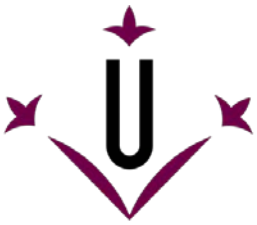

High-carotenoid biofortified maize is an alternative to color additives in poultry feed

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Abstract

Skin color in poultry is achieved by the addition of natural or synthetic pigments to feed. Crops used routinely in feed formulations offer an alternative cost-effective strategy to replace color additives if they are biofortified with sufficient levels of carotenoids. We tested the hypothesis that high-carotenoid (HC) maize, which was genetically engineered to accumulate high levels of β-carotene, lutein and zeaxanthin in the endosperm, can replace carotenoid additives in poultry feed by performing two feeding trials using diets incorporating different maize lines with diverse carotenoid compositions: control (wild-type M37W, the parental line), HC, and standard yellow commercial maize supplemented with color additives (marigold flowers and red paprika extracts). The effects of dietary treatments on growth performance, health parameters, color evolution and carotenoid distribution were determined. We found that chickens fed on the HC diet grew normally and developed similar pigmentation to animals fed on a commercial diet supplemented with color additives, although yellowness was significantly higher in the commercial diet due to the high concentration of yellow xanthophylls. Lightness scores in chickens fed on the control, HC and commercial diets were 45.88 ± 1.31, 44.32 ± 1.10 and 44.29 ± 0.99, respectively, in breast muscle, and 51.62 ± 1.33, 49.66 ± 0.96 and 50.10 ± 1.16, respectively, in thigh muscle. Redness scores in chickens fed on the control, HC, and commercial diets were 0.36 ± 0.26, 3.25 ± 0.29 and 3.58 ± 0.29, respectively, in breast muscle, and 1.28 ± 0.37, 4.79 ± 0.39 and 4.85 ± 0.34, respectively, in thigh muscle. Yellowness scores in chickens fed on the control, HC, and commercial diets were 2.45 ± 0.47, 7.61 ± 0.64 and 9.66 ± 0.73, respectively, in breast muscle, and 3.38 ± 0.64, 10.00 ± 1.10 and 12.64 ± 0.97, respectively, in thigh muscle. High-carotenoid maize is therefore a cost-effective alternative to feed supplementation in the poultry industry.

Keywords

Chicken, biofortification, carotenoids, metabolic engineering, pigments.

1. Introduction

Skin color is the first quality attribute of poultry meat that is evaluated by consumers. A golden skin color is preferred by consumers, especially in North America and the Asia-Pacific markets, because this is associated with a normal state of health (Williams, 1992). Skin pigmentation is affected by genotype, the quantity and dietary source of pigments, and the health of the birds, among other factors (Sirri et al., 2010). In poultry meat, skin color is provided by carotenoid pigments present in the feed that are deposited in the skin and subcutaneous fat (Pérez-Vendrell et al., 2001). Both natural and synthetic pigments can be used as feed additives in the poultry industry to achieve the level of skin pigmentation desired by consumers, but this increases the production costs (Castañeda et al., 2005; Tarique et al., 2013).

Carotenoids are ubiquitous isoprenoid-based natural pigments that can be classed as carotenes, which contain only hydrogen and carbon, or xanthophylls, which also contain oxygen (Britton, 1995). Some carotenoids have pro-vitamin A (PVA) activity, including α-carotene, β-carotene and β-cryptoxanthin, whereas others have no PVA activity but can act as antioxidants (Farré et al., 2010). Carotenoids improve disease resistance in birds and mammals (Surai, 2002) and can also modulate the immune system (Chew and Park, 2004). Chickens, like most other animals, cannot synthesize carotenoids *de novo* and must obtain them from their diet (Breithaupt, 2007; Nogareda et al., 2016). Vitamin A and carotenoid metabolism in chickens is closely related to the equivalent processes in humans, so chickens are also susceptible to vitamin A deficiency with similar symptoms (NRC, 1994; Pretorius and Schönfeldt, 2013).

The value of the global carotenoids market was an estimated $US 1.5 billion in 2014, and is expected to reach nearly $US 1.8 billion in 2019 (BCC Research, 2015). This

increasing demand for dietary pigments in the food/feed industry makes it necessary to find cost-effective alternatives. Novel strategies have been developed recently to enhance carotenoid levels in staple crops (Bai et al., 2011; Farré et al., 2014). The South African white-endosperm maize inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase 1 (PSY1), has been used as a basis for the development of high-carotenoid (HC) maize, which expresses PSY1 and the bacterial enzyme carotene desaturase (CrtI) and therefore accumulates high levels of β-carotene, lutein and zeaxanthin (Zhu et al., 2008).

To address our hypothesis that HC maize is a suitable replacement for carotenoid additives in poultry feed, we performed two animal feeding trials to evaluate three diets containing different types of maize: control (wild-type M37W), HC, and standard yellow commercial maize supplemented with color additives (marigold flowers and red paprika extracts). Chickens fed on these diets were compared in terms of productivity and health parameters, including coloration and carotenoid distribution.

2. Materials and methods

2.1. Diet preparation

The South African white maize inbred M37W and its engineered derivative HC (Zhu et al., 2008) were grown in an experimental field in Northeastern Spain (Lleida, Catalonia) for two consecutive seasons. They were used to prepare the diets together with standard yellow commercial maize provided by the feed industry. The diets were prepared at the Mas de Bover Research Center (IRTA, Reus, Spain) and formulated according to National Research Council (NRC) recommendations (NRC, 1994).

Maize-supplemented diets were prepared by mixing ~50 kg of milled maize with a commercial poultry diet and adjusting the other ingredients to maintain nutritional

93 balance (Table 1). Vitamin A is included to ensure normal growth and development,
94 and was present in all the diets: 10,000 IU/kg (3 mg retinol/kg) for starter diets and
95 8,000 IU/kg (2.4 mg retinol/kg) for grower diets (Yuan et al., 2014). Three diets were
96 prepared: control diet (M37W maize), high-carotenoid diet (HC maize), and commercial
97 diet supplemented with color additives (standard yellow maize). The control diet was
98 prepared before the carotenoid-enriched diets to prevent cross-contamination. Taking
99 into account the pigment characteristics of the standard yellow commercial maize, the
100 quantity of color additives was calculated using standard industry methods (Amaya et
101 al., 2014). The commercial diet was therefore supplemented with 31 mg/kg yellow
102 xanthophylls (marigold flower extract; Capsantal EBS-40-NT) and 3 mg/kg of red
103 xanthophylls (red paprika extract; Capsantal FS-20-NT), both provided by Industrial
104 Técnica Pecuaria S.A. (Barcelona, Spain). The humidity, protein, fat, fiber and ash
105 content of the blended maize grains and formulated diets was measured according to
106 European Commission Regulation No. 152/2009 (European Commission, 2009).
107 Mycotoxin levels were determined using enzyme immunoassay kits (Ridascreen®, R-
108 Biopharm AG, Darmstadt, Germany).

109 *2.2. Bird management and experimental design*

110 In each trial, 1-day-old male broiler chickens of the Ross 308 strain purchased from a
111 commercial hatchery were initially weighed, wing-banded for individual identification
112 and randomly allocated into pens in the Animal Research Center of the University of
113 Lleida. Six broilers were allocated to each of four pens per treatment, i.e. 24 broilers per
114 treatment. Pens were separated by solid partitions to avoid cross-contamination caused
115 by feed spreading. The broilers were presented with starter feed (52% maize, 36%
116 soybean meal and 7% soybean oil) on days 0–8 and grower feed (58% maize, 18%
117 soybean meal, 16% soybean hull and 4% soybean oil) on days 9–35. Feed and drinking

water were provided *ad libitum*. No medication was administered during the feeding trial. Management parameters (temperature, humidity, lighting and ventilation) were monitored and changed according to the age of the chickens, following NRC guidelines (NRC, 2011).

Animals were handled according to Directive 2010/63/EU (European Parliament, 2010) and Commission Recommendation 2007/526/EC (European Commission, 2007), as well as the best practices recommended by the International Life Sciences Institute (ILSI, 2007). All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida and the Catalan Government (reference numbers DAAM 7672 and DAAM 7743).

2.3. Growth performance

Body weight and feed weight were determined every 7 days (and on day 9, when starter diet was changed to grower diet). Average daily gain (ADG), body weight gain, feed intake and feed conversion ratio (FCR) were calculated at the end of the trial. All chickens were humanely euthanized on day 35 and gross necropsy was carried out. Blood samples were randomly collected in 3 chickens of each pen for biochemical and hematological analysis, and serum was separated and frozen for carotenoid analysis. Pre-chilled whole liver, spleen and bursa of Fabricius were collected and weighed. Pre-chilled samples of liver were freeze-dried (fd) for carotenoid analysis.

2.4. Carotenoid analysis

Total carotenoids were extracted from freeze-dried samples in 20 mL methanol containing 12% KOH at 65 °C for 1 h. Lipophilic compounds were partitioned into 30% diethyl ether in petroleum ether, the upper phase was collected and the solvent was evaporated under a stream of nitrogen gas at 37 °C. For separation by high-performance

liquid chromatography (HPLC), samples were re-dissolved in 100 µL methanol/dichloromethane (50:50 v/v) and a 20-µL aliquot was injected immediately. Compounds were separated on a 15-cm Nucleosil C18 3-µm column with an acetonitrile, methanol and 2-propanol mobile phase (85:15:5 v/v/v) at 20 °C. Samples were monitored with a Kontron 440 photodiode array detector with online registration of the spectra and were identified by comparison against authentic reference compounds (Sandmann, 2002).

2.5. Colorimetric analysis

The color was measured using the CIELab trichromatic system (CIE, 2004) as lightness (L*), redness (a*) and yellowness (b*) with a CM-700d compact portable colorimeter (Konica Minolta, Tokyo, Japan). Coordinate L* represents lightness ranging from 0 (black) to 100 (white), a* indicates the red/green component and b* indicates the yellow/blue component. The range of both chromatic components is between −128 and 128 (Sharifzadeh et al., 2014). The illuminant D65 and a 10° viewing angle were used for all measurements. The measuring area was changed according to the footpad size, using a mask of 3-mm-diameter during the first week and a mask of 8-mm-diameter from then onward. During the feeding trial, the color was measured weekly in the footpad with prior cleaning if necessary. After slaughter, the color was measured in pre-chilled footpad, breast skin, and breast and thigh muscles.

2.6. Statistical analysis

Experimental data were analyzed using JMP® Pro 12 (SAS institute, 2015) and differences were considered significant at the 5% level of probability. The experimental unit was the pen, except for carotenoid levels, and the MIXED procedure was applied, including fixed effect of dietary treatments, feeding trial (as there were only two levels)

and their interaction, and random effect of pens: $Y_{ijke} = Diet_i + Trial_j + Diet_i*Trial_j + Pen_k + \varepsilon_{ijke}$. For statistical analysis of carotenoids, analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test was used, and for color evaluation, pairwise correlations were used. Variables expressed in percentages were normalized using the arcsine of the square root of the probability.

3. Results

3.1. Compositional analysis

A standard compositional analysis for humidity, protein, fat, fiber and ash content revealed that the diets were not substantially different, although protein levels were slightly lower in the commercial diet (Table 1). Carotenoid analysis of the grower diets (used from days 9–35) showed that the HC diet had the highest levels of zeaxanthin (10.33 µg/g fd diet formulation), β-carotene (3.04 µg/g fd) and β-cryptoxanthin (2.09 µg/g fd), whereas the commercial diet supplemented with color additives had the highest lutein levels (23.69 µg/g fd) due to the presence of marigold extract (Table 2). Capsanthin was only detected in commercial diet supplemented with color additives due to the presence of paprika extract.

3.2. Productivity and health parameters

According to the statistical analysis, there was no pen effect on any of the criteria evaluated. Chickens fed on the different diets had a similar body weight gain profile ($p > 0.05$) (Supplementary Figure S1). Despite there was no treatment effect, chickens from the first experiment were heavier than chickens from the second one. There were no significant differences ($p > 0.05$) in the overall body weight gain, ADG and feed

intake whereas the FCR was significantly higher in the commercial diet ($p < 0.05$) (Table 3). The weight of the liver, spleen and bursa of Fabricius was similar among chickens fed on the three diets ($p > 0.05$) (Supplementary Table S1).

The analysis of biochemical parameters revealed that bilirubin levels were significantly higher ($p < 0.05$) in chickens fed on the HC and commercial diets compared to those fed on the control diet. Creatinine levels were significantly lower ($p < 0.05$) in chickens fed on the HC diet compared to those fed on the control diet whereas animals fed on the commercial diet had significantly lower ($p < 0.05$) albumin levels compared to those fed on the control diet (Supplementary Table S2). The analysis of hematological parameters indicated that the commercial diet resulted in a significantly higher ($p < 0.05$) lymphocyte count than the HC diet group, although the proportion of lymphocytes did not differ significantly among the three diet groups ($p > 0.05$) (Supplementary Table S3).

3.3. Carotenoid and retinol levels

Carotenoid results reported here belong to the second animal feeding trial. Chickens fed on the HC diet had the highest retinol levels in liver, whereas chickens fed on the commercial diet supplemented with color additives had the highest retinol levels in the serum (Supplementary Figure S2). Nevertheless, these differences were only statistically significant in the liver ($p < 0.05$). Chickens fed on the HC diet had significantly higher levels of zeaxanthin ($p < 0.05$) and β-cryptoxanthin ($p < 0.001$) in the liver compared to the other diets, whereas chickens fed on the commercial diet supplemented with color additives had significantly higher levels of lutein ($p < 0.001$). The levels of zeaxanthin in the livers of chickens fed on the control and HC diets were 1.30 ± 0.05 and 7.19 ± 1.22 μg/g fd, respectively. The levels of lutein in the livers of

chickens fed on the control, HC and commercial diets were 0.61 ± 0.02, 1.76 ± 0.26 and 15.40 ± 1.44 µg/g fd, respectively. Finally, the levels of β-cryptoxanthin in the livers of chickens fed on the HC and commercial diets were 7.42 ± 0.51 and 0.26 ± 0.05 µg/g fd, respectively. Zeaxanthin was not detected in chickens fed on the commercial diet and β-cryptoxanthin was not detected in chickens fed on the control diet.

We did not detect any β-carotene in the liver or serum of any chickens in any of the diet groups. There were no significant differences in the levels of lutein in serum from chickens fed on any of the diets ($p > 0.05$). The serum lutein levels were 0.11 ± 0.00 µg/mL in chickens fed on the control and HC diets and 0.29 ± 0.11 µg/mL in chickens fed on the commercial diet. There were no significant differences in the levels of zeaxanthin ($p > 0.05$) between chickens fed on the HC (0.19 ± 0.00 µg/mL) and commercial (0.2 ± 0.01 µg/mL) diets whereas zeaxanthin was not detected in serum from chickens fed on the control diet. β-Cryptoxanthin was not detected in the serum of any chickens in any of the diet groups.

3.4. Color parameters

Skin color evolution was evaluated every week using the CIELab trichromatic system according to lightness (L*), redness (a*) and yellowness (b*). There were already significant differences ($p < 0.001$) in the redness and yellowness parameters 1 week into the feeding trial (Fig. 1). There was a continuous increase in those parameters in chickens fed on both the HC and commercial diets. The footpad color of chickens fed on the HC diet had a significantly lower lightness value ($p < 0.05$) than the control diet, but the footpad color of chickens fed on both the HC and commercial diets had significantly higher redness ($p < 0.001$) and yellowness ($p < 0.001$) values than chickens fed on the control diet. After slaughter, these results were corroborated by measuring

237 the color in footpad, breast skin, and breast and thigh muscles (Table 4). Chickens fed
238 on the HC and commercial diets showed the highest redness values in breast and thigh
239 muscles ($p < 0.001$), whereas the highest yellowness values ($p < 0.001$) were observed
240 in chickens fed on the commercial diet supplemented with color additives.

241 There was a correlation in yellowness between breast and thigh in chickens fed on all
242 diet groups: control ($R^2 = 0.09$, $p < 0.05$), HC ($R^2 = 0.10$, $p < 0.05$) and commercial (R^2
243 $= 0.14$, $p < 0.05$) diets. There was also a correlation in yellowness between breast skin
244 and breast muscle in chickens fed on the HC ($R^2 = 0.129$, $p < 0.05$) and commercial (R^2
245 $= 0.109$, $p < 0.05$) diets but not in those fed on the control diet ($p > 0.05$). There was no
246 correlation between the breast and thigh muscles in terms of lightness or redness in any
247 of the diet groups ($p > 0.05$). There was also no correlation in terms of redness between
248 breast skin and breast muscle in any of the diet groups ($p > 0.05$), and a correlation in
249 lightness was only found in chickens fed on the control diet ($R^2 = 0.09$, $p < 0.05$). The
250 color differences among chickens fed on the different diets are summarized in Figure 2.

251

252 **4. Discussion**

253 Chickens, like most other animals, must obtain carotenoids from their diet because they
254 cannot synthesize them naturally (Breithaupt, 2007). However, the crops typically used
255 in commercial poultry feed (e.g. maize and soybean) do not supply sufficient
256 carotenoids to achieve the skin pigmentation desired by many consumers in North
257 America and the Asia-Pacific markets, and synthetic or natural carotenoids are therefore
258 routinely added to feed formulations (Castañeda et al., 2005; Williams, 1992). Larger
259 quantities of color additives are required for today's poultry genotypes because they
260 have been bred to grow rapidly, and this increases the cost of feed. Novel crops such as

HC maize, which accumulates high levels of β-carotene, lutein and zeaxanthin, could therefore provide an alternative to color additives in the poultry industry. We tested the impact of a poultry diet based on HC maize by measuring its impact on productivity and health parameters, color development and the distribution of carotenoids compared to equivalent diets based on the near-isogenic wild-type maize inbred M37W (which was used as the background to create the transgenic HC variety used in this study) and a commercial maize diet supplemented with color additives.

There were no significant differences in productivity parameters when we compared chickens fed on the control and HC diets. The FCR, which is defined as the weight of feed in kg required to produce a weight gain of 1 kg in a living animal, was significantly lower in the HC diet than in the commercial diet supplemented with color additives. The similar performance of the HC and control diets suggests that efficient FCR may be a property specific to the genetic background of these lines (M37W maize, the control maize used as a basis for the development of HC) rather than the carotenoid content, given that the commercial diet is based on a mixture of yellow maize varieties and that feed supplementation with pigments does not appear to influence feed consumption or body weight (Liu et al., 2008; Pérez-Vendrell et al., 2001). Previous experiments have shown that broilers fed on the HC diet had a heavier bursa of Fabricius than chickens fed on the control diet which may reflect their better immunomodulatory response to vaccination against infectious bursal disease (Gumboro) (Nogareda et al., 2016). However, we observed no statistically significant difference in the weight of this organ among chickens fed on the control, HC and commercial diets most likely because unlike the earlier experiments, chickens were not vaccinated in these trials. Bilirubin levels were higher in the chickens fed on the carotenoid-enriched diets and highest in those fed on the commercial diet, which may reflect the onset of subclinical inflammation. Both

286 bilirubin and carotenoids have immunomodulatory effects and this may explain the
287 differences in lymphocytes levels in chickens fed on the three different diets (Koutsos et
288 al., 2003; Y. Liu et al., 2008; Rajput et al., 2013).

289 Mycotoxin analysis (data not shown) indicated that mycotoxins were below the
290 maximum levels set for aflatoxin B_1 in poultry feed (0.02 mg/kg) and the guidance
291 values set for other mycotoxins by the European Union (European Commission, 2013,
292 2006, 2003, 2002).

293 Carotenoid analysis indicated that feed composition plays an important role in the final
294 carotenoid content of the different tissues. The HC diet contained the highest levels of
295 zeaxanthin, β-carotene and β-cryptoxanthin, whereas the commercial diet supplemented
296 with color additives contained the highest levels of lutein. Similar proportions of these
297 carotenoids were found in the chicken livers, with the exception of β-carotene, which
298 was not detected in livers from any of the diet groups, reflecting its conversion into
299 retinol for storage. The levels of β-cryptoxanthin, which also has PVA activity,
300 remained high in livers from chickens fed on the HC diet. This suggests the preference
301 of the enzyme β-carotene 15,15'-dioxygenase 1 for β-carotene as a substrate (Kim and
302 Oh, 2010, 2009). Furthermore, β-cryptoxanthin levels in livers from chickens fed on the
303 HC diet were higher than those originally present in the HC feed, suggesting that the
304 polar nature of this molecule may facilitate more efficient transfer from the feed to the
305 chicken tissues (Liu et al., 2012). A high concentration of β-cryptoxanthin was detected
306 in yolks from hens fed on a diet based on maize biofortified with β-cryptoxanthin (Liu
307 et al., 2012), and the β-cryptoxanthin concentration in yolks and chicken livers was
308 higher than the initial β-cryptoxanthin content in the feed in hens fed on a biofortified
309 orange-maize diet (Heying et al., 2014). Even so, β-cryptoxanthin was not detected in
310 livers from chickens fed on the control diet or was found at low levels in livers from

311 chickens fed on the commercial diet (0.26 ± 0.05 μg/g fd). This probably reflects the
312 conversion of β-cryptoxanthin into retinol because those diets contained less β-carotene
313 than the HC feed. Alternatively, the difference in bioavailability may indicate a food
314 matrix effect, in which carotenoids provided as intrinsic components are released more
315 slowly and therefore absorbed over a longer duration for more efficient assimilation.
316 Non-provitamin A carotenoids supplied to hens as biofortified maize are more
317 efficiently assimilated than the same carotenoids provided as a commercial maize diet
318 (Moreno et al., 2016).

319 Only lutein and zeaxanthin were detected in the serum, which may reflect the
320 preferential transport of these carotenoids in the circulation. Chicken serum contains
321 more HDL than LDL, and β-carotene is mainly carried by LDL whereas lutein and
322 zeaxanthin are carried by HDL (Heying et al., 2014; Parker, 1996).

323 Chickens fed on the HC diet accumulated more retinol in the liver, but not in the serum.
324 The liver is a major storage tissue for vitamin A whereas the serum is the mobile phase
325 for carotenoids (Jlali et al., 2012). Our results are consistent with a previous study
326 showing that chickens fed on the HC diet had 1.72-fold more retinol in the liver than
327 chickens fed on the control diet and 1.42-fold more than those fed on the commercial
328 diet with color additives (Nogareda et al., 2016). Here we found that chickens fed on the
329 HC diet had 1.34-fold more retinol in the liver than chickens fed on the control diet and
330 1.28-fold more than those fed on the commercial diet with color additives. Despite the
331 slightly smaller fold change, our absolute retinol levels were higher for all treatments
332 compared to the previous study (Nogareda et al., 2016), which may reflect the storage
333 conditions of the feed which may lead to the loss of carotenoids over time
334 (Jintasataporn and Yuangsoi, 2012).

335 Consumers usually reject or accept poultry meat based on its appearance, color being
336 one of the major contributing components to this quality attribute. Skin color is crucial
337 for the marketing of whole parts whereas meat color is important for the marketing of
338 skinless cut up pieces (Fletcher, 2002). The chicken is the only poultry species in which
339 pigmentation of products can be desired by consumers, although preferences vary by
340 region and by culture (Grashorn, 2016). Some consumers can perceive the yellow color
341 of chicken meat negatively because it is associated with an old bird or rancidity while
342 others can consider it positively because it is associated with better health (Grashorn,
343 2016; Kennedy et al., 2002).

344 The intensity of the skin color was measured using the CIELab trichromatic system
345 (CIE, 2004) to provide values for lightness (L*), redness (a*) and yellowness (b*). The
346 redness and yellowness values were higher in chickens fed on carotenoid-enriched diets
347 compared to control diet, and the values in the footpad were higher than those in the
348 breast and thigh muscles after slaughter. Higher pigmentation levels in shank compared
349 to breast were previously reported in experiments in which broilers were fed on diets
350 supplemented with natural and synthetic pigments (Liu et al., 2008). The color
351 parameters vary according to the tissue, e.g. lower yellowness was detected when the
352 target tissue was the fat vein (Castañeda et al., 2005), and the production conditions,
353 e.g. higher yellowness was found when broilers were reared under intensive conditions
354 (Sirri et al., 2010).

355 Previous experiments have shown that the levels of lutein were 2-fold higher in breast
356 muscle from chickens fed on the HC diet compared to the control, whereas there were
357 no differences in fat, breast and thigh skin, and thigh muscle between the two diets. A
358 more substantial difference was found in zeaxanthin levels, which were 7-fold higher in
359 fat and thigh muscle, 11-fold higher in thigh skin, 20-fold higher in breast skin, and 22-

360 fold higher in breast muscle from chickens fed on the HC diet compared to the control.
361 β-Carotene levels were 11-fold higher in breast muscle from chickens fed on the HC
362 diet compared to the control whereas it was not detected in fat, breast and thigh skin,
363 and thigh muscle in any case. However, β-carotene epoxides were found in those tissues
364 in chickens fed on the HC diet, suggesting that β-carotene was accumulated initially but
365 it was metabolized into downstream derivatives. Finally, β-cryptoxanthin was only
366 detected in breast muscle from chickens fed on the HC diet (Nogareda et al., 2016).

367 Chickens fed on the commercial diet supplemented with color additives had higher
368 breast and thigh yellowness compared to those fed on the HC diet, which can be
369 explained by the presence of the lutein-rich marigold flower extract in this formulation
370 (Breithaupt et al., 2003). Lutein increases the yellowness of chicken meat, as reported
371 for broilers fed on a diet supplemented with 200 mg/kg lutein (Rajput et al., 2013).
372 Yellowness is a good indicator of yellow xanthophyll content in the feed (Pérez-
373 Vendrell et al., 2001). Despite the better absorption of synthetic pigments (apo-ester and
374 canthaxanthin), it seems that natural pigments (marigold flowers and red paprika
375 extracts) are more efficiently distributed into tissues, because natural pigments
376 increased skin yellowness more efficiently than synthetic pigments (Castañeda et al.,
377 2005).

378 A positive correlation between skin and raw breast meat yellowness was observed when
379 Ross 508 broilers were reared under commercial conditions and fed on diets
380 supplemented with xanthophylls (Bianchi et al., 2007). A slight correlation was
381 observed in the carotenoid-enriched diet groups under our experimental conditions, but
382 higher yellowness was found in breast skin than in breast meat in those groups.
383 Yellowness in breast and thigh muscles was also correlated in experiments in which
384 Ross 308 and Ross 508 broilers were reared under intensive conditions, suggesting that

a single measurement in one tissue is adequate to assess color development (Sirri et al., 2010). Despite there was a correlation between the yellowness values of breast and thigh muscles in our study, the correlation values were not as high as those reported by these authors.

The results obtained from the analysis of color parameters suggest that breast and thigh muscles are appropriate target tissues to measure the pigmentation of broilers intended to be processed into retail cuts. Taking into account yellowness and redness, the meat obtained from chickens reared on the HC diet was similar in appearance to the meat obtained from chickens reared on the commercial diet supplemented with marigold flowers and red paprika extracts. Thus, HC maize is a suitable feed component to achieve the golden skin color which is desired by the consumer in some markets, avoiding or reducing the need for supplementary pigments and thus reducing feed costs.

5. Conclusions

High-carotenoid maize had no adverse effects on poultry, and it resulted in similar growth and health parameters to its near isogenic wild-type line and the commercial maize supplemented with color additives, in addition to providing PVA carotenoids. Chickens fed on the HC diet developed similar pigmentation to those fed on the commercial diet supplemented with color additives, although the latter had greater yellowness values due to the high levels of lutein in the feed. We conclude that HC maize is a suitable alternative to color additives in the poultry production industry.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by La Caixa (Recercaixa project PC084082 VitaMaize: High quality and safe food through antioxidant fortified maize), the Spanish Ministry of Economy and Competitiveness (BIO2014-54426-P; BIO2014-54441-P, FEDER funds) and the Catalan Government (2014 SGR 1296 Agricultural Biotechnology Research Group). The authors would like to thank the University of Lleida for a PhD fellowship awarded to J. Díaz-Gómez, IRTA-Mas de Bover (Reus, Spain) for diet formulation and manufacture, ITPSA (Barcelona, Spain) for providing the color additives, and Antonio Michelena and Jaume Lloveras for their work in the experimental field.

Supplementary information accompanies this paper.

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Table 1. Composition of starter (days 0–8) and grower (days 9–35) diets and chemical analysis of the grower diets.

Ingredient (g/kg)	Starter control	Starter high-carotenoid	Starter commercial	Grower control	Grower high-carotenoid	Grower commercial
Control maize	522.00	0	0	582.00	0	0
High-carotenoid maize	0	522.00	0	0	582.00	0
Commercial maize	0	0	522.00	0	0	582.00
Soybean meal 47.5%	361.00	361.00	361.00	178.50	178.50	178.50
Soy hulls	0	0	0	158.50	158.50	158.50
Soybean oil	68.00	68.00	68.00	42.00	42.00	42.00
Monocalcium phosphate	15.23	15.23	15.23	12.28	12.28	12.28
Calcium carbonate	14.55	14.55	14.55	10.97	10.97	10.97
Sodium chloride	3.47	3.47	3.47	3.17	3.17	3.17
Sodium bicarbonate	2.33	2.33	2.33	1.19	1.19	1.19
Choline chloride 60%	0.51	0.51	0.51	0.42	0.42	0.42
L-Lysine chloride 79%	2.69	2.69	2.69	2.77	2.77	2.77
DL-Methionine 99%	3.26	3.26	3.26	3.33	3.33	3.33
L-Threonine 98%	0.42	0.42	0.42	0.47	0.47	0.47
Vitamin-mineral premix [1]	3.03	3.03	3.03	3.03	3.03	3.03
Vitamin premix starter [2]	3.03	3.03	3.03	0	0	0
Vitamin premix grower [3]	0	0	0	1.04	1.04	1.04
Coccidiostat (cygro)	0.51	0.51	0.51	0	0	0
Coccidiostat salinomycin 12%	0	0	0	0.52	0.52	0.52
Mixture of color additives	0	0	0	0	0	0.93
Chemical analysis (g/kg)						
Crude protein				218.50	219.60	190.50
Crude fiber				45.30	40.80	41.00
Crude fat				99.20	99.40	91.70
Ash				48.10	57.40	54.60
Moisture				143.90	102.90	124.00

[1] Vitamin-mineral premix: vitamin D_3 1,700 IU/kg; vitamin B_1 2 mg/kg; vitamin B_2 6.4 mg/kg; vitamin B_6 3 mg/kg; vitamin B_{12} 0.02 mg/kg; vitamin E 50 mg/kg; vitamin K 3 mg/kg; folic acid 1 mg/kg; nicotinic acid 40 mg/kg; panthotenic acid 11.7 mg/kg; biotin 0.1 mg/kg; cooper 6 mg/kg; zinc 54 mg/kg; iron 40 mg/kg; manganese 77 mg/kg; selenium 0.45 mg/kg; iodine 2.28 mg/kg; BHT antioxidant 125 mg/kg.

[2] Vitamin premix starter: vitamin A 10,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

[3] Vitamin premix grower: vitamin A 8,000 IU/kg; vitamin D_3 300 IU/kg; vitamin B_2 1.6 mg/kg; vitamin E 20 mg/kg.

Table 2. Carotenoid composition (µg/g freeze-dried feed) of the grower diets (used on days 9–35) in the first and second feeding trials. Values are means ± standard errors (n = 3). Means within a row with no superscript in common are significantly different ($p < 0.05$). nd: not detected.

1st feeding trial	Control diet	High-carotenoid diet	Commercial diet + color additives
Violaxanthin	0.13± 0.00 [c]	2.77 ± 0.31 [a]	0.9 ± 0.08 [b]
Lutein	1.05 ±0.01 [c]	3.69 ± 0.47 [b]	25.77 ± 0.96 [a]
Zeaxanthin	0.40 ± 0.01 [c]	10.85 ± 1.18 [a]	5.68 ± 0.42 [b]
β-cryptoxanthin	0.25 ± 0.01 [c]	3.15 ± 0.37 [a]	0.4 ± 0.03 [c]
β-carotene	0.07 ± 0.01 [b]	3.02 ±0.31 [a]	traces
2nd feeding trial	**Control diet**	**High-carotenoid diet**	**Commercial diet + color additives**
Violaxanthin	nd	0.11 ± 0.01	nd
Lutein	1.18 ± 0.03 [c]	3.45 ± 0.12 [b]	21.61 ± 0.59 [a]
Zeaxanthin	1.55 ± 0.02 [c]	9.80 ± 0.58 [a]	4.99 ± 0.08 [b]
β-cryptoxanthin	nd	1.04 ± 0.06 [a]	0.84 ± 0.02 [b]
β-carotene	nd	3.06 ± 0.17 [a]	1.67 ± 0.00 [b]

Table 3. Analysis of broiler production parameters: initial and final body weight, average daily gain (ADG), feed intake and feed conversion ratio (FCR). Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Initial body weight (g)	40.65 ± 1.12	40.84 ± 1.01	41.11 ± 1.14
Final body weight (g)	1831.35 ± 69.26	1837.31 ± 56.13	1796.76 ± 57.12
ADG 1st week (g)	15.84 ± 1.21	16.44 ± 0.82	16.12 ± 1.02
ADG 2nd week (g)	38.91 ± 3.10	37.27 ± 2.81	36.59 ± 2.42
ADG 3rd week (g)	69.34 ± 3.60	67.09 ± 3.27	66.30 ± 3.68
ADG 4th week (g)	82.10 ± 4.22	80.75 ± 4.57	78.53 ± 4.40
ADG 5th week (g)	49.62 ± 4.31	55.10 ± 4.03	53.27 ± 4.70
ADG final (g)	51.16 ± 1.97	51.33 ± 1.60	50.16 ± 1.62
Feed intake (g)	2918.17 ± 90.99	2849.02 ± 29.19	2999.77 ± 93.86
FCR	1.63 ± 0.02 [a, b]	1.59 ± 0.01 [b]	1.67 ± 0.02 [a]

Table 4. Color determination after slaughter in footpad, breast skin, breast muscle and thigh muscle of chickens fed on the three diets. Color determined using the CIELab trichromatic system as lightness (L^*), redness (a^*) and yellowness (b^*). Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

		Control diet	High-carotenoid diet	Commercial diet + color additives
Footpad	L*	73.94 ± 4.31 [a]	66.36 ± 4.15 [b]	68.82 ± 3.27 [a, b]
	a*	1.22 ± 0.69 [b]	11.55 ± 1.14 [a]	12.63 ± 1.00 [a]
	b*	16.77 ± 2.61 [b]	37.48 ± 4.30 [a]	45.75 ± 4.75 [a]
Breast skin	L*	64.03 ± 1.55 [a]	60.85 ± 1.55 [b]	61.88 ± 1.62 [a, b]
	a*	0.01 ± 0.60 [b]	1.91 ± 0.70 [a]	2.10 ± 0.85 [a]
	b*	0.69 ± 1.43 [c]	8.60 ± 2.56 [b]	13.55 ± 2.06 [a]
Breast muscle	L*	45.88 ± 1.31	44.32 ± 1.10	44.29 ± 0.99
	a*	0.36 ± 0.26 [b]	3.25 ± 0.29 [a]	3.58 ± 0.29 [a]
	b*	2.45 ± 0.47 [c]	7.61 ± 0.64 [b]	9.66 ± 0.73 [a]
Thigh muscle	L*	51.62 ± 1.33 [a]	49.66 ± 0.96 [b]	50.10 ± 1.16 [b]
	a*	1.28 ± 0.37 [b]	4.79 ± 0.39 [a]	4.85 ± 0.34 [a]
	b*	3.38 ± 0.64 [c]	10.00 ± 1.10 [b]	12.64 ± 0.97 [a]

Supplementary Table S1. Effects of feed diets on the relative organ weight of broilers (expressed as % relative to whole live animal weight). Values are means ± standard errors (n = 8).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Liver	1.95 ± 0.10	2.02 ± 0.12	2.08 ± 0.10
Spleen	0.13 ± 0.02	0.13 ± 0.02	0.14 ± 0.01
Bursa of Fabricius	0.16 ± 0.03	0.15 ± 0.02	0.16 ± 0.03

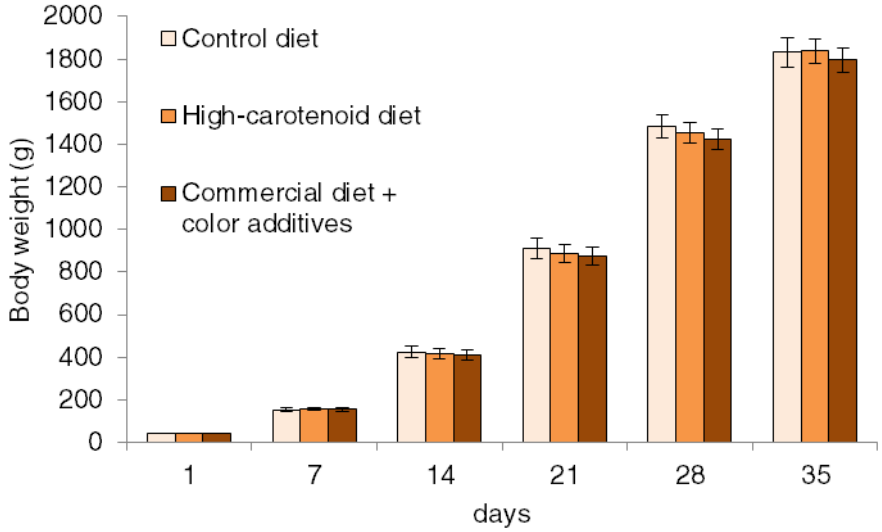
Supplementary Table S2. Biochemical values after slaughter measured in blood samples from chickens fed on the three diets. Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$).

	Control diet	High-carotenoid diet	Commercial diet + color additives
Glucose (mg/dL)	228.38 ± 6.02	225.81 ± 5.49	225.90 ± 5.85
Calcium (mg/dL)	102.76 ± 1.96	102.71 ± 1.86	103.28 ± 1.95
Total protein (g/dL)	33.17 ± 1.18	32.77 ± 1.01	33.21 ± 1.21
Aspartate transaminase (IU/L)	295.90 ± 43.76	255.45 ± 14.76	248.69 ± 14.92
Total Bilirubin (mg/dL)	0.11 ± 0.02 [b]	0.18 ± 0.02 [a]	0.20 ± 0.02 [a]
Potassium (mEq/L)	5.85 ± 0.58	5.88 ± 0.41	5.68 ± 0.36
Creatinine (g/dL)	0.25 ± 0.01 [a]	0.23 ± 0.01 [b]	0.24 ± 0.01 [a, b]
Phosphorus (mg/dL)	8.02 ± 0.38	8.00 ± 0.24	7.74 ± 0.21
Albumin (mg/dL)	12.17 ± 0.33 [a]	11.74 ± 0.43 [a, b]	11.55 ± 0.29 [b]
Alanine transaminase (IU/L)	2.55 ± 0.61	2.52 ± 0.34	2.38 ± 0.22
Lactate dehydrogenase (IU/L)	3896.52 ± 627.96	3255.06 ± 371.85	2881.38 ± 367.00
Uric acid (mg/dL)	4.34 ± 0.51	4.93 ± 0.98	3.74 ± 0.63
Total Cholesterol (mg/dL)	162.34 ± 5.86	164.77 ± 6.86	165.00 ± 6.97
Alkaline phosphatase (IU/L)	4884.24 ± 969.88	5123.55 ± 1065.15	5316.83 ± 1424.52
Gamma-glutamyl transpeptidase (IU/L)	25.83 ± 1.41	25.42 ± 1.83	24.03 ± 1.82
Sodium (mEq/L)	152.17 ± 1.01	152.68 ± 0.92	151.66 ± 0.76
VLDL (mg/dL)	8.59 ± 1.16	8.84 ± 0.69	10.34 ± 2.05
HDL Cholesterol (mg/dL)	108.31 ± 3.47	105.10 ± 4.42	108.59 ± 4.23
LDL Cholesterol (mg/dL)	60.23 ± 6.89	63.81 ± 7.23	59.25 ± 6.90

Supplementary Table S3. Hematological values after slaughter measured in blood samples from chickens fed on the three diets. Values are means ± standard errors (n = 8). Means within a row with no superscript in common are significantly different ($p < 0.05$). The H/L ratio was calculated from the mean values of the heterophils and lymphocytes.

	Control diet	High-carotenoid diet	Commercial diet + color additives
Hematocrit (%)	0.38 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
Hemoglobin g/dL	12.47 ± 0.34	12.53 ± 0.40	12.04 ± 0.36
Total leukocytes/µL	13800.00 ± 2119.26	14216.67 ± 1866.95	17378.95 ± 3383.96
Eosinophils (%)	4.58 ± 1.37	4.96 ± 1.06	5.26 ± 1.12
Basophils (%)	9.47 ± 1.60	10.83 ± 2.47	9.79 ± 2.15
Lymphocytes (%)	39.53 ± 5.00	33.71 ± 3.55	40.89 ± 5.02
Monocytes (%)	8.16 ± 1.56	10.38 ± 2.26	7.32 ± 1.98
Heterophils (%)	36.37 ± 3.52	40.13 ± 3.51	36.74 ± 4.47
Eosinophils/µL	649.58 ± 259.69	754.08 ± 221.84	966.63 ± 324.11
Basophils/µL	1422.21 ± 375.01	1633.92 ± 479.15	1675.74 ± 477.68
Lymphocytes/µL	5493.63 ± 894.69 [a, b]	4658.46 ± 636.71 [b]	6860.84 ± 1448.54 [a]
Monocytes/µL	1111.16 ± 304.45	1357.42 ± 294.57	1268.63 ± 418.28
Heterophils/µL	5039.21 ± 898.30	5812.79 ± 1063.36	6607.11 ± 1694.76
H/L (1/1)	1.04 ± 0.27	1.39 ± 0.29	1.16 ± 0.35

Supplementary Figure S1. Body weight gain profile of chickens fed on the three diets for 5 weeks. Values are means ± standard errors (n = 8).



Supplementary Figure S2. Retinol levels in serum (µg/mL) and liver (µg/g freeze-dried –fd–) measured in the second feeding trial in chickens fed on the three diets. **a)** Serum (n = 6); **b)** liver (n = 8). Values are means ± standard errors. Means within each graph with no superscript in common are significantly different ($p < 0.05$).