30 cycles) and analysed by agarose gel electrophoresis, and visualised using a Gel Doc station (Bio-Rad, Hercules, CA, USA).

Experiment 2

To examine the effect of in ovo and dietary lutein on the abundance of inflammatory cytokine mRNA in laying hens, a 2 x 2 x 2 factorial arrangement of the same treatments as Experiment 1 was used. Single comb white Leghorn chicks (Hyline Y-strain, n = 80/ egg yolk level) were randomly assigned to one of eight pens in a Petersime battery brooder (n = 10/ pen), and then each pen was randomly assigned to one of two diet lutein treatments as described above. At 21 days post-hatch, chicks received one of two LPS treatments: half of the chicks within each pen were not vaccinated (n = 5/pen, control), and half of the chicks within each pen (n = 5/pen) were vaccinated with 100 μg LPS/kg BW intravenously (i.v.).

At 2 h post-LPS injection, 3 chicks/LPS treatment/ pen were killed by cervical dislocation and whole spleens and livers were removed, freeze-clamped in liquid N_2, and stored at -80 °C prior to mRNA analysis. At 24 h post-vaccination, 2 chicks/LPS treatment/pen were bled via cardiac puncture into heparinised tubes for plasma isolation and then killed. The left liver lobe was removed and stored at-80 °C prior to analysis. Dependent variables measured were quantitative splenic and hepatic mRNA abundance of β-actin, iNOS, IL-1, IL-6 and TGFβ_2 at 2 h post-injection, as inflammatory mediator mRNA abundance increases linearly between 1 and 3 h post-LPS injection (Hussain and Qureshi, 1997; Sijben et al., 2001). Tissues sampled at 24 h post-

vaccination were analysed for plasma and liver lutein and zeaxanthin, and plasma haptoglobin (or more accurately, haptoglobin-like protein in chickens (Wicher and Fries, 2006).

To measure the abundance of mRNA for inflammatory mediators (Experiment 2), total RNA was isolated from spleens and livers (RNAgents Total RNA Isolation System; Promega), according to manufacturer's instructions. Optical density at 260 nm was used to determine RNA concentrations. Each total RNA sample (2 lg) was reverse transcribed according to manufacturer's instructions (Promega Reverse Transcription System #A3500). Quantitative real-time PCR analysis of spleen and liver IL-1β, IL-6, TGFβ_2, iNOS and β-actin mRNA was performed using the Roche Lightcycler (Roche Diagnostics #2 011 468, Mannheim, Germany). Each 20 µl PCR reaction volume contained 2 µl of RT product, 2 µl SYBR Green 1 (Roche Diagnostics #2 158 817) and the appropriate volumes of DEPC water, 25 mm MgCl2 and primers to optimise PCR conditions (Table 2). Single band PCR products for each gene were sequenced (Davis Sequencing, Davis), and each product had >98% sequence homology with the corresponding chicken gene sequence (BLAST nucleotide-nucleotide sequence search, NCBI). PCR cycle conditions for all primer pairs consisted of an initial melting step at 95 °C for 2 min, followed by 40 cycles of denaturation, annealing and extension. After the 40 cycles were completed, a melting curve analysis was performed to confirm that a single gene product was amplified, by heating samples at 65 °C for 30 s and then increasing the temperature at a linear rate of 20 °C/s to 95 °C while continuously monitoring fluorescence. Relative quantitation (i.e., change in abundance of target gene relative to untreated control) of mRNA abundance was achieved using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Although it was our intention to use β-actin mRNA as a reference gene to control for mRNA isolation and loading, we were unable to use it for this purpose because LPS significantly reduced hepatic β-actin mRNA expression ($p \leq 0.05$). Therefore, cytokine mRNA quantity was normalised to the average CT values for control (no LPS injection) animals for each tissue and genes examined (equation = $2^{Ct\ LPS - Ct\ Control}$). Cytokine data are presented as fold change normalised to data from C- chicks fed 0 mg lutein.

Measurement of inflammatory status

Plasma haptoglobin-like protein activity was analysed as a marker of the acute phase response (Experiment 1 and 2), as this protein is a positive acute phase protein, and plasma levels increase following LPS injection (Takahashi et al., 1997). Plasma haptoglobin-like protein was measured according to manufacturer instructions, using a commercial kit based on a colorimetric assay (Phase Haptoglobin Kit; Tridelta Diagnostics, #TP801, Morris Plains, NJ, USA).

Measurement of carotenoids (Experiment 2)

Tissues and diets were thawed, weighed, homogenised, and lutein plus zeaxanthin were extracted as previously described (Koutsos et al., 2003a), and analysed at 464 nm using a UV/vis spectrophotometer (BioTek Synergy 2, Winooski, VT, USA).

Statistical analysis

A one-way ANOVA was used to analyse hatching weights as affected by in ovo lutein. A two-way ANOVA was used to measure body weights prior to LPS vaccination. A three-way ANOVA was used to analyse all other data by general linear model (imp software, SAS, Cary, NC, USA). Variances were all confirmed to be homogeneous prior to ANOVA. When variances were not homogeneous (for plasma lutein plus zeaxanthin in Experiment 2 only), data were transformed by square root prior to analyses. Dependent variables were examined for the main effect of in ovo lutein level, diet lutein level, LPS treatment, and their interactions. Three-way interactions that did not approach significance ($p > 0.25$) were removed from the model. When main effects or interactions were significant, differences between means were identified using Tukey's LSMEANS comparisons. Differences between means were considered significant at $p < 0.05$.

Results

Experiment 1

Hatching weight was affected by *in ovo* lutein levels; chicks from C+ eggs hatched at a lower weight than did chicks from C- eggs ($p < 0.01$; 42.99 g vs. 44.82 ± 0.19 g pooled SEM). However, there was no effect of *in ovo* lutein or diet lutein on BW at day 11 or 12 post-hatch ($p > 0.20$).

Neither *in ovo* lutein level nor diet lutein level affected spleen weight at either 2 or 24 h post-injection ($p > 0.20$). At 2 h post-injection there was no effect of LPS treatment, *in ovo* lutein level or diet lutein level upon liver weight ($p > 0.20$). At 24 h post-vaccination, liver weights were affected by the main effect of inflammatory treatment (increased with LPS treatment; 2.68% vs. 3.24% body weight ± 0.17; $p = 0.03$), as well as by egg carotenoid level (increased with C— eggs; C+ egg = 2.93%, C— egg = 3.3% ± 0.17; $p < 0.01$). Additionally, liver weights were affected by an LPS x diet interaction ($p = 0.04$), in which chicks fed 0 mg dietary lutein had LPS-induced increases in liver weights, while chicks fed 40 mg dietary lutein did not (Fig. 1).

Vaccination with LPS also increased plasma haptoglobin-like protein levels at 24 h post-injection ($p < 0.05$; LPS mean = 0.36, control mean = 0.3 ± 0.02 mg/ml), but neither *in ovo* nor diet lutein exposure affected plasma haptoglobin-like protein levels ($p > 0.2$ for each). Qualitative (conventional) PCR confirmed that IL-6 mRNA was present in splenic samples taken at 2 h post-vaccination; however, IL-6 mRNA was also present in control. We were unable to conduct quantitative PCR on these samples, so a second experiment was conducted.

Experiment 2

In contrast to experiment 1, chicks from C+ eggs hatched at a higher BW than did chicks hatched from C— eggs ($p < 0.01$; 46.1 g/bird vs. 44.5 g/bird ± 0.25 g/bird). However, there was no effect of *in ovo* lutein or diet lutein on BW at day 7, 14 or 21 post-hatch ($p > 0.1$ for all).

Plasma L + Z (days 21 post-hatch) were significantly affected by *in ovo* lutein, diet lutein and LPS treatment ($p < 0.05$ for each main effect). Chicks fed 40 mg lutein and hatched from C+ eggs had higher plasma L + Z than those hatched from C— eggs ($p = 0.03$; plasma L + Z for C+ eggs = 26.6 ± 5.5 nmol/ml; for C— eggs = 9.5 ± 2.4 nmol/ml). Similarly liver L + Z was significantly increased by feeding 40 mg lutein ($p < 0.01$; liver L + Z for 0 mg lutein = 0.01 ± 0.001 nmol/g; for 40 mg lutein = 1.42 ± 0.32 nmol/g) and was significantly reduced by LPS treatment ($p = 0.05$; 60.1% reduction). Additionally, a diet lutein x LPS interaction ($p < 0.01$) demonstrates that chicks fed 40 mg but not 0 mg lutein had LPSinduced reductions in plasma L + Z ($p < 0.05$, 54% reduction vs. 26% increase respectively). Plasma haptoglobin-like protein was not affected by *in ovo lutein* ($p = 0.77$) or diet lutein ($p = 0.17$) but was significantly increased by LPS ($p < 0.01$).

Splenic iNOS mRNA abundance was increased by LPS treatment ($p < 0.01$; 3,134-fold increase), but was not affected by *in ovo* lutein ($p = 0.72$) or by diet lutein ($p = 0.38$). Similarly, liver iNOS mRNA abundance was increased by LPS treatment ($p < 0.01$; 2300-fold increase), but was not affected by in ovo lutein ($p = 0.21$) or diet lutein ($p = 0.47$).

Splenic IL-6 mRNA abundance was increased by LPS treatment ($p < 0.02$; 15,642-fold increase) and IL-6 levels were not detected in control chicks. Additionally, an *in ovo* lutein x diet lutein interaction ($p < 0.03$) demonstrates that C— chicks fed 40 mg lutein had increased IL-6 mRNA expression when compared with C+ chicks fed 40 mg lutein ($p < 0.05$; Fig. 2). Hepatic IL-6 mRNA abundance was also increased by LPS treatment ($p < 0.01$; 5459-fold increase), but was not affected by *in ovo* lutein ($p = 0.65$) or dietary lutein ($p = 0.27$).

Splenic and hepatic IL-1 mRNA abundance were increased by LPS treatment ($p < 0.05$ for each main effect; 1898- and 972-fold increase respectively), but were not affected by in ovo lutein or dietary lutein ($p > 0.10$ for each). Splenic and hepatic TGFβ mRNA abundance was not affected by LPS treatment, *in ovo* lutein or dietary lutein ($p > 0.20$ for each).

Discussion

In experiment 1, C+ chicks hatched at a lower BW, whereas in experiment 2, C+ chicks hatched at a higher BW. However, as egg weights were not measured, it is difficult to pinpoint the basis for changes in hatching weight, although incubator environment, hen age or other factors may have influenced the hatching weight response to lutein (Luquetti et al., 2004). In both experiments, overall growth as measured by BW at day 11 and 12 (Experiment 1) and day 7, 14, and 21 (Experiment 2) was not influenced by either in ovo or dietary lutein, which further supports the conclusion that lutein included in the diet or egg at the tested levels does not significantly affect BW or growth of chicks. Plasma and liver lutein and zeaxanthin levels were affected by diet and by in ovo carotenoid level, as expected based on previous research (Koutsos et al., 2003a). Therefore, chicks fed no dietary carotenoids and chicks hatched from carotenoid-deplete eggs would have had reduced tissue carotenoid levels during the LPS challenge.

The acute phase response to LPS results in increased hepatic protein synthesis and as a consequence increased liver weight relative to body weight (Klasing and Austic, 1984; Johnson,

1998). In experiment 1, liver weights were generally increased by LPS challenge, but in chicks fed 40 mg lutein, liver weights were not increased as much as they were in chicks fed 0 mg lutein, suggesting that dietary lutein acted to reduce the magnitude of the hepatic acute phase response at 24 h post-challenge. This is similar to the effect of feeding a lutein-free diet to growing meat-type birds (Koutsos et al., 2006). In addition to effects of LPS on liver weights, liver weights were generally greater in chicks hatched from carotenoid-deplete (C—) eggs, despite similar body weights at this age. This difference might be explained by increased oxidative stress in lutein-depleted chicks, as well as by some of the additional anti-inflammatory properties of lutein that have been demonstrated in mammalian systems (Jin et al., 2006; Wang et al., 2006; Rafi and Shafaie, 2007). Finally, in experiment 2, lutein and zeaxanthin were depleted in livers of chicks fed 40 mg lutein and challenged with LPS, which is in agreement with the previous research (Koutsos et al., 2003b).

Unlike changes in liver weight in response to a lack of dietary lutein, production of the acute phase haptoglobin-like protein was not affected by in ovo or dietary lutein in either experiment 1 or 2, whereas previous research found a modulatory effect of dietary lutein upon plasma haptoglobin-like protein levels in fast growing meat-type chickens (Koutsos et al., 2006). This is somewhat unexpected, given that egg-type birds tend to have stronger magnitude of inflammatory immune responses than meat-type birds, although haptoglobin was a parameter measured (Leshchinsky and Klasing, 2001).

Splenic and hepatic iNOS mRNA abundance in experiment 2 was affected by LPS treatment, but was not affected by *in ovo* or dietary lutein. This is contrary to research showing that *in vitro* exposure of mouse macrophages to lutein reduces iNOS mRNA and protein expression (Jin et al., 2006; Rafi and Shafaie, 2007). However, Selvaraj and Klasing (2006) have recently demonstrated that the interaction of dietary fat level and dietary lutein level are responsible for modulation of iNOS activity. Specifically, when fatty acid exposure is low, lutein exposure increases iNOS mRNA abundance, but when fatty acid exposure is higher, lutein exposure reduces iNOS mRNA abundance, and these effects are mediated by PPARy/RXR homodimers. Similar responses were also seen in vivo; meat-type birds fed low (3%) dietary fat generally had higher macrophage nitrite production when compared with birds fed higher (6%) dietary fat. In birds fed 6% fat, there was no difference in macrophage nitrite production when fed 0, 25 or 50 mg dietary lutein/kg diet (Selvaraj et al., 2005). In the current experiment, dietary fat levels were —6%, thus a lack of change in iNOS mRNA abundance is similar to the response in fast-growing meat type birds fed diets containing similar lipid and lutein levels.

Finally, in experiment 2, IL-6 mRNA abundance after an LPS challenge was the greatest in chicks hatched from C— eggs compared with those hatched from C+ eggs, despite being fed 40 mg lutein. These data support the change in liver weights seen in experiment 1, and provide further experimental evidence to support enhanced inflammation in chicks not exposed to lutein during embryonic development. Similarly, in mice, lutein exposure lowered the concentration of IL-6 in addition to that of other cytokines via modulation of the I-KB/ NF-KB pathway (Jin et al.,

2006). Given the demonstrated interaction of lutein and specific fatty acids on regulators of the NF-KB pathway (Selvaraj and Klasing, 2006), it would be interesting to examine the effects of lutein and PUFA during avian embryogenesis on gene expression when fatty acid exposure via yolk is high.

In summary, these experiments demonstrate that carotenoid exposure in ovo and in the diet post-hatch reduce parameters of inflammation in the spleen (e.g. IL-6 mRNA abundance), and in the liver (e.g., change in liver weight during acute phase response) of slow-growing egg-type chicks. These results are likely mediated via effects on oxidative stress and resulting changes in the I-KB/ NF-KB pathway, or via direct effects on gene expression via the PPARy/ RXR pathway. These data support the previous observations in fast-growing meat type chickens as well as in free-living birds.

Acknowledgements

We would like to thank Guochen Hu, Jake Olson, Ashley Palmer and Aleece Diaz for assistance with animal care in these trials.

References

Goodwin, T. W., 1986: Metabolism, nutrition, and function of carotenoids. Annual Review of Nutrition 6, 273–297.

Gray, D. A., 1996: Carotenoids and sexual dichromatism in North American passerine birds. American Naturalist 148,453–480.

Hussain, I.; Qureshi, M. A., 1997: Nitric oxide synthase activity and mRNA expression in chicken macrophages. Poultry Science 76, 1524–1530.

Jin, X. H.; Ohgami, K.; Shiratori, K.; Suzuki, Y.; Hirano, T.; Koyama, Y.; Yoshida, K.; Ilieva, I.; Iseki, K.; Ohno, S., 2006: Inhibitory effects of lutein on endotoxininduced uveitis in Lewis rats. Investigative Ophthalmology and Visual Science 47, 2562–2568.

Johnson, R. W., 1998: Immune and endocrine regulation of food intake in sick animals. Domestic Animal Endocrinology 15, 309–319.

Klasing, K. C.; Austic, R. E., 1984: Changes in protein synthesis due to an inflammatory challenge. Proceedings of the Society for Experimental Biology and Medicine 176, 285–291.

Koutsos, E. A.; Calvert, C. C.; Clifford, A. J.; Klasing, K. C., 2003a: Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (Gallus gallus domesticus). Journal of Nutrition 133, 1132–1138.

Koutsos, E. A.; Calvert, C. C.; Klasing, K. C., 2003b: The effect of an acute phase response on tissue carotenoid levels of growing chickens (Gallus gallus domesticus). Comparative Biochemistry and Physiology. Part A, Molecular Integrative Physiology 135, 635–646.

Koutsos, E. A.; Lopez, J. G. C.; Klasing, K. C., 2006: Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (Gallus gallus domesticus). Journal of Nutrition 136, 1027–1031.

Koutsos, E. A.; Lopez, J. C. G.; Klasing, K. C., 2007: Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (Gallus gallus domesticus). Comparative Biochemistry and Physiology B. 147, 87–92.

Laurin, D. E.; Klasing, K. C., 1987: Effects of repetitive immunogen injections and fasting versus feeding on iron, zinc, and copper metabolism. Biological Trace Element Research 14, 153–165.
Leshchinsky, T. V.; Klasing, K. C., 2001: Divergence of the inflammatory response in two types of chickens. Developmental and Comparative Immunology 25, 629– 638.
Livak, K. J.; Schmittgen, T. D., 2001: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Luquetti, B. C.; Gonzales, E.; Bruno, L. D. G.; Furlan, R. L.; Macari, M., 2004: Egg traits and physiological neonatal chick parameters from broiler breeder at different ages. Brazilian Journal of Poultry Science 6, 13–17.
Lyon, B. E.; Eadie, J. M.; Hamilton, L. D., 1994: Parental choice selects for ornamental plumage in American coot chicks. Nature (London) 371, 240–243.
McGraw, K. J.; Ardia, D. R., 2003: Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. American Naturalist 162,704–712.
NRC, 1994: Nutrient Requirements of Poultry. 9th edn. National Academy Press, Washington, D.C.
Rafi, M. M.; Shafaie, Y., 2007: Dietary lutein modulates inducible nitric oxide synthase (iNOS) gene and protein expression in mouse macrophage cells (RAW 264.7). Molecular Nutrition &Food Research 51, 333–340.
Saino, N.; Ninni, P.; Calza, S.; Martinelli, R.; De Bernardi, F.; Moller, A. P., 2000: Better red than dead: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. Proceedings. Biological sciences / The Royal Society 267, 57–61.
Saino, N.; Ferrari, R.; Romano, M.; Martinelli, R.; Moller, A. P., 2003: Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. Proceedings. Biological Sciences 270, 2485–2489.
Selvaraj, R.; Klasing, K. C., 2006: Lutein and eicosapentaenoic acid interact to modify iNOS mRNA levels through the PPARy/RXR pathway in chickens and
HD 11 cell lines. Journal of Nutrition 136, 1–7.
Selvaraj, R. K.; Koutsos, E. A.; Calvert, C. C.; Klasing, K. C., 2005: Dietary lutein and fat interact to modify
macrophage properties in chicks hatched from carotenoid-deplete or replete eggs. Journal of Animal Physiology and Animal Nutrition 90, 70–80.
Sijben, J. W.; Schrama, J. W.; Parmentier, H. K.; van der Poel, J. J.; Klasing, K. C., 2001: Effects of dietary polyunsaturated fatty acids on in vivo splenic cytokine mRNA expression in layer chicks immunized with Salmonella typhimurium lipopolysaccharide. Poultry Science 80, 1164–1170.
Stradi, R., 1995: The Colour of Flight: Carotenoids in Bird Plumage. Solei Gruppo Editoriale Informatico, Milan.
Straus, D. S.; Glass, C. K., 2007: Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. Trends in Immunology 28, 551–558.
Takahashi, K.; Ohta, N.; Akiba, Y., 1997: Influences of dietary methionine and cysteine on metabolic
responses to immunological stress by Escherichia coli lipopolysaccharide injection, and mitogenic response in broiler chickens. British Journal of Nutrition 78,
815–821.

Wang, M.; Tsao, R.; Zhang, S.; Dong, Z.; Yang, R.; Gong, J.; Pei, Y., 2006: Antioxidant activity, mutagenicity/ anti-mutagenicity, and clastogenicity/anti-clastogenicity of lutein from marigold flowers. Food and Chemical Toxicology 44, 1522–1529.

Wicher, K. B.; Fries, E., 2006: Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. Proceedings of the National Academy of Sciences of the United States of America 103, 4168–4173.

Table 1 Basal diet composition for growing chicks*

Ingredient	g/kg diet
Soybean meal	294.0
Rice flour	450.0
Cornstarch	86.0
Cellulose	70.0
Vegetable oil	57.0
Dicalcium phosphate	17.4
Calcium carbonate	15.0
Sodium chloride	3.3
Vitamin mix†	2.5
Mineral mix†	2.5
Choline	1.0
Methionine	0.7
Threonine	0.6
Isoleucine	0.5
Calculated composition	
Crude protein (%)	17.0
Crude fat (%)	6.0
Crude fibre (%)	6.5
Calcium (%)	1.05
Phosphorus (non-phytate, %)	0.40

*Chicks were provided *ad libitum* access to diet, which was formulated to meet or exceed all NRC requirements for growing egg-type chicks (NRC, 1994).

†Vitamin mix provided (per kg final diet): thiamin HCl (1.8 mg/kg), riboflavin (3.6 mg/kg), calcium pantothenate (12.5 mg/kg), niacin (25 mg/kg), pyridoxine HCl (3 mg/kg), folacin (0.6 mg/kg), biotin (0.2 mg/kg), vit B12 (10 μg/kg), retinyl palmitate (6.3 mg/kg), cholecalciferol (0.5 mg/kg), α-tocopherol acetate (20 mg/kg), vitamin K (0.5 mg/kg), ethoxyquin (125 mg/kg). Mineral mix provided sodium selenite (200 μg/kg), copper sulphate (16 mg/kg), zinc sulphate (156 mg/kg), manganese sulphate (170 mg/kg), and iron sulphate (920 mg/kg).

Table 2 PCR reagents and conditions for the assessment of mRNA levels for β-actin, iNOS, IL-1, IL-6 and TGFβ_2* in growing chickens

	Reagents			PCR conditions†	
Gene	DEPC water (μl)	$MgCl_2$ (mm)	Primer mix (μm)	40 cycles	Primer sequence and predicted product size
β-actin	8.8	5	2	95 °C/1 s 63 °C/5 s 72 °C/16 s	5′: CTGACACCACACTTTCTACAATG, 3′: GATCTTCATGAGGTAGTCCGTCAG; 350 bp
IL-1	8.8	5	2	95 °C/1 s 57 °C/4 s 72 °C/15 s	5′: ATGTCGTGTGTGATGAGCG, 3′: CTTGTAGGTGGCGATGTTGA; 330 bp
IL-6	10.8	5	1	95 °C/1 s 59 °C/4 s 72 °C/20 s	5′: GATGTGCAAGAAGTTCACCG, 3′: TGGCAGATTGGTAACAGAGG; 455 bp
iNOS	11.6	4	1	95 °C/1 s 59 °C/4 s 72 °C/20 s	5′: CTCATTCTCCAAGCAAACGG, 3′: ACAATCCACACCCAATCAGC; 435 bp
TGFβ_2	11.6	4	1	95 °C/1 s 57 °C/5 s 72 °C/10 s	5′: ATGGACGGATGACAAGAAGG, 3′: TGGAGCGTAACTGTGGTGAA; 244 bp

*Primer sequences were based upon Genbank sequences: β-actin #L08165, IL-1 #AJ245728, IL-6 #AJ250838; iNOS #U46504, TGFβ2 #X59080.
†PCR conditions for each cytokine consisted of a melt cycle of 95 °C for 120 s, followed by 40 cycles of denaturation, annealing and extension. After 40 cycles, a melting curve analysis ensured that a single product was produced for each sample.

Fig. 1 Dietary lutein exposure reduces the lipopolysaccharide (LPS)-induced increase in liver weight. Chicks (n = 6 per treatment) were offered diets containing 0 or 40 mg lutein/kg diet for 12 days post-hatch, and either not injected (control) or injected with LPS (100 μg/kg BW i.v.). Tissue weights were measured at 24 h post-injection. [a–b]Bars with different superscripts are significantly different ($p < 0.05$).

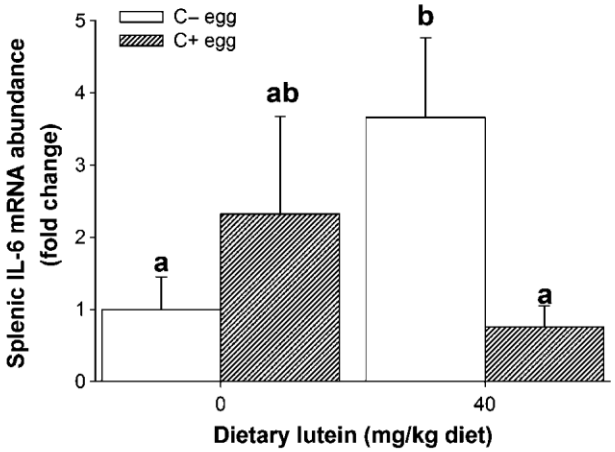

Fig. 2 Embryonic carotenoid exposure affects the lipopolysaccharide (LPS)-induced increase in splenic IL-6 mRNA abundance. Chicks (n = 8 per treatment) were hatched from carotenoid-deplete (C–) or carotenoid-replete (C+) eggs, and at 12 days post-hatch either not injected (control) or injected with LPS (100 μg/kg BW i.v.). mRNA abundance was measured at 2 h post-injection. [a–b]Bars with different superscripts are significantly different ($p < 0.05$).