Assiut Vet. Med. J. Vol. 43 No. 85, April 2000

Dept. of Nutrition and Deficiency Diseases,
Fac. Vet. Med., Beni-Suef, Cairo University.

EFFECT OF CERTAIN PIGMENTS ON PERFORMANCE AND IMMUNE RESPONSE OF CHICKS AS A MODEL

(With 5 Tables and 11 Figures)

By

H.M. ABD EL-HAFEEZ; SAMAR S. TAWFIK ; ELHAM S.E. SALEH; HANAA. I. FAHEEM* and S. Kh. ABD EL–GHAFFAR**

* Dept. of Zoology, Fac. of Science, Beni-Suef, Cairo University .
** Dept. of Pathology and Clinical Pathology, Fac. Vet. Med., Assuit University.

(Received at 30/3/2000)

تأثير بعض الصبغات على الأداء والاستجابة المناعية في الدجاج كنموذج

حسن محمود عبد الحفيظ ، سمر سيد توفيق ، الهام صالح السيد صالح ، هناء ابراهيم قهيم ، ساري خليل عبد الغفار

أجري في هذه الدراسة اختبار تأثير إضافة صبغات الكاروتينويد المصنعة والطبيعية على علائق الدواجن من الجنس المحلي (الفيومي) على كفاءة أداء النمو وكذلك الاستجابة المناعية . تم استخدام تركيزين (٢٠ ، ٢٠٠ مجم / كجم) من الصبغات المصنعة وهي الكنثازانثين وكذلك حمض الكاروتينويك ايثيل استر ، أضيفا على العليقة الاساسية للكتاكيت من عمر يوم (لمجاميع ٢ ، ٣ ، ٤ ، ٥) ولمدة عشرة أسابيع . مصادر الصبغة الطبيعية التي استخدمت في هذه الدراسة كانت حشيشة الذهب والعصفر وأضيفا على العليقة الأساسية للكتاكيت بمعدل ٣,٥ ، ٣٥ جم / كجم من المصدر الأول و ٢٠ جم / كجم من المصدر الثاني وغذيت المجاميع ٦ ، ٧ ، ٨ من عمر يوم ولمدة عشرة أسابيع . قدرت المقاييس الخاصة بتقييم أداء النمو وكذلك معدلات استهلاك الطعام وكفاءة التحويل الغذائي ، ووجد أن إضافة الكنثازانثين بكلا المعدلين تحسن كل المقاييس بينما حمض الكاروتينويك له تأثير ايجابي فقط بالمستوي عالي التركيز. تم الحصول على المقاييس المناعية بتقدير كل من بروتين السيرم الكلي ومكوناته ، قيمة الأجسام المناعية لفيروس مرض النيوكاسل باستخدام اختبار تثبيط التخثر الدموي والاوزان النسبية لكل من البرسا والطحال والغدة التيموسية بالإضافة إلى دراسة المناعة البائولوجية لنفس الأعضاء سابقة الذكر وتحسس تزايد الخلايا الليمفاوية من نوعي T , B . وقد أظهرت النتائج أن المستويات العالية من الصبغات

المصنعة أكثر فاعلية في رفع كفاءة نشاط الجهاز المناعي وذلك عن طريق اثبـــات ارتفـــاع مستوي الجاما جلوبيولين والتغيرات الإيجابية في معدلات أوزان كل من البرســـا والطحـــال والغدة التيموسية. وأيضا زيادة كثافة أعداد الخلايا الليمفاوية وارتفاع نسبة الأجسام المناعيـــة في اختبار تثبيط التخثر الدموي . ويتضح من التأثير الإيجابي لصبغات الكاروتينويد على كل من أداء النمو واستجابة الجهاز المناعي للدواجن كنموذج أنه يمكن تطبيق ذلك على الطيـــور والحيوانات وكذلك الإنسان .

SUMMARY

The effect of supplementing the diets of native breed–chicks with carotenoids, synthetic or natural, on performance and immune response was tested. Two concentrations (20 & 200 mg/Kg) of each of the synthetic pigments canthaxanthin and B-apo-8 carotenoic acid ethyl ester were added to a basal diet and fed to one–day old chicks (groups 2, 3, 4, & 5) for 10 weeks. The natural pigment sources marigold and safflower were added to the basal diet at the rate of 3.5 & 35 g/Kg for the first and 20 g/Kg for the second and fed to the chick groups 6, 7, & 8. The chicks were 320 in number and equally distributed into the eight experimental groups including the control one which received no supplemental pigments. The growth performance, feed intake and feed conversion efficiency were assessed. Canthaxanthin improved all parameters at both levels while apo – carotenoic acid showed a positive effect only at the high level . The natural pigments had no promoting effect especially the marigold high level. The immunological assays were achieved by determining the total serum protein and its fractions; antibody titers to the Newcastle disease virus using hemagglutination inhibition test (HI); relative weights of bursa , spleen & thymus ; in addition to the immunopathological studies of the same organs detecting the B & T lymphocyte proliferation. The results indicated that the high levels of the synthetic pigments were more effective in enhancing the immune functions proved by the heightened gamma–globulin; bursa, spleen and thymus weight change; intensed lymphocytic population; and high HI titers. The demonstrated effect of carotenoids on both performance and immune system in chicks could be also true for other birds and animals including man.

Key words: Pigments; immune response, chicks.

INTRODUCTION

Enhancing the ability of man & animals to resist and respond to disease organisms is one of the most important goals of animal research. Disease resistance usually involves the maintenance of protective barriers against pathogen entry and a well-functioning immune system.

There is an increasing evidence of the positive influence of plant pigments on human health and animal resistance, and a heightened interest is developing. Plant pigments which play important roles in plant metabolism and visual attraction include carotenoids , anthocyamins and other flavonoids, betalains, and chlorophylls (Young and Britton, 1993). Food crops containing these pigments are believed, since 1970s, to function as "chemopreventers" by providing protection against certain forms of cancer in human (Paganini-Hill et al., 1987; Ziegler, 1989 ; Giovannucci et al., 1995) , in rats and mice (Krinsky, 1989 ; Palozza and Krinsky , 1992) , and a reduction of cardiovascular disease (Gey et al., 1993; Gaziano, 1994). The important characteristic common to all chemopreventers is their antioxidant capacity. The antioxidant properties of carotenoids which protect chlorophylls from photo-oxidation apparently may also protect humans from carcinogens and heart disease.

There is also a growing evidence from a large number of epidemiological, animal and clinical intervention, studies that carotenoids enhance immune function in human (Alexander et al., 1985; Zhang et al., 1991 and Omaye et al., 1996), rats (Bendich and Shapiro, 1986; Bendich, 1989) and chicks (Haq and Bailey, 1995; Haq et al., 1996; Okotie–Eboh et al., 1997). The theoretical background behind the idea that carotenoids might have beneficial health effects is in part that related to their ability to quench singlet oxygen (DiMascio et al., 1991), and they may act as chain–breaking antioxidants under physiological conditions of oxygen tension (Meydani et al., 1994 and Omaye et al., 1996).

Because of the complexity of large scale efficacy testing of natural compounds in foods , dietary supplementation with plant pigments and other chemopreventers has not yet been undertaken in man, while some of the fat-soluble carotenoids are suitable for the coloring of the margarine and cheese, and by using exceptionally fine suspensions of them for the aqueous solutions. In poultry it is well documented that skin, shank, foot-pad and egg yolk color are dependent

upon the dietary content of carotenoids (Food and Drug Administration, 1984; Lipstein, 1984). It was reported by the Food and Drug Administration (1984) that improved pigmentation is an effect closely associated with the improved feed efficiency and increased rate of weight gain claims and is attributed to improved absorption and subsequent deposition of the xanthophylls and carotenoids in carcass (skin/fat) and egg yolk.

There are more than 600 naturally occurring carotenoids, but of these only a few can be metabolized to vitamin A. Of all the carotenoids, B–carotene is the most widely distributed in plants and considered safe for human and animal consumption, with little toxicity being reported, even at pharmacological doses (Omenn et al., 1996 and Hennekens et al., 1996). Only a few carotenoids are utilized by the broilers and layers for their pigmentation. Pigmenting carotenoids are mainly found in these members of the group which have no vitamin A activity at all and are transferred unchanged to the tissues. The hydroxycarotenoids are known as oxycarotenoids or xanthophylls (Seddon, 1994) which are pigmenting carotenoids.

The most important natural pigment – containing materials used in poultry food are yellow maize, alfalfa and grass meal in addition to the various products made from these materials. Marigold meal has been shown to be an effective xanthophyll-source as it contains from 6000 to 10000 mg/Kg (Lipstein, 1984 and Food and Drug Administration, 1984). Some plants containing pigments other than carotenoids have been claimed to have some beneficial effects on health as safflower and its meal which contain carthamin, carthamone and caryophyllene. These phytochemicals were found to have diuretic, anti – inflammatory , calming and soothing effects on pain and bleeding in rat , dog and human (Raintree Nutrition, 1996 and Akihisa et al., 1996). The synthetic xanthophylls such as B-apo-8-carotenoic acid ethyl ester, canthaxanthin and Zeaxanthine have been widely used in poultry rations.

It was the object of the test reported here to investigate natural and synthetic pigments under local conditions, by using it as a performance – improving additive for an Egyptian fowl breed, as a model, with particular reference to its immune-promoting effect. Two pure synthetic pigments canthaxanthin and B-apo-8-carotenoic acid ethyl ester and two natural pigment sources marigold and safflower were used, each at levels several folds that required for pigmentation, in order to establish the most effective rate for immunity stimulation.

The two synthetic pigments are marketed, as carophyll red for canthaxanthin and carophyll yellow for the B-apo-8-carotenoic acid ethyl ester. The pigments are added as 10 % in a starch-coated matrix of gelatin, carbohydrates, ethoxyquin and ascorbyl palmitate are added as antioxidants (Hoffmann La Roche company). They are added to the poultry diets at the rate of 70 & 80 mg/ kg diet in respective order and in maximum, and when the diets are completely free of pigments (company recommendation).

The chicks were used as an experimental model because they are useful for immunological investigations and have an immune system capable of producing high levels of antibodies as reported by Patterson et al. (1962) as a result of serum protein injection.

MATERIALS and METHODS

Birds:

Three hundred and twenty dual purpose chicks, of the local Fayoumi breed (BB), were procured from the El-Azab farm of Fayoum province, Ministry of Agriculture, Egypt. The chicks were nearly of a uniform weight, averaging 30g, where they were randomly distributed into eight equal experimental groups.The chicks were floor reared in a hygienic pen, of eight compartments, bedded with wheat straw. Fresh and clean water were supplied ad libitum throughout the experimental period which lasted for 10 weeks.

The birds were individually weighed and food consumption was recorded every second week. During the experiment the chicks of the eight groups were equally cared for, and clinical signs of significant importance, if any, were recorded.

At the age of 4, 8 & 10 weeks, five chicks were randomly chosen from each group for slaughtering and immunity studies. For the induction of an immune response in the eight bird groups, they were vaccinated biweekly with NDV (Newcastle Disease Virus). Live virus Lasota strain, Bioteke, pharmaceutical research and production lab. Italy, was administered in the drinking water.

Diets:

Two basal diets, starter and grower, were formulated to satisfy the needs recommended and stated by the NRC (1994) for white – egg laying strain as that of the experimental breed have not been estimated yet .The starter diet was fed for the first 6 weeks of age, while the

grower one for the last 4 weeks of the experimental period. The physical and the calculated chemical composition of the formulated diets are illustrated in Table (1).

As to the pigments tested the two synthetic and available pigments, canthaxanthin and B-apo-8-carotenoic acid ethyl ester, were nominated, while marigold petal and safflower were the two natural pigment sources tried. The pure pigments were obtained from Hoffmann La Roche company Egyptian agency and the natural sources from the herbs' shops where they were cleaned and finely ground to be mixed as meals. Each of the two synthetic pigments and the marigold petal meal was tried in two divergent concentrations; 20 mg/Kg diet and its 10 – fold 200 mg, as an extreme level, for the synthetic supplements (chick groups number 2,3,4 & 5 in respective order); and 3.5 g/Kg and its 10 – fold 35g for the marigold (groups number 6 & 7). The more costly safflower was tried at only one concentration level, 20 g/Kg diet (group 8). The design of supplementation is pointed to in Table 2 footnotes. It is worth to note that the levels of pure pigments added equal 2. 5 & 25 times that of the maximum recommended by Hoffmann La Roche to be added to diets for body & egg pigmentation. The first group of chicks was considered as control and fed the basal diets without any supplementary pure or natural pigments.

Immunological Assays:

(A) Serum:

A blood specimen was collected from each of the slaughtered chicks, in the eight groups, at the aforementioned settled ages, making a sum of 120 sample. The blood samples were allowed to clot at ambient temperature, centrifuged for 10 minutes at 3000 rpm, and serum from each sample was extracted. The serum samples (1 ml /vial) were kept frozen at – 20 C until immunity parameters were measured.

Total protein: It was determined colorimetrically using Biuret reaction (Henry, 1964 and Gornall, 1968).

Protein fractions: They were determined using the Helena procedure or cellulose acetate electrophoresis (Kohln, 1958). This method depends on electron distribution of structural subgroups of proteins at a given pH (8.8). Serum proteins are fractionated on the basis of their electrical charge into 5 classical fractions: albumin; alpha 1 (α_1), alpha 2 (α_2), beta (β) and gamma ($\wp$) globulins (Ritzmann and Daniels, 1982 and Killing, 1980). The cellulose acetate strips were fixed after being used and then scanned for obtaining the estimated values of the

different fractions using a computerized system.

Immunoglobulins are synthesized by the cells of the reticuloendothelial system in response to a variety of antigenic stimuli that are ever present in the interior and the exterior environment of all living organisms. In the electrophoretic pattern of fractionation, part of beta and essentially all of the gamma globulins are immunoproteins (Torres-Medina et al., 1971). The albumin /globulin (A/G) ratio was also calculated.

Haemagglutination test (HI): It was applied as a rapid means for measuring the immune response of the birds to Newcastle vaccination. The antibody titers in response to NDV were assessed by the serial dilution technique of the serum using physiological saline (0.65% NaCl) as described by Beard (1989).

(B) Immunopathological Examination:

Organs: From the bird carcasses in the different groups the spleen, thymus and bursa of Fubricious, the organs having immunological importance, were removed, weighed and their relative weights (%) were calculated at the end of the 4^{th}, 8^{th} &10^{th} week.

Lymphocytic population: The organs were stored in Zinker's formal solution (2.5g potassium dichromate, 4.0g mercuric chloride, 1.0g sodium sulfate in 1 liter of distilled water, and 0.5 ml formaline added just before use) for immunopathological examination.

From each of the different samples several sections were made and stained with heamatoxlin and eosin for routine histological examination (cited by Bancroft and Stevens, 1977). Lymphocytes were detected by using the following methods:

- Alkaline phosphatase reaction for the detection of activated B-lymphocytes (Gomori method, 1952) .
- Non-specific esterase activity for the detection of T-lymphocytes (Lodja et al., 1976).
- Alkaline phosphatase and non–specific esterase reactions as indicators for activated B & T – lymphocytes, respectively (El-Sherry et al., 1994 and Inone et al., 1988).

Statistical Analysis:

All the data obtained were statistically analyzed using PC-STAT (1985) in order to test the significancy of the differences between groups and evaluate the calculated means.

Table 1: The composition of the diets fed during the experimental period

Ingredients		Diets	
		Starter (0– 6 wks.)	Grower (6 – 10 wks.)
Physical composition:			
Yellow corn, ground	%	66.60	70.45
Soybean meal (44 %)	%	20.30	20.70
Wheat bran, course	%	6.00	5.20
Fish meal, herring (71%)	%	4.00	-
Dicalcium phosphate	%	1.70	2.00
Limestone, ground	%	1.00	1.25
Common salt	%	0.40	0.40
Premix *		+	+
Calculated chemical composition & energy value #:			
Crude protein	%	18.14	16.18
Methionine and cystine	%	0.63	0.55
Lysine	%	0.98	0.82
Calcium	%	0.90	0.82
Phosphorus, available	%	0.41	0.36
ME (K cal / Kg)		2852	2866

* MUVCO broiler mineral and vitamin premix used at the rate of 1 Kg / ton diet .
# The chemical composition was calculated on " as-fed " basis .

RESULTS and DISCUSSION

Performance:

The performance of the chicks in the different groups as reacting to the pigment addition was evaluated through the body weight development during the experimental period , food intake , and efficiency of conversion. The data were summarized and represented by that recorded at the age stage 4, 8 & 10 weeks (Table 2).

Growth rate: The fowl breed BB appears to be more suitable for egg production than for broiler production as in the first group the birds showed an average daily gain of only 8.8 g. The second group fed on the 20 mg canthaxanthin-supplemented diets achieved a body weight 1.2 times that of the control at the end of the experiment, while the other supplementations whether synthetic or natural achieved mostly 1.05 to

1.07 times. Group 7 supplemented with 35 g marigold was the only deviant and scored the lowest body weight (93 % of the control).

Making consideration for the average initial weight of the chicks in each of the eight groups, it was found that all the groups surpassed the control one in times of initial weight duplication with the groups 2, 4 & 6 the highest – 3, 5 & 8 the lowest – and group 7 nearly was equal to that of the control. Eventually it could be concluded that the 35 g – marigold has no beneficial effect on body weight and the decreased final weight is an expected consequent of a lowered initial one. It might be either the high fiber content of the diet, due to the addition of a large percentage of marigold, nullified the advantage of its high pigment content, or the high percentage of natural pigments reversely caused a decrease in weight. This fact is also noted with the synthetic pigments in its respective groups.

The promoting effect of pigments is not simply proportional to the amount added but it is the kind of the carotenoid and its most effective proportion.

The pigments are better to be added in a concentrated form, otherwise the plant material and its high fibre content may negatively antagonize. Lipstein (1984) previously concluded that the utilization of the xanthophylls from the natural ingredients is lower than the synthetic ones .

Food intake and efficiency of utilization: It seems that the improving effect of pigments on performance, translated as body gain, was effected through increased food consumption and / or increased conversion efficiency.

Calculating the amount of food consumed in the different groups in comparison to group 1, it was found that the addition of synthetic pigments did not affect the rate of food consumption except when canthaxanthin was added at the rate of 20 mg/Kg. But addition of either canthaxanthin or B-apo-8- carotenoic acid ethyl ester at the two addition extremes pointed to increased feed utilization efficiency (3.22 to 3.35 Kg food /Kg gain compared with 3.51 in the first group). The carotenoic acid surpassed canthaxanthin in relation to the efficiency of feed utilization and appeared to be the least costly from this point of view.

The natural pigments added as marigold and safflower increased the food consumption, except when the bulky nature of the plant interferes with, but on the other hand marigold added at 3.5 g/Kg diet did not have any beneficial effect on utilization efficiency, while on the

contrary marigold at 35 g/Kg and safflower at 20 g/Kg diet had bad effects.

For a conclusive recommendation it could be stated, as far as it is tested, that supplementation with synthetic pigments improved performance, weight or food conversion efficiency, if added at a rate of 20 mg/Kg. Increasing the amount added will not add extra – effect but on the contrary may antagonize the good effect. Addition of natural sources if increases body weight or food consumption may cost much through decreasing the efficiency of conversion.

It is worthy here to point to the mention of Food & Drug Administration, Center for Veterinary Medicine (1984) relating to the improved feed efficiency and increased rate of weight gain which is attributed to increased absorption and deposition of xanthophylls and carotenoids in different tissues.

Immunological Assays:

Total serum protein and its fractions: The serum protein in the different groups did not get affected by pigment supplementation whether of synthetic or natural origin. It seems that it is controlled by a physiological mechanism resistant to dietary change, and so much greater constancy prevails. It is the level of dietary protein which may have an influence (Leveille and Sauberlich, 1961). In our study the level varies from 3.43 to 5.05 / dl at the age of 4 weeks, 3.27 to 4.06 at 8 weeks, and 2.92 to 4.05 at 10 weeks pointing to its declining trend with age (Table 3).

The albumin did not differ among groups and had a trend to decrease with age as it ranged from 1.81 to 2.26 at 4 weeks of age, 1.33 to 1.64 at 8 weeks, and 1.24 to 1.74 g/dl at 10 weeks. The same was with-globulin where no change noted due to pigment supplementation, and the level decreases with age from 0.83–1.25 at 4 weeks to 0.33 – 0.77 at 10 weeks. It is the B-and- globulins which changed inversely and the change due to the pigment supplementation is significantly clear at the age of 8 weeks in the beta and early at the age of 4 weeks in the gamma.

Collectively the synthetic pigments decreased the beta – globulin and increased the gamma while no effect for the natural pigments was noted. Increasing the amount of pigments added increases the proportion of gamma globulin in the serum protein but achieving the maximal effect, which is 120 – 150 % of that of the 20 mg/Kg needs 10 fold – addition (200 mg/Kg), thus it is too costly to expend.

As the ℘- globulin increased on the expense of the beta due to the pigment supplementations, the A/G ratios remained non-significantly different (Table 3).

Immune response to NDV: According to Beard and Wilkes (1973), the HI test is generally considered to be a reliable, economical, and rapid means of measuring the immune response of poultry to Newcastle vaccination. From this experiment we were able to detect positive significant differences ($P<0.05$) at HI titers in response to synthetic pigment supplementations at the 10 th week of age (Table 3).

The immuno-enhancing effect of synthetic pigments was confirmed by this test, especially the high level of canthaxanthin (200 mg/Kg) followed by the high one of apo–carotenoic acid which recorded the highest values of HI titers. The lower values of both of the synthetic pigments were significantly higher than the control and the other groups (6, 7, & 8). McWhinney et al. (1989) reported that dietary canthaxanthin significantly increased the antibody titers of chicks. The natural pigments had suppressing effect on HI titer values especially the high level of marigold and safflower.

Organs:. The bursa, spleen and thymus are the most reactive organs for any immune stimuli (Nobuma et al., 1994). This fact was tried to be tested as a result of pigment addition and the data obtained are displayed in (Table 4).

The relative weights of bursa and spleen, at the age of 4 weeks, were nearly equal 0.34 and 0.38% respectively (control group). The bursa increases with age as it reached at the age of 10 weeks to about 1.7 times that at 4 weeks, while the spleen decreases in weight and reached only 0.8 time. Thymus relative weight, on the other hand, varies from 0.6 – 0.8 %, it seems that age has no clear effect on its weight.

The relative weights of the organs did got effected by the pigments added, early and after 4 weeks of feeding in spleen and thymus, while the bursa showed a reaction after 10 weeks. The bursa decreased in weight by 16–33%, indicating reactivity (Nobuma et al., 1994), in all the groups except group 7 and the most significant effects were recorded for the 20 mg addition of canthaxanthin and B-apo-carotenoic acid and the 200 mg addition of canthaxanthin. As related to the spleen the synthetic pigments were lonely having a positive effect and the weight decreased by about 17–27% of that of the control group, especially with the 200 mg canthaxanthin group. Haq and Bailey (1995) reported that birds hatched from breeders fed B – carotene and

canthaxanthin had significantly lower spleen weights than the control. The synthetic pigments in the present work kept on affecting the thymus but only at the exaggerated level of addition, and showing an increase in weight of 30% in canthaxanthin and 23% in B-apo-8- carotenoic acid. It can be summarized that the organs immunologically react to the added pigments by reducing its weight in bursa and spleen, and increasing it in thymus. The effect is more clear with the synthetic ones especially at the high levels, while the natural pigments may have an effect on bursa , reverse effect on thymus (decreasing its weight), but no effect on spleen.

Lymphocytic population: The intensity of lymphocytic population in spleen, thymus and bursa was estimated using the different aforementioned reactions, and is shown in Table 5. It is clear that there is an increase in the splenic B & T lymphocytes in groups 3 & 5 which fed the high levels of canthaxanthin and carotenoic acid respectively. Both groups also showed the highest lymphocytic proliferation in thymus and bursa. On the other hand the second and fourth group fed the low levels of the same synthetic pigments showed high splenic B cells & bursa cells in the second and high splenic T cells & thymus cells in the fourth. The natural pigments had no positive effect on the reactivity of the organs except the 3.5 g marigold addition which showed high bursal cells and the 20 g safflower which had negative effect on the splenic B cells.

The positive effect of canthaxanthin on lymphocyte response was reported by Bendich & Shapiro (1986) in mice and rats, and of carotenoids, in general, on immune function in broilers during aflatoxicosis by Okotie-Eboh et al. (1997). The figures 1 to 11 demonstrated this positive effect in comparison with the negative control group.

As a collective end result, this work adds more confirmatory information on the positive effect of carotenoids, synthetic or natural, on performance and immune function. The high performance represented by high rate of growth, increased food intake and efficiency of conversion. The high levels were more effective in enhancing immune functions proved by the heightened $\wp$-globulin; bursa, spleen and thymus weight change; intensed lymphocytic population and high HI titers. The immune system-stimulating effect of carotenoids which is demonstrated in chicks can be also true for the other birds and animals including man. Enriching the food articles of animal origin, in man diets by pigments may give the same beneficial effect. In relation to animals,

to reduce the cost, the high potentiating levels can be only used in case of need as in time of vaccination or in stress.

ACKNOWLEDGMENT

Special thanks to Hoffmann La Roche Inc., Giza, Tammouh, cooperative with Rovogypt for providing the synthetic pigments (canthaxanthin and apo-8- carotenoic acid ethyl ester) used in this study.

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Table 2: Chick performance in the eight groups.

Age in Weeks	Groups *							
	Control	Synthetic pigment groups				Natural pigment groups		
	1	2	3	4	5	6	7	8
Body Weight (g)								
Initial	30.4	30.0	29.7	28.5	30.6	29.0	28.0	31.0
4	220.53 c ± 4.04	250.73 a ± 2.71	235.13 b ± 2.44	245.82 a ± 2.95	237.24 b ± 2.78	228.72 b ± 2.28	219.79 c ± 2.51	225.31 c ± 2.34
8	495.35 c ± 11.74	595.05 a ± 5.77	552.80 b ± 11.90	505.67 c ± 13.31	562.95 b ± 10.31	512.05 c ± 13.00	460.61 d ± 6.00	501.25 c ± 5.86
10	646.75 c ± 8.40	778.00 a ± 6.38	688.31 b ± 7.50	684.50 b ± 5.02	690.06 b ± 5.22	692.20 b ± 4.83	602.22 d ± 5.72	679.47 b ± 4.35
Times the initial	21.27	25.93	23.17	24.02	22.55	23.87	21.51	21.92
Feed Intake (g)								
0 – 4	588.43	595.39	585.52	588.89	559.89	560.19	609.66	587.75
4 – 8	981.32	1229.33	1106.80	892.59	1104.08	1038.13	945.55	1036.59
8 – 10	593.96	677.10	505.00	681.30	457.56	734.81	607.46	776.40
Total (0-10)	2163.71	2501.82	2207.32	2162.78	2121.53	2333.13	2162.67	2400.74
Feed Conversion Efficiency								
0 – 4	3.10	2.70	2.85	2.71	2.71	2.81	3.18	3.01
4 – 8	3.57	3.55	3.48	3.44	3.39	3.66	3.93	3.76
8 – 10	3.92	3.70	3.73	3.81	3.60	4.08	4.29	4.36
Total (0-10)	3.51	3.34	3.35	3.29	3.22	3.52	3.77	3.71

* Group 1: no additives , 2 & 3 : canthaxanthin 20 & 200 mg/ Kg respectively 4 & 5: carotenoic acid 20 & 200 mg/Kg respectively, 6 & 7: marigold petal meal 3.5 & 35 g / Kg respectively and safflower meal 20 g/ Kg diet.

- Values (mean ± SE) in the same row which are not significantly different are followed by the same superscript ($P < 0.05$) .

Table 3: Blood proteins and antibody titers to the Newcastle disease virus

Age in Weeks	Groups *							
	Control	Synthetic pigment groups				Natural pigments		
	1	2	3	4	5	6	7	8
Total protein (g/dl)								
4	4.09 ± 0.56	5.05 ± 0.43	4.58 ± 0.86	4.84 ± 0.73	3.96 ± 0.67	4.18 ± 0.39	3.43 ± 0.39	4.84 ± 0.54
8	3.88 ± 0.10	3.087 ± 0.49	4.06 ± 0.40	3.36 ± 0.25	3.91 ± 0.43	3.30 ± 0.45	3.75 ± 0.21	3.27 ± 0.49
10	3.41 ± 0.42	3.42 ± 0.29	3.79 ± 0.49	2.92 ± 0.48	3.67 ± 0.36	3.34 ± 0.12	3.26 ± 0.35	4.05 ± 0.067
Albumin (g / dl)								
4	1.81 a ± 0.16	2.26 a ± 0.22	2.01 a ± 0.42	2.17 a ± 0.27	1.95 a ± 0.28	2.14 a ± 0.27	1.33 b ± 0.08	1.91 a ± 0.16
8	1.56 ± 0.05	1.62 ± 0.18	1.64 ± 0.44	1.41 ± 0.14	1.81 ± 0.28	1.33 ± 0.28	1.34 ± 0.11	1.35 ± 0.15
10	1.24 ± 0.25	1.42 ± 0.08	1.74 ± 0.26	1.24 ± 0.20	1.28 ± 0.21	1.35 ± 0.10	1.26 ± 0.21	1.50 ± 0.31
α-Globulin (g/dl)								
4	0.87 a ± 0.12	1.03 a ± 0.23	0.89 a ± 0.24	1.01 a ± 0.21	0.83 a ± 0.24	1.00 a ± 0.15	0.54 b ± 0.10	1.25 a ± 0.15
8	0.58 ± 0.12	0.73 ± 0.14	0.43 ± 0.11	0.56 ± 0.04	0.66 ± 0.14	0.55 ± 0.13	0.69 ± 0.20	0.59 ± 0.15
10	0.51 ± 0.03	0.51 ± 0.07	0.42 ± 0.06	0.33 ± 0.07	0.64 ± 0.06	0.66 ± 0.12	0.62 ± 0.11	0.77 ± 0.22
β -Globulin (g/dl)								
4	0.89 ± 0.20	0.64 ± 0.10	1.09 ± 0.15	0.87 ± 0.17	0.64 ± 0.20	0.87 ± 0.14	0.67 ± 0.13	1.14 ± 0.24
8	1.28 a ± 0.11	0.56 c ± 0.01	0.82 b ± 0.22	1.05 a ± 0.14	1.03 a ± 0.06	1.01 a ± 0.14	0.99 a ± 0.20	0.94 a ± 0.18
10	1.31 a ± 0.42	0.47 c ±0.18	0.39 c ± 0.18	0.58 b ± 0.10	0.57 b ± 0.11	0.82 a ± 0.16	1.00 a ± 0.32	1.51 a ± 0.37
℘-Globulin (g / dl)								
4	0.52 b ± 0.13	1.12 a ± 0.27	0.95 a ± 0.31	0.72 a ± 0.17	0.53 b ± 0.10	0.80 a ± 0.14	0.90 a ± 0.07	0.54 b ± 0.20
8	0.46 ± 0.08	0.97 ± 0.24	1.00 ± 0.30	0.37 ± 0.12	0.42 ± 0.08	0.42 ± 0.20	0.72 ± 0.40	0.39 ± 0.14
10	0.35 c ± 0.02	1.03 a ± 0.11	1.23 a ± 0.20	0.77 b ± 013	1.18 a ± 0.27	0.51 c ± 0.17	0.36 c ± 0.08	0.27 d ± 0.03
A \ G ratio								
4	0.75 a ± 0.03	0.82 a ± 0.09	0.69 a ± 0.07	0.93 a ± 0.13	0.98 a ± 0.17	0.84 a ± 0.14	0.65 b ± 0.07	0.67 b ± 0.05
8	0.68 ± 0.02	0.71 ± 0.08	0.84 ± 0.18	0.78 ± 0.16	0.87 ± 0.12	0.68 ± 0.12	0.59 ± 0.09	0.73 ± 0.06
10	0.65 ± 0.21	0.72 ± 0.05	0.86 ± 0.08	0.83 ± 0.73	0.53 ± 0.07	0.71 ± 0.11	0.82 ± 0.34	0.66 ± 0.20
Antibody titers (log- titers)								
4	3.33 ± 0.67	2.33 ± 0.67	3.67 ± 1.20	3.00 ± 0.10	3.33 ± 0.57	3.33 ± 0.34	3.00 ± 0.58	2.00 ± 0.58
8	4.25 ± 1.03	4.00 ± 0.10	3.67 ± 0.67	4.75 ± 1.38	4.25 ± 1.03	5.75 ± 1.38	3.67 ± 0.33	3.67 ± 0.33
10	4.50 b ± 0.65	5.25 b ±0.48	7.00 a ± 0.55	5.67 a ± 0.33	6.67 a ± 0.33	4.53 b ± 0.96	3.67 c ± 067	3.00 d ± 0.58

* Group 1: No additive, 2 & 3: canthaxanthin 20 & 200 mg / Kg respectively . 4 & 5: carotenoic acid 20 & 200 g/Kg respectively, 6 & 7: marigold petal meal 3.5 & 35 g/Kg respectively and safflower meal 20 g / Kg

- Values (mean ± SEM) in the same row which are not significantly different are followed by the same superscript (P< 0.05).

Table 4: Bursa, spleen, and thymus relative weight (%) in the different chicken groups

Age in Weeks	Groups							
	Synthetic pigments					Natural pigments		
	1	2	3	4	5	6	7	8
Relative bursa weight								
4	0.34 ± 0.08	0.31 ± 0.02	0.33 ± 0.03	0.34 ± 0.06	0.30 ± 0.04	0.25 ± 0.01	0.34 ± 0.03	0.35 ± 0.06
8	0.60 ± 0.06	0.55 ± 0.08	0.46 ± 0.04	0.47 ± 0.03	0.47 ± 0.07	0.48 ± 0.06	0.61 ± 0.03	0.57 ± 0.07
10	0.57 a ± 0.04	0.38 c ± 0.01	0.43 c ± 0.04	0.43 c ± 0.04	0.46 b ± 0.06	0.47 b ± 0.04	0.58 a ± 0.04	0.48 b ± 0.03
Relative spleen weight								
4	0.38 a ± 0.01	0.29 b ± 0.05	0.28 b ± 0.01	0.37 a ± 0.03	0.31 b ± 0.06	0.43 a ± 0.04	0.41 a ± 0.03	0.43 a ± 0.01
8	0.31 ± 0.01	0.33 ± 0.02	0.31 ± 0.03	0.31 ± 0.02	0.25 ± 0.06	0.34 ± 0.02	0.34 ± 0.01	0.31 ± 0.01
10	0.30 a ± 0.02	0.25 b ± 0.02	0.22 c ± 0.01	0.23 b ± 0.01	0.25 b ± 0.02	0.30 a ± 0.01	0.30 a ± 0.01	0.32 a ± 0.01
Relative thymus weight								
4	0.68c ± 0.07	0.79 a ± 0.04	0.92 a ± 0.06	0.87 a ± 0.08	0.90 a ± 0.04	0.69 c ± 0.04	0.54 c ± 0.02	0.73 b ± 0.05
8	0.79 b ± 0.05	0.83 b ± 0.07	0.91 a ± 0.08	0.80 b ± 0.09	1.06 a ± 0.06	0.60 c ± 0.03	0.61 c ± 0.01	0.60 c ± 0.01
10	0.60 b ± 0.05	0.61 b ± 0.04	0.97 a ± 0.04	0.61 b ± 0.05	0.74 a ± 0.04	0.51 c ± 0.09	0.54 c ± 0.08	0.39 c ± 0.02

- Values means ± SE.
- Values in the same row which are not significantly different are followed by the same superscript (P<0.05).

Table 5: Relative intensity of lymphocytic population in the different groups at the end of the experiment.

Lymphocytes	*Groups*							
	Control	*Synthetic pigments*				*Natural pigments*		
	1	2	3	4	5	6	7	8
Spenic B cells	-	+++	++++	++	+++	++	++	-
Splenic T cells	++	++	++++	+++	+++	++	++	++
Thymus cells	++	++	++++	+++	+++	++	++	++
Bursa cells	++	+++	++++	++	+++	+++	++	++

The lymphocytic population intensity was referred to as - ve , + ve , ++ ve, +++ ve and ++++ ve for negative , low , moderate , high and very high intensity respectively

Figure 1

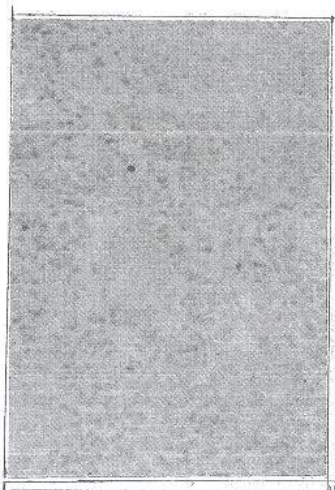
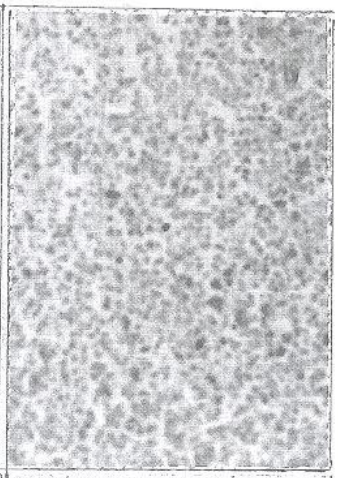


Figure 2



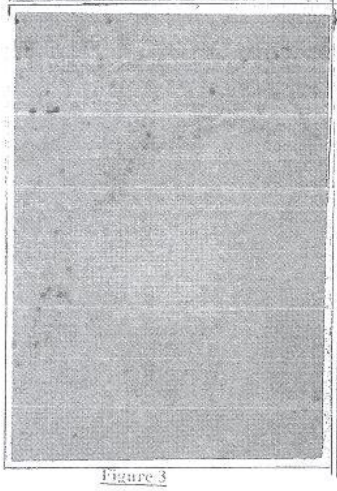


Figure 3

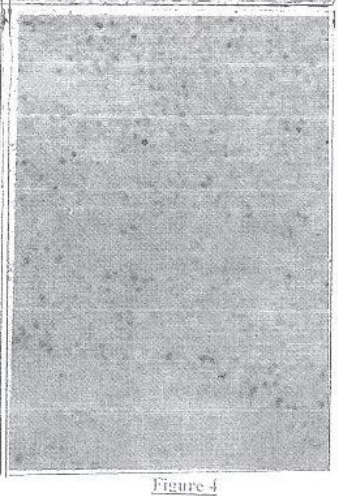


Figure 4

Figure 5

figure 6

Figure 7

Figure 8

Figure 9

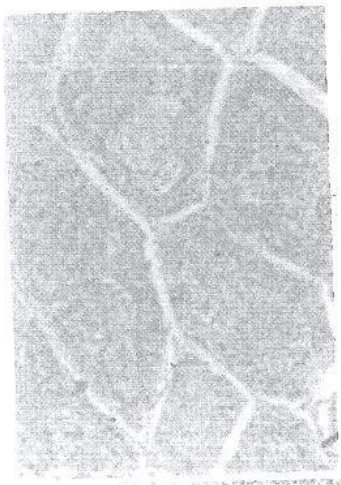
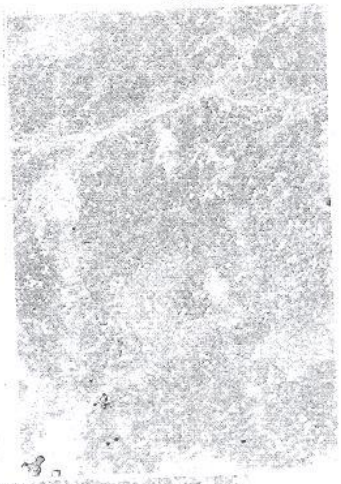


Figure 10



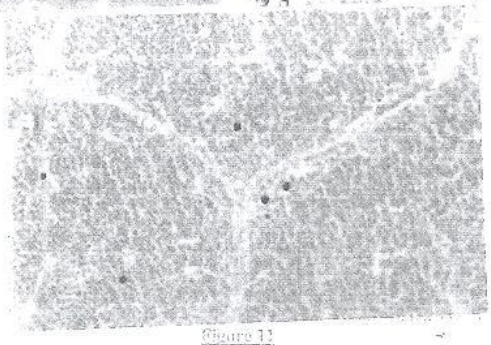


Figure 11