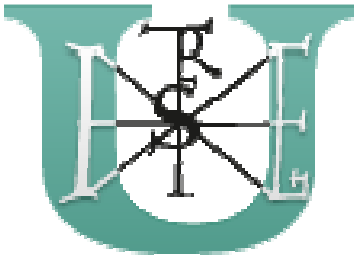

SZENT ISTVÁN UNIVERSITY

THE DYNAMICS OF INFILTRATION OF IMMUNOGLOBULIN (IGY) AND ITS INDUCTIVE VITAMINS (A, E AND CAROTENOIDS) WITHIN THE YOLK

Thesis of Dissertation

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1. PREPARATION, GOALS TO ACHIEVE

The transport of substance, energy and information between the egg-laying mother and the offspring, the multideutoplasmic ovula and its layers are created within a very short time period. In case of laying hen the formation (growth) of yolk consists of a couple of days. The formation of the yolk layers (cytoplasm, shell membrane, eggshell) during the ovulation consists of little bit more than 24 hours. During this time period an amount of material, energy and information transport and storage is being processed, which is enough for the in ovo evolution (21 days inside a laying hen, 16-18 days inside a Japanese quail), what is more, in case of certain materials it can cover the needs of the first postnatal days as well.

These factors are especially important in the case of the immunoglobulins which provide so-called yolk immunity. Due to the fact that the fraction and function of these antibodies partly differ from that of the mammals they are thus referred to as IgYs. This term is used not only in the embryonal but also the early postembryonic evolution, and also in the analysis of the vitamins as well, which have an important role in the later life. Such are the members of the vitamin A family, the retinoids, which sustain the differentiation, the protection of the layers and the immunity; the tocopherols with vitamin E effect, which sustain the antioxidant protection, the stability of the membrane and several other substances among the carotenoids, which on the one hand are of provitamin character (beta-ringed) and have an antioxidant-like structure.

Ovulation inside the egg-laying birds, similar to mammals, is caused by the follicle stimulating hormone (FSH). The minuscule follicles of the sole (left) ovary begin to grow following the sexual maturity, and in the reproductive periods. During this growth cytokinesis is being processed within the layers of the follicle walls. In case of birds no liquid is created within the follicle, unlike in the mammals. Following the meiosis the so-called complex yolk lipoproteins are settled next to the oocyte instead. This is how yolk is created. Carotenoids, especially oxycarotenoids (lutein, zeaxanthin) cause the yellow colour. Based on the quantity the intensity of the yellow colour can differ as discerned with the naked eye. The occurrence of this colour can be well distinguished in the little white and yellow follicle status during the follicle growth.

Of course feeding plays a crucial part in the infiltration of carotenoids and other lipids. This is especially valid for those lipids, which do not develop within the bird’s fat metabolism. Such are the carotenoids and partly vitamin A and E as well. This is only partly valid for the vitamin A vitamin retinoids, since it can develop from beta-ringed carotenoids within the organism. This again calls attention to the role of carotenoid supply.

Another important factor is that materials can only infiltrate into the egg during the development of the egg parts. Hence the yolk substance infiltrate from the beginning of the ovulation, which consists of no more than 15-17 days. The other large substance, the egg white is developed within 3 hours while being in the magnum. Hence during the development there is a limited time for the egg-layer to provide its offspring with the energy and material needed for the embryonic development. There are also substances within the egg that are not only important during the embryonal in ovo phase, because the baby chicken also needs them after the hatching. To these substances belong the

immunoglobulins, carotenoid and other fat soluble, the so-called inductive vitamins (A, E) which I shall analyse in this paper.

The role of both the carotenoids and that of vitamin A and E within the immune system is well known. Nevertheless the clarification of contradictory data in the scientific literature is still in progress. Birds are special in the receipt, infiltration and use process of material on the hen-egg-offspring axis.

Our goals are to clarify the difference between the immune system of chicken and mammals and the interaction of vitamins within the immunsystem. Our main goal is to get to know the dynamics of immunoglobulin infiltration in the case of different vitamin supply.

In our analysis we aim to explore the immune response readiness of tocopherols / retinoids and some carotenoids on our model animals: the Japanese quail and the chicken.

Questions to consider:

- Can a specific antibody be detected also in the “white” follicle or is it only to be found in the “yellow” one?
- When a well-defined antigen is immunised into the quails the increased level amount of vitamin E, carotenoids (beta-carotene, lutein, lycopene is used solely or combined, can this have an influence on the infiltration of vitamin A and E and that of carotenoids into the yolk?
- In what amount does the sole and combined dosage of vitamin E, carotenoid (beta-carotene, lutein, lycopene) in the feeding influence the level of yolk immunity?
- How do these treatments influence the brooding and the level of yolk immunity in the hatching baby chicken?

This complex interaction will be examined based upon the IgY and vitamin content of the developing follicles. Our further aim is to examine the accumulation of different vitamin doses within the yolk, as well as that of the relation between these and the IgY titer: on the one hand the competition between the fat soluble factors, on the other the immunmodulant effect.

We shall also record the dynamics of IgY infiltration into the yolk after the immunisation and its interaction with the deposition of the examined vitamins.

The results can be used in the production of the poultry industry, since these correlations could optimize the immunobiological status of the economic livestock.

2. MATERIAL AND METHOD

2.1 The examination of IgY transport: record of initial status with commercial fodder. (1. experiment)

In the case of chicken the formation of the yolk occurs within a couple of days, this short time period is crucial for the in ovo and the postnatal development of the offspring with regard to the accumulation and storage of the needed substances. Namely the transport of these substances between the mother and the offspring play an important role in the so-called yolk immunity, which is provided by the IgY in the yolk, which is similar to the IgG in the mammals. After the immunisation this policlonic antibody can be produced by the hen during the whole hatching period. The greatest

advantage of IgY usage is that it can be removed from the egg relatively easily, without blood-letting. Other advantages are also known such as relative temperature and pH stability, the interaction between the Fc receptors and the complement system is omissible, etc.

The IgY transport can be divided into two, well-isolable processes. During the first process the antibody infiltrates from its production place (IgY-producing clones) through the blood transport to the ovary follicles. The second process is the penetration from the yolk bag into the embryo. Our analysis is based upon this system.

2.1.1. Examinations carried out during the follicle development in Japanese quails

We began our analysis with the examination of the IgY transport into the follicles. We have determined the IgY and carotenoid content of the follicles within the different states of the follicular hierarchy: little white, little yellow, big yellow, and preovular. We bought the test "Tápiófürj" Japanese quails from a breeder in Tápiógyörgye. At the breeding place the animals were fed with normal, commercial fodder with *ad libitum* access. They were provided with drinking water on a regular basis. Our goal was to examine the development of the follicle system with normal, commercial fodder feeding.

Following the lege artis extermination of 4 active Japanese quail laying hens we measured the diameter and the weight of the stuffed ovary follicles. We prepared a homogenatum from the follicles and analysed the carotenoid, retinoid and tocopherol with reversed- phase high performance liquid chromatography (HPLC), and IgY with enzyme-linked immunosorbent assay (ELISA) method.

2.1.2. Analysis of the embryo within the incubated chicken egg and examination of the chickling

We monitored the in ovo transport of carotenoid, retinoid, tocopherol and IgY transport within incubated chicken eggs. The chicken eggs originated from Transylvanian Naked Neck hens which were bred at the Centre of Gene Preservation for Domestic Animals (Haszonállat-génmegőrzési Központ) in Gödöllő. Similarly to the previous record, we examined the transport with normal feeding.

The eggs were incubated in a table incubator, we took 5-5 samples of them on the 0th, the 7th, 14th and 19th day, equally 5 samples were taken of the baby chicken 5 days after the hatching. We weighed the eggs, the embryos and the yolk bag, as well as the liver of the embryos and that of baby chicken, after their lege artis extermination.

We examined the carotenoids and retinoids in the blood (serum) and the homogenatum prepared from physiological salt solution with reversed-phase HPLC, and IgY with ELISA method.

2.2. The effect of carotenoid supplement on the IgY transport

2.2.1. The effect of xanthophyll supplement (2. experiment)

We divided the mature Japanese quail hens into two groups (n=10-10). We fed the members of the control group with commercial fodder. We fed the other (Capsantal complimentary group) with the same fodder mixed with 1000 ppm xanthophyll supplement (Capsantal EBS 40 NT Copharm: active ingredients 40g / kg xanthophyll with 82% lutein content). During the 6-week-experiment the birds were fed and watered ad libitum.

Both groups have been immunised. The antigen used during the experiment consisted of purified goat red blood cells (gRBC), the 5% suspension of which was prepared with physiological salt solution to which a tannic acid of 1:125 000 dilution was added. We then solved as much BSA in this mixture until we reached a 100 μg / animal concentration. We injected the goat red blood cells and BSA mixture (gRBC-BSA) into the pectoral muscle, the quails were thus immunised at the beginning of the experiment and on the 4th week.

Blood was drawn from the wing vein (*vena subcutanea ulnaris*) on a weekly basis. During the experiment the eggs were collected every day following the second immunisation. Following the *lege artis extermination* of the active Japanese quail laying hens we measured the diameter and the weight of the stuffed ovary follicles. We examined the carotenoids and retinoids concentration in the blood and the follicles with reversed-phase isocratic HPLC, and the IgY in the follicles, yolk and blood circulation with ELISA method. We also measured the yolk and skin surface colour. The egg colours were compared with the use of Yolk Colour Fan (YCF). We defined the colour of the egg and that of the skin surface with colorimetric method, with a manual reflected Micromatch ™ Plus (Sheen Ltd., UK) spectrophotometer which compares the result to the CIELab scale.

2.2.2. The effect of carotenoid-free fodder mixed with lycopene, lutein, beta-carotene and vitamin A supplement (3. experiment)

We divided the 8-weeks-old quails into 7 five-membered-groups. A rooster was included to each group in order to ensure the egg fertility. We fed the first group with commercial fodder. The other groups were fed with fodder prepared from the researchers previous experiments (Réthy and co, 2005): rice-based, carotenoid-free fodder was mixed with 15 000 NE retinol / fodder kg, equivalent beta-carotene (BC, group 2), lutein (LU, group 3), lycopene (LI, group 4), all three carotenoids (BC+LU+LY, group 6), and vitamin A (group 7). Group 5 was fed with supplement and carotenoid free fodder.

The fodder mixture was provided in small (a couple of days') rations in order to avoid the deterioration of the supplement carotenoids. The supplements were stored according to the package label: airtight, in a dark cool place.

Table 2.1.

Supplements used in the experimental fodder

	producer	active agent concentration	g/fodder kg
Beta-carotene	DSM	10%	0,4
Lutein	DSM	5%	0,8
Lycopene	DSM	10%	0,4
A-vitamin	Vitafort		0,3268

Table 2.2.

Supplements fed to the groups in the third experiment

group	raw feed	supplement	Measured quantity per groupsg
1	normal hen feed	-	-
2	rice based fodder	BC	0,4 g/ kg
3	rice-based fodder	LU	0,8 g/kg
4	rice-based fodder	LI	0,4 g/ kg
5	rice-based fodder	-	
6	rice-based fodder	BC LU LI	0,12 + 0,24 + 0,12 g/kg
7	rice-based fodder	A-vitamin	0,6556 g/2 kg

During the experiment the birds were fed and watered *ad libitum*.

The quails were immunised through the pectoral muscle. We followed the protocol of previous immunisation experiments. The antigen used during the experiment consisted of a mixture of purified goat red blood cells (gRBC) and Bovin serum albumin (BSA) with a concentration of 100 μg / animal. Blood draw took place every week, eggs were collected as well. The eggs laid between the 14th and the 21st day of the experiment have been incubated.

We collected 3 incubated egg samples per group on the 14th day. We weighed the eggs, the embryos and the yolk bag, as well as the liver of the embryos and that of baby chicken.

We examined the carotenoids and retinoids in the blood (serum) and the homogenatum prepared from physiological salt solution and IgY with ELISA method.

3. Incubation method used

Incubations were carried out with ME3M (MainoEnrico-Ariano Di Maino Roberto C.S.N.C) type table incubation machine. We carried out the chicken egg incubation following Bogenfürst's (1994) description, whereas quail egg incubation was based upon the experiences of Mrs. Sinkovics (1973) and that of the research centre (Kerti and Bárdos, 1997).

4. Analytical method used

4.1. Specific IgY Examination

The quality and quantity report on the specific antibodies were carried out from the blood serum, yolk and the liver with ELISA method (Losonczy et al, 1999). Colour development was not done with the original description's OPD (o-phenylenediamine-dihydrochloride) chromogene substrate but with our previous experiment's TMB (3,3', 5, 5'-tetramethylbenzidine) (Jung et al., 2009).

Blood samples

We applied serums in our researches. Following the blood draw we kept the sample on room temperature till complete clotting (max. 1-2 hours). The congealment was separated from the test tube's wall with needle or mandrin, then it got centrifuged. The blood serum samples were refrigerated to -20 Celsius.

Yolk samples

The fraction of egg white and yolk was separated, then we took a 1g yolk sample, we diluted it with 1 ml physiological water and refrigerated it to -20 Celsius.

Liver samples

Following the dissection of the blood-let quails we removed and weighed the liver then refrigerated it to -20 Celsius.

There is considerable difference in the vitamin A concentrates among the lobes at multilobed species (i.e. pig, dog). This can be explained with the intralobular vascular system. Poultry have only two (rarely three) liver lobes. The liver lobe blood vessels originate from a common stem, they form a more simple net thus the blood flow is more balanced (Bárdos, 1991). We carried out our measurements on the right, bigger liver lobe. Before the analysis we homogenised 1 g from the melted tissue using potter device, then we centrifuged it (at 4 Celsius, for 20 minutes). We carried out ELISA measurements on the supernatant.

4.2. Carotenoids, Retinoids and Tocopherol Analysis

We took samples from the blood serum, the yolk and the liver. We analysed the carotenoid and retinoid content of the yolk, yolk, liver and serum samples with reversed-phase isocratic HPLC (rpHPLC) method (Kerti and Bárdos, 2006) in every experiment.

The samples have been prepared according to their type.

Serum Sample Preparation

After the blood draw we acted as described under 4.4.1., separated the congealment with centrifugation and store it at -20 Celsius temperature. We melted the refrigerated samples and put a dose of 250 µl into a 4ml centrifuge tube, then added 250 µl 10 % ascorbic acid solution followed by 500 µl ethanol. After a 30-second-long continuous mixing 1000 µl hexane was added and we repeated the continuous mixing for the same amount of time. After a 10-minute-long centrifugation we began our rpHPLC analysis by pipetting 400µl from the pure supernatant into the Eppendorf tube and condensed it with nitrogen streaming in 4-5 minutes. Before injecting it onto the HPLC tower we put it into 100 µl mixture of ethanol dioxane of a 1:1 proportion, then after a short continuous mixing 150 µl acetonitrile was added to it.

Preparation of the other animal tissues (yolk, liver) for the carotenoid and retinoid analysis

During the dissection we have removed the liver and the yolk bag, weighed them and stored them at -20 Celsius temperature. We took 0.5 g from the egg samples and added 1ml

10% ascorbic acid solution to them and stored them at -20 Celsius temperature. During continuous mixing the melted sample has been homogenised with a glass homogeniser and 3ml extraction mix (10:6:6:7 hexane: acetone: absolute ethanol: toluol) was added to it. After a 10-minute-long centrifugation we began our rpHPLC analysis by pipetting 200µl from the pure supernatant into the Eppendorf tube and proceeded as it has been described under the serum section.

We took a 0.3 g sample of the refrigerated liver then added a 3ml extraction mix and 1ml ethanol to it. After a 10-minute-long centrifugation we began our rpHPLC analysis by pipetting 200µl from the pure supernatant into the Eppendorf tube and proceeded as it has been described under the serum section. Before injecting it onto the HPLC tower we put it into 200 µl mixture of ethanol dioxane of a 1:1 proportion, then after a short continuous mixing 300 µl acetonitrile was added to it.

We injected 20 µl of the pure extract onto the C18 Rocket Platinum tower (100A 3µ 53 mm x 7 mm) (Alltech, USA). The HPLC system consisted of a PU-980 pump and a UV-2077, 4 channel detector (Jasco, Japan). We pumped the dynamic phase (1% acetonitrile: tetrahydrofuran: methanol: ammonium-acetate solution – 684:220:68:28) with a 1ml / min speed. The maximum values were detected based upon these standards: tocopherol 290nm, retinoid 325 nm, carotenoids 450 nm, lycopene 505 nm, dissolutions have been taken into consideration, the concentrations were measured with ChromPass (Chromatography Data System, JASCO HPLC Japan) programme.

4.3. Egg and Skin Surface Examination with CIELab Method

The yolk colour of the fresh eggs has been defined with Yolk Colour Fan (YCF - DSM) and with colorimetric method with a Micromatch™ Plus photo meter (Sheen Ltd. UK) which compares it with the CIELab scale. The colour change of the skin surface was measured in the same way (Szabó and co, 2007). This system characterises the reflected colour with the use of coordinates (L*, a*, b*) in a 3D-spectrum. The colours are represented on two horizontal, perpendicular axes, where the values can be red (a* = 0-+100) – green (a* = 0- -100), yellow (b* = 0-+100) or blue (b* = 0- -100). The vertical axis shows the lightness (L* which varies from 0 (black) to 100 (white).

We can measured the color of the carotenoids there in to the fat of inner skin surface deposited.

5. Statistical methods

We took an average (x) and a standard deviation estimation (±s) of the measurements (i.e. weight, absorbency, concentration unit). The results were evaluated with paired and two-sample t-test, whereas the relation between the data has been examined through the Pearson product-moment correlation coefficient (r) in MS Office Excel 2010 format.

When analysing the group average values we used Tukey's test in ANOVA variation analysis) and Dunett's test (GraphPad Prism ver. 5.0 for Windows).

A value smaller than $p<0.05$ (5%) has been set as significant.

3. RESULTS

3.1. Az The examination of IgY transport: record of initial status with commercial fodder. (1. experiment)

3.1.1. Examinations during the development of follicels in Japanese quails

From the F7 time period on an abrupt follicle weight gain and a gradual diameter increment can be noted. The storage of carotenoid (lutein LU and BC), retinoid (ROL and retinyl palmitate RP) and tocopherol (TF) in the yolk becomes rather significant in the F7 time period.

BC, LU and the retinoids (ROL and RP) reach their concentration peak within the F3 follicle size. When Japanese quails are fed with commercial fodder, a close connection ($r>0.7$) among the tocopherol, carotenoid (BC, LU) and retinol level of their ovular follicles can be recorded. These connections were all significant ($p<0.05$).

An abrupt IgY-titer increment can be seen in the F10 – still white – follicles, this process shows some fluctuation but practically stays on the same level until the ovulation. No simultaneity can be detected in the concentration change of carotenoids and in the change of the immunoglobulin titer with regard to the follicle size analysis.

3.1.2. Analysis of the Embryo within the Incubated Chicken Egg and Examination of the Chickling

In the samples from the different development phases (from 0th, 7th 14th 19th day and from the 5th day after the hatching) the amount of the examined substances (BC, LU, ROL, RP, tocopherol) decreases simultaneously with the yolk infiltration. The highest amount of these substances in the yolk can be noted before the incubation. Although on the 5th day in the incubation their amount decreases, however, their concentration reaches its peak in the absorbing yolk bag by then.

When examining the IgY presence in the yolk with ELISA method, we could determine an increment in the amount until the 19th day, from which on this concentration tendency begins to decrease. Parallel to this the IgY level increases in the serum.

During the incubation the concentration of carotenoids (LU, BC), retinoids (ROL, RP) and vitamin E is almost constant, their amount increases in the embryo liver.

3.2. The effect of xanthophyll supplement (2. experiment)

The main component (82%) of the supplement Capsantal (Copharm, Gr) is a xanthophyll (oxycarotenoid), the lutein. In the Capsantal supplement receiving group the lutein

concentration in the Japanese quail serums and the eggs showed increment compared to the control group members. The difference between the two groups is significant ($p<0.05$ *).

No difference was detected in the serum, egg and follicle retinoid (ROL) concentration between the two groups ($p>0.05$). This means that xanthophylls show no provitamin A activity, because there is an oxo-group (substituent) in their terminal ring. The beta-ringed structure is essential at the retinoids, that is, at the provitamin A carotenes as well.

Capsantal is the natural extract of the Mexican Marigold (tagetes erecta), it contains 1 % natural beta-carotene (http://www.copharm.gr), and therefore we could use Capsantal as a beta-carotene source next to lutein.

In the Capsantal supplement receiving group the beta-carotene concentration was higher in the eggs ($p<0.05$**).The concentration of the natural BC from the normal commercial fodder and the main ingredient (82%) of the Capsantal supplement: lutein is similar to that of inside the yolk ($p>0.05$).

The average IgY titer in the serum (after the booster dose $p<0.05$) and in the egg ($p<0.05$***) was higher in the supplement receiving group than in the control group. From the 3th week on, the IgY concentration is on a constant level inside the egg, even after the booster dose, this is where the most considerable difference can be detected in the serum.

From the F8 follicle size on an abrupt IgY titer increment can be detected in both groups, though this process shows some fluctuation but practically stays on the same level with no significant differences. The carotene concentration (LU, BC) shows significant ($p<0.05$*) increment in the F8 follicles compared to the control group. The Capsantal supplement has also an effect on the follicle immunoglobulin level. rLU=0.6240 (ns), rBC=0.6356 ($p=0.0483$*), rRP=0.5203 (ns).

YCF showed an increment in the yolk colour intensity already by the 2nd week in the Capsantal supplement receiving hen group, the average colour values became significantly ($p<0.05$) higher all through the experiment. When measuring the skin colour intensity the L and b* values showed significant difference in the CIElab.

3.3. The effect of carotenoid-free fodder mixed with lycopene, lutein, beta-carotene and vitamin A supplement (3. experiment)

With regard to the IgY titer in the serum, there is significant ($p<0.05$) difference among the 2. (BC) **, the 3. (LU) *, the 4. (LI) **, and the 7. (vitamin A) group ** compared to the 1. (commercial fodder receiving) one. As for the egg immunoglobulin titers there is significant ($p<0.05$) difference among the 2. (BC) **, the 3. (LU) ***, the 4. (LI) *group, compared to the first one.

The storage of carotenoid (BC, LU, LI), retinoid (ROL, RP) and tocopherol into the follicle becomes important at the F7 (ø=5 mm, 0.07g) sized follicles.

No significant difference can be drawn in the follicle immunoglobulin titer of the groups.

There is no close connection between the supplement (BC, LU, LI, BC+LU+LI and vitamin A) and the measured IgY titer. As opposed to that inside the serum and the yolk all carotenoids showed close connection with the immunoglobulin production, but there were also differences. So was the case with the 6. group (BC+LU+LI supplement) where there was a closer correlation compared to the other ones where only one type of carotenoid was fed to the animals.

Our experiment showed no close connection between vitamin E and carotenoids, however close correlation was detected between tocopherol and the retinoids. In the BC+LU+LI supplement receiving group the egg E vitamin concentration was significantly ($p<0.05$*) higher than that of the other groups.

4. NEW SCIENTIFIC RESULTS

1.
Int he ovarian follicles of with commercial hen fodder fed Japanese quails can be observed a significant positiv strong relationship between tocopherols and carotenoids (BC, LU) and retinol levels.

2.
On Japanese quials carried out carotenoid transport studies turn out, that the major carotenoid accumulation starting with the F7 follicle size (x=5 mm, 0,07-0,09 g), however dont be detected synchronism between the accumulation of IgY and carotenoids, which is explained with the different transport mechanisms.

3.
The accumulation of IgY into the follicle occurs in earlier time (3-4 days) compared to the start of carotenoid deposition into the follicle. The IgY titer of smaller follicles F10 (x = 0.05 g = 2 mm Ø) has been launched for a significant increase.

4.

The addition of natural xanthophylls extract to Japanese quail feed has effect on the level of follicle immunoglobulins. r_{LU}=0,6240 (ns), r_{BC}=0,6356 (p=0,0483*), r_{RP}=0,5203 (ns). The most powerful and significant correlation was observed for beta-carotene.

5.
In our experiment all the supplemented carotenoids (BC, LU, LI) are strong positive correlation with the production of immunoglobulin in the antigen BSA-gRBC immunised Japanese quail IgY immunoglobulin production. The most pronounced correlation was observed in the group BC +LU + LI supplemented.

5. CONCLUSION

5.1. The examination of IgY transport: record of initial status with commercial fodder. (1. experiment)

5.1.1. Examinations Carried out During the Follicle Development in Japanese Quails

The lipid transport into the follicles can only be processed with the help of the small, very low-density lipoprotein fraction (VLDLy) (Walzem and co, 1999) which is produced under estrogen effect in the liver only in the egg-laying period. “Y” stands for “yolk” indicating the very-low-density lipoprotein (VLDL) transport into the yolk. Though this transport is constant, the follicle growth, that is, the yolk bag penetrability enables to a certain extent a smaller, then a larger yolk storage.

Our carotenoid transport research with Japanese quails showed that a significant carotenoid, retinoid and tocopherol concentration increment (x=0.4g, ø=5 mm) can be detected in the F7 follicles.

The increment of vitamin E level and concentration is followed by a change of carotenoids and retinoids in the follicles, close connection (r>0.7) shows itself with normal commercial fodder feeding.

Since the IgY transport does not depend on the VLDLy transport there is no simultaneity in their detection within the follicles. According to our results the IgY titer already begins to grow significantly in smaller follicles (x=0.006g, ø=2 mm). Thus the significant immunoglobulin transport precedes the beginning of carotenoid deposition in the follicles by several days. Since it is known that the primordial follicle needs 15-17 days to develop into a ovulation-ready (F1) (Perry and co, 1983), the difference means 3-4 days.

The fact that the IgY titer measured in the F10 follicles practically remains the same indicates that the IgY deposition occurs simultaneously with the weight gain.

We experienced a more significant change in the amount than in the concentration increment of the carotenoids, retinoids and tocopherol.

These two facts exclude the mathematical justification of the correlation, which is biologically relevant due to the fact that the transport and the mediated follicle deposition of these two substances (lipoids and proteins) into the follicles are different.

5.1.2. Analysis of the embryo within the incubated chicken egg and examination of the chickling

During the incubation the yolk carotenoids (LU, BC), retinoids (ROL, RP) and vitamin E concentration is almost constant, the substances will be used gradually by the developing embryo. They are emptied from the yolk gradually and thus emerge in the embryo’s liver in a higher amount where they will be stored for their future function.

The not yet absorbed yolk of hatched birds shows an abrupt concentration increment, which provides useful source for the baby chicken. This concentration increment is due to the thickening of the yolk and not because of the substance deposition (Gregorits and co, 2009).

Thus IgY transport still takes place into the not yet absorbed yolk of the baby chicken. The IgY amount increases gradually in the yolk. The concentration tendency changes in the samples of the 19th day, the immunoglobulin level begins to decrease. Parallel to this the IgY level increases in the serum which is a sign of the antibody transport. In this way the antibodies infiltrate into the circulation and thus through passive immunity the baby chicken organism is capable of humoral reactions against antigens, for which it has a specific IgY reserve.

5.2. The effect of carotenoid supplement on the IgY transport

5.2.1. The effect of xanthophyll supplement (2. experiment)

We experienced a LU saturation in the eggs. This phenomenon – above 350 ppm no deposition is built into the yolk – was also experienced by Leason and co (2004). In our research the 1000 ppm Capsantal supplement – with its 82% LU content – represented a value of more than the double of that of the saturation.

Based on our researches we came to the conclusion that BC has a limiting effect on the LU accumulation in the egg, nevertheless it is to be noted that the supplement had no effect on the proportion of the BC-LU concentration: the LU concentration did not exceed that of the BC.

This can be explained by the fact that Capsantal is the natural extract of the Mexican Marigold (tagetes erecta), with a 1 % natural beta-carotene content (http://www.copharm.gr). It is evident that compared to the control value the 1000 ppm 1% content represents a significant increment. Besides it is to be excluded that in the presence of the polar xanthophyll it has a positive effect on the apolar BC storage, this can be effective at the membrane deposition for instance (Bárdos and co, 2011).

Our research showed a moderate increment in the humoral immune response in the lutein supplement receiving Japanese quails' serum and eggs because we measured higher IgY titers compared to the red blood cell-BSA-antigen complex. No significant follicle difference could be detected between the two groups.

According to Perez-Vendrell and co (2001) when applying the CIELab system measurements on the pectoral area the colour space's b* value can well indicate the xanthophyll presence in the fodder. Our experiment showed significant difference between the b* values in the case of Japanese quails. The average yolk colour values of the control group was in the 4-6 YCF range, whereas the xanthophyll supplement–fed group showed a range of 12-14 after the 2nd feeding week. This proves that the natural xanthophyll absorbs, metabolises and deposes well into the Japanese quail organs. The rpHPLC measurement shows that the beta-carotene and retinoid concentration in the yolk is higher in the lutein

supplement-fed group. No sources were found about this beta-carotene – lutein interaction. In human beta-carotene and lutein connection researches a conclusion was made that the common use of beta-carotene and lutein causes beta-carotene level decrement (van der Berg, 1999).

The present examinations proved that lutein which is the main source of oxycarotenoids extracted from the Mexican Marigold can not only paint the Japanese quail yolk and the skin surface yellow but can also improve the immune responsiveness.

5.2.2. The effect of carotenoid-free fodder mixed with lycopene, lutein, beta-carotene and vitamin A supplement (3. experiment)

Researches on the interactions in the hen carotenoids show that the higher amount of beta-carotene in the lutein zeaxanthin has an unfavourable effect on the accumulation of the other carotenoids (van der Berg, 1999, Wang, 2010).

With the use of the simple (group 2: rice-based fodder +BC) or combined (group 6: rice-based fodder+BC+LU+LI) supplement no BC was detected in the follicles, however, ROL and RP was present in higher concentration. It is to be assumed that with the use of carotene-free fodder the ß-carotene provitamin activity was more detectable, and ROL turns into RP during the deposition into the follicle, which provides the baby chicken with vitamin A during the incubation. But in the case of the serum and the yolk this phenomenon cannot be observed: BC can be detected in both group 2 and 6.

When analysing the connection between carotenoids and vitamin E we could detect a less close connection than with retinoids. The combined BC+LU+LI supplement results in the closest correlation in the examined carotenoids.

Our research shows the immunostimulative effect of carotenoids by pointing to the fact that the immunoglobulin titer was higher in the serum and yolk of the carotenoid free fodder with beta-carotene, lutein and lycopene supplement-fed group than that of the commercial fodder-fed one. When examining the immunoglobulin level of the follicles in the different groups we could point to the tendency that the normal commercial fodder-fed and the fodder with simple or combined carotenoid supplement-fed groups had a higher IgY titer than group 5 with carotenoid free fodder, the difference was not significant. We can settle that the deposition of carotenoid (BC, LU, LI), retinoid (ROL, RP) and tocopherol into the follicles becomes significant at the F7 follicle size (ø=5 mm, 0,07g).

A closer correlation can be shown in the connection between carotenoids and the immunoglobulin titer of the group which was fed with all 3 types of carotenoids (BC+LU+LI) than in the ones with simple supplements. It seems that the combined carotenoid supplement had a greater effect on the IgY production of the immunocompetent cells. Similar to our previous experiment there was no significant difference in the IgY titer of the groups, which shows a kind of regulation of the follicle deposition. This cannot be explained with the results of our past experiments. As we could see it from the results of the first experiment, there was no close correlation between the carotenoids and the immunoglobulin titers.

In general we can say that the immunisation efficiency is highly influenced by the proper breeding and feeding circumstances, the satisfactory health condition of the organism, in order to achieve and maintain this proper energy, that is, fodder supply, balanced antioxidant supply (carotenoids, vitamin A and E) is needed. Before immunisation the above should be well considered.

Other poultry species, hybrids are different in growth, which demands biological needs to be fulfilled. These are supported by the biological supplements (carotenoids, vitamins) in the fodder. Antigens have a constant effect on the organism. Protection against these demands substance and energy. Protection against immunisation (pathogens) demands more activity from the organism. In order to immunise efficiently we need to provide the organism with carotenoids, vitamin A and E before taking any action. The immunoresponsiveness of such organism is more efficient. In case the organism is attacked by an antigen through a pathogen, it can protect itself with a response reaction with the help of immunisation and can thus eliminate it.

10. PUBLICATIONS

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