Accepted Manuscript

Maternal effects and β-carotene assimilation in Canary chicks

Aurélie Tanvez, Mathieu Amy, Olivier Chastel, Gérard Leboucher

PII: S0031-9384(08)00349-1
DOI: doi: 10.1016/j.physbeh.2008.11.001
Reference: PHB 8697

To appear in: *Physiology & Behavior*

Received date: 12 November 2007
Revised date: 3 November 2008
Accepted date: 5 November 2008

Please cite this article as: Tanvez Aurélie, Amy Mathieu, Chastel Olivier, Leboucher Gérard, Maternal effects and β-carotene assimilation in Canary chicks, *Physiology & Behavior* (2008), doi: 10.1016/j.physbeh.2008.11.001

Maternal effects and β-carotene assimilation in Canary chicks

Aurélie Tanvez[1†], Mathieu Amy[1], Olivier Chastel[2] & Gérard Leboucher[1]

[1] Laboratoire d'Éthologie et Cognition Comparées, Université Paris X-Nanterre, BSL, 200 avenue de la république, 92001 Nanterre, France

[2] Centre d'Études Biologiques de Chizé, CNRS UPR 1934, 79360 Beauvoir sur Niort, France

[†] Corresponding author:

E-mail : aurelietanvez@gmail.com

Phone: +33 1 40 97 74 84

Fax: +33 1 40 97 74 74

Running head : Maternal effects and β-carotene.

ABSTRACT

TANVEZ, A. AMY, M. CHASTEL, O. AND LEBOUCHER, G. Maternal effects and β-Carotene assimilation in Canary chicks. PHYSIOL BEHAV. Carotenoids are pigments responsible for the red, orange and yellow coloration of plants and animals. They may be beneficial in two ways; they have a powerful antioxidant activity, and they can behave as an immunostimulant. Animals however cannot synthesize carotenoids *de novo*, they must obtain them through their diet. In our experiments on Canaries, we investigated how mothers transfer their dietary carotenoid-related benefits to their offspring; either through the egg, or through the diet (during chicks' feeding). Female Canaries were allowed to access β-carotene enriched food during egg formation and/or chicks feeding. We sorted the chicks into four groups using the period when they assimilated the *beta*-carotene as a variable. The four groups were: (i) before hatching (from yolk), (ii) after hatching (from maternal feeding), (iii) before and after hatching, or (iv) never. Colorimetry and HPLC analysis from sub-samples of yolks confirmed the maternal transfer of dietary carotenoids to the yolk. Our results show that benefits from maternal dietary carotenoids are transferred to the chicks, but according to the period when they are assimilated by the chicks, the physiological effects are different. It was found that the chicks growth was enhanced when carotenoids were assimilated both before and after hatching. However an increase in cellular immunity efficiency only occurs when the assimilation takes place after hatching.

Keywords : Maternal effects, carotenoids, β-carotene, cellular immunity, egg, *Serinus canaria*.

INTRODUCTION

It has been stated that maternal effects occur when the phenotype of a mother or the environment she experiences causes phenotypic effects in her offspring [1]. In birds, these maternal effects can be observed at different stages of the reproductive period. Hence, the choice of where to nest, when to nest, how much to invest in egg composition or in chicks rearing can have profound effects on future chicks' phenotype [2]. In the last decade, many research studies on maternal effects have focused on egg and moreover on yolk composition. Indeed, it has been shown that yolk contains maternal hormones [3] the concentration of which vary according to the maternal characteristics or maternal environment (see [4] for a revue on testosterone variation in yolk). More recently, studies have shown that yolk carotenoid variation is under the control of maternal nutritional environment [5, 6, 7].

Carotenoids are pigments responsible for the red, orange and yellow coloration of many plants, invertebrates, fishes, amphibians, reptiles, and birds [8]. Among others, a large number of secondary sexual ornaments are carotenoid-based signals [9]. They are of interest since animals can not synthesize them *de novo* [10], they must acquire them through their diet [8]. Besides their importance in animal signaling, these pigments are also powerful immunostimulant molecules [11, 12, 13]. This capacity has been experimentally demonstrated by several authors. For instance, when Zebra finches, *Taeniopygia guttata*, are given the opportunity to access carotenoid enriched food they show an improved cellular [14] and humoral immunity [15]. In European Blackbirds, *Turdus merula*, males with more orange beaks; coloration due to carotenoid assimilation; are the ones with better cellular immunity [16]. Finally, European Greenfinches, *Carduelis chloris*, with the brightest yellow breast feathers also present a higher cellular immunity [17]. Also males with a carotenoids enriched diet, have a stronger immune response than those with a non-enriched diet [18]. Moreover, carotenoids also have an antioxidant ability which can help to protect tissues against oxidative stress [19, 5]. Carotenoids are found in egg yolk [20], and since embryos and newly hatched are very sensitive to oxidative stress [21], it has been suggested that carotenoids may play a protection role during the early stages of the chicks' life [22]. The most antioxidant carotenoid is the β-carotene [23, 24, 19]. Nevertheless, carotenoids' antioxidant efficiency has been questioned by Hartley and Kennedy. They argue that carotenoids may be victims of oxidative stress, and they may only be good indicators of the level of oxidative stress in the tissues [25].

In females, carotenoids are deposited in the integument (i.e., beak, tarsus or feathers [8]), the plasma and also in the eggs [6]. Embryos and newly hatched chicks are very sensitive toward oxidative stress [21]; chicks hatching from eggs containing higher amounts of carotenoids could benefit from their antioxidant activity [24]. Furthermore, yolk contains maternal IgG which are also protected against catabolism by carotenoids [26]. So, in order to enhance their chicks survival, females should depose an optimal amount of carotenoids in their eggs, but carotenoids are scarce and the amount of carotenoid ingested is limited [27, 28]. Females need them for their somatic maintenance but they also have to deposit them into their egg yolk; consequently, the possibility of a trade-off between maternal fitness and the prospects for their offspring has been proposed [5, 7] . Studies of this yolk component are thus good candidates to evaluate maternal effects. Little is known about the mechanisms underlying the assimilation and utilization of carotenoids in embryos and newly hatched chicks, or the optimal time frame for the mothers to provide their chicks with these molecules.

In this experiment, we aimed to study two different periods when maternal effects can occur: during egg formation, and during chick rearing. We investigated whether carotenoid-related benefits can be inherited by offspring through the egg, or whether they must be acquired anew through the diet by each generation. To this purpose, we fed females with β-carotene enriched food (the most common carotenoid [29]) during egg formation and/or during chicks feeding. We then recorded developmental and physiological data from the chicks, including factors such as growth, immunity and survival. Our hypothesis is that the maternal nutritional environment can have an influence on chicks' development. We propose that females having access to β-carotene enriched food, will lay eggs containing higher amounts of carotenoids this will lead to more resistant chicks. The effect will be accentuated when access to the enriched diet is extended during the chicks' rearing.

METHODS

Subjects and housing conditions

Subjects

The subjects of this study were common domesticated male and female Canaries (*Serinus canaria*, 73 males and 73 females), hatched and bred in our laboratory. Before the experiment, they were housed in single-sexed aviaries in a short daylight photoperiod (8L : 16D). Four days before the beginning of the experiment, males and females were paired aimlessly and were housed in individual cages (38 X 33 X 26 cm). The pairs were then assigned randomly to the 4 experimental groups. The first day of the experiment, the photoperiod was switched to long days (16L : 8D). The cages had each been provided with a nest bowl and the Canary pairs were supplied with cotton string placed in a dispenser. Molting occurs mainly in photorefractory birds (when switching from long days to short days)[30, 31]. Indeed no molting was observed during our experiments, excluding any impact on carotenoid allocation in feathers. When laying was complete, the males were removed from the cage, and only the females incubated the eggs and fed the chicks. Canary is an altricial species where chicks remain in the nest for at least 15 days after hatching [32], permitting us to totally control the maternal nutritional environment of the chicks before fledging.

Diet

All birds were fed with the same regular diet with the exception of eggfood, which was supplemented or not with β-carotene (depending on the group). The diet consisted on seeds (mainly canary grass, *Phalaris canariensis*, and rape, *Brassica rapa*) and water *ad libitum*, CéDé eggfood (wheat flour, eggs, sugar, honey, hemp seed, niger seed, broken hulled oats, poppy seed, vitamins, minerals, amino acids: lysin - methionine, yeast) everyday, and fruits and vegetables twice a week.

For the β-carotene enriched diet, 10 mg of 10% β-carotene solution (Rovimix) was added to the daily 5 g of eggfood. We did not remove any carotenoids naturally present in the birds diet. So, the regular diet contained natural amounts of carotenoids and the enriched diet the same amounts plus the β-carotene supplement.

Experimental groups

Our experimental groups differed based on the period when the supplemented β-carotene diet was administrated. The feeding period was either Before hatching (B) or After hatching (A) and food quality was either β-carotene enriched ($_C$) or non β-carotene enriched ($_N$). So, according to the feeding period and to the food quality, we obtained 4 different experimental groups : B_CA_C (females accessing β-carotene enriched food Before and After chicks hatching), B_CA_N, B_NA_C and B_NA_N. Figure 1 describes the different feeding periods according to the experimental group.

For yolk composition analysis, only the mother's diet occurring prior to egg laying had to be taken into account. We pooled together data from groups which mother's diet was similar during egg formation. So, data from groups B_NA_N and B_NA_C were pooled into group B_N and the ones from groups B_CA_N and B_CA_C into group B_C.

A number of individuals were needed for each experimental group so it was not possible to conduct the experiment in a single session. Two separate sessions were conducted. Within each of them, we introduced a control group (group B_NA_N) in order to verify the homogeneity of the results. The first session was composed of experimental groups B_CA_N (n=*17*) and B_NA_N (n=*13*), and the second session of B_CA_C (n=*15*), B_NA_C (n=*15*) and B_NA_N (n=*13*) groups.

Chick growth and immune response

Daily measurement of the chicks' weight was made from the day they hatched until fledging (±0.01mg).

The cellular immune response was estimated on the day the chicks fledged using PHA-P method [33]. On day D_t, the wing web thickness was measured (± 0.01 mm) using a spessimeter (Mitutoyo). Then, 0.2 mg of *Phaseolus vulgaris* Phytohemagglutinin (PHA-P, Sigma) dissolved in 0.04 mL of Phosphate Buffer Saline (PBS, Sigma) was injected on the left wing, and 0.04 mL of PBS without PHA-P was injected in the right wing. Twenty four hours later (D_{t+1}) the thickness of these 2 wing webs were again measured. In order to estimate the cellular immune response, the Wing Web Index (WWI) was calculated according to the following formula :

$$WWI = (D_{t+1} - D_t)_{Thickness\ left\ wing} - (D_{t+1} - D_t)_{Thickness\ right\ wing}$$

Egg sampling and analysis

Sampling

Within each experimental group 8 pairs were not given the opportunity to raise chicks. Their eggs were collected the day they were laid and replaced by dummy eggs. They were weighed and immediately stored at -20°C. Before being assayed (by colorimetry or High Performance Liquid Chromatography method), the frozen eggs were thawed and the yolk and albumen were separated.

Colorimetry

Colorimetry is often used to estimate variation of carotenoid concentration; for instance, it has already been employed to estimate the coloration of birds' feathers [28], beak [6, 14], mouth [34], or tarsus [6].

Here, we used colorimetry in order to estimate the variation of yolk carotenoid concentration. After separation from the albumen, all the yolks were placed individually in ependorf tubes and homogenized.These tubes were scanned (Agfa SNAPSCAN 1212) and their color was computer-analyzed. The mean value between ten HST coordinates (Hue, Saturation and Tone) recorded from each yolk was considered for analysis.

High Performance Liquid Chromatography

17 yolk samples, 8 from mothers fed with regular food (B_NA_N and B_NA_C) and 9 from mothers fed with β-carotene enriched food (B_CA_N and B_CA_C) were assayed for β-carotene using High Performance Liquid Chromatography. These samples were also previously tested with the colorimetry assay.

Carotenoid extraction was performed according to the following method. An aliquot of yolk was diluted in 1 mL of NaCl solution (5%) and vortexed for 10 s, then 1 mL of ethanol was added and vortexed for 20 s, then 1 mL of hexane was added and homogeneized for 20 s. The solution was centrifuged for 5 min (3000rt/min) and the hexane phase was collected and the extraction was repeated another time. Hexane extracts were combined and evaporated under N_2. The dried extract was dissolved in 300 μL of methanol : dichloromethane 1:1 (v/v), then vortexed for 20 s and centrifuged for 5 min (13000rt/min). The supernatant was used for β-carotene determination.

β-carotene concentration was quantified using high performance liquid chromatography using a phase sep Nucleosil, 5mum C18 reverse phase column (25 cm X 0.3 cm) with a mobile phase of methanol, using detection by absorbency at 458 nm.

Statistics

Parametrical analyses were performed to analyze yolk composition (colorimetry), hatching probability, chick growth and chick immunity. Two Way ANOVAs for Repeated Measures were used to analyze yolk colorimetry data, Two Way ANOVA for chick growth and One way ANOVA was used for yolk composition (HPLC), hatching probability and chick immune response. All *Post hoc* analyzes were performed using Tukey tests.

When normality test failed, the data was log transformed (HPLC results). When log transformation failed, we used non-parametrical statistical tests. This proved to be the case for the chick survival data. We therefore performed a Kruskal-Wallis ANOVA on ranks.

All statistical analyses were computed using SigmaStat version 2.03 (SPSS Inc., Chicago, IL).

RESULTS

Yolk composition

Regarding colorimetry, for each of the 3 color components considered, statistical analysis show significant differences between B_C and B_N groups, but no effect of laying order. There was also no interaction between the two factors (two way RM ANOVAs results are presented on table 1). So, there is an effect of mothers' diet on yolk coloration: females eating a β-carotene enriched diet laid eggs with a lower hue, a higher saturation, and a lower tone than females provided with regular food.

HPLC performed on a sub-sample also showed an effect of mothers' diet on β-carotene yolk composition. Eggs laid by females on the β-carotene enriched diet contained significantly more β-carotene than eggs laid by females on the regular diet (One Way ANOVA, $F_{(1)}$=5.223, p=0.037, N=17).

Eggs hatchability

Hatchability was calculated as the ratio of the number of eggs hatched vs. the number of eggs laid for each female. Our results show no significant differences between the hatching probability of eggs laid by females of the B_C group (0.609±0.0983) compared to those of the females of the B_N group (0.636±0.0757), one Way ANOVA, $F_{(1)}$=0.0483, p=0.828, N=22.

Chick survival

Chick survival rates were obtained by calculating the ratio of the number of chicks fledged vs. the number of chicks hatched for each clutch. The mean survival rate was 0.867±0.0358. Kruskal-Wallis ANOVA on ranks was completed using the mean survival rate of each group. It showed no effect on the survival rate of any of the 4 experimental groups (p_{exact}=1, p_{est}=0.392, $H_{(3)}$=3, N=4).

Chick growth

We studied the evolution of the 117 chicks' weight during their first 20 days of life according to their experimental group. Two way ANOVA shows an effect based on the experimental group, as well as the chicks age. There is also a significant interaction between these two factors (group, $F_{(3)}$=84.740, p<0.001, age, $F_{(19)}$=655.107, p<0.001, interaction, $F_{(57)}$=1.501, p=0.010). Tukey *post hoc* analyses show significant differences between most of our experimental groups (B_CA_C vs B_CA_N, p<0.001; B_CA_C vs B_NA_N, p<0.001; B_CA_C vs B_NA_C, p<0.001; B_NA_C vs B_CA_N, p<0.001, B_NA_C vs B_NA_N, p<0.001; but B_NA_N vs B_CA_N, p=0.133). Significant results regarding the age effect and the interaction between the two factors, are presented in figure 2. These results show that chicks hatched from the B_CA_C group (females fed with β-carotene enriched food throughout the experiment) grow faster than chicks from the B_CA_N group (β-carotene supply before hatching) from days 7 to 19, and chicks from the B_NA_N group (regular food) from days 7 to 18. Differences between chicks from the B_CA_C group and B_NA_C group; and between the B_NA_C group and B_CA_N group occurs only sporadically. Thus, it seems that it is mostly the food's quality during rearing which is important for chicks' growth and that yolk carotenoids concentration has a very marginal effect.

Chick cellular immunity

At fledging, our results show significant differences on chicks' cellular immunity according to their experimental groups. One Way ANOVA, $F_{(3)}$=15.541, p<0.001, N=106. Tukey tests indicates that significant differences occurs between B_NA_C vs B_NA_N, B_NA_C vs B_CA_N, B_CA_C vs B_NA_N and B_CA_C vs B_CA_N (p<0.001 in all cases). Chicks from B_NA_C and B_CA_C groups have a higher immune response than chicks from groups B_NA_N and B_CA_N. These results are presented in figure 3. It shows that only the carotenoids' food quality provided by the mothers after hatching affects chicks' cellular immunity efficiency.

DISCUSSION

Our experiment was designed to study the effects of carotenoid maternal diet enrichment on egg composition and on chick development. Maternal transfer of carotenoids from the diet into the yolk has already been observed in species such as the lesser black-backed Gull, *Larus fuscus*. In this species, females fed with a diet enriched with various carotenoids (β-carotene, canthaxanthin, lutein and zeaxanthin) lay eggs with an overall higher carotenoid concentration [6, 35]. Recent studies have also demonstrated that maternal carotenoid diet affects deposition of carotenoids into tissues of growing nestlings in chicken [36] and that lutein acquired by diet affects immune response of growing chicks [37]. In our experiment, when the mothers have access to enriched-β-carotene food, their eggs present a lower hue (i.e. they are more orange than yellow), a higher saturation (i.e. they are more contrasted), and a higher tone (i.e. they are brighter) than the eggs laid by the females fed with the regular food. This color analysis cannot presume of which yolk component concentration has changed, however, as carotenoids are responsible for yellow, orange and red colors [8], we can suppose that the color modification observed can be related to an increase of carotenoid concentration. This statement is supported by previous studies using colorimetry methods to evaluate carotenoid concentration variations [28, 6, 14, 34]. We cannot however speculate which type of carotenoid has increased in the yolk. Indeed, Blount and co-workers have showed that an increase in certain carotenoid concentrations in a mother's diet does not provoke an increase of the same carotenoid within the yolk [6]. The authors suggested that this difference can be due to the mothers' own carotenoids metabolism. β-carotene is present in egg yolks of the common moorhen, *Gallinula chloropus*, the American coot, *Fulica americana*, and the black-backed Gull, *Larus fuscus* [38]. It has not however been found in the domestic hen eggs, *Gallus gallus* [39]. Our HPLC analysis confirm the presence of β-carotene in the Domesticated Canary yolk and that females supplemented with *beta*-carotene lay eggs more concentrated in this type of carotenoid. Thus, the transfer of *beta*-carotene from maternal diet to the eggs can be validated.

Carotenoids are powerful antioxidant [24] and immunostimulant molecules [11]. If mothers can transfer dietary carotenoids to their eggs, we can expect their newly hatched to undergo some benefit from this transfer. In Barn swallows, *Hirundo rustica*, a carotenoid inoculation into eggs (Lutein) results in better cellular immunity in 12-days-old chicks [40]. It was also showed that in grey Partridge, *Perdix perdix*, the eggs laid by females fed with supplemental β-carotene present a higher hatching rate [41]. In contrast with these previous results our experiment did not reveal any positive effect of *beta*-carotene supplementation on egg hatchability.

β-carotene supply also has a positive effect on chicks' cellular immunity. But this time, it seems that only the diet occurring after hatching is important since the only differences obtained are between the chicks fed with β-carotene after hatching and the ones fed with regular food whatever their eggs' composition. This result confirms that the immunostimulant effect of β-carotene already observed in adults [11, 12, 42, 14, 16] and in precocial chicks [43]. A study by [14] shows that Zebra finches chicks fed during the first 15 days of their life with an enriched diet (more proteins, carotenoids and E and A vitamins) have higher plasma antioxidant concentration when adults, indicating a probable long term effect of these molecules.

Similarly, neither egg carotenoid concentration nor carotenoid feeding quality after hatching affected chicks survival. In the present experiment, the chicks of all four experimental groups had similar survival rates. This lack of difference could be attributed to our experimental conditions: all our birds were raised in a laboratory and thus suffer from less bacterial and viruses infections than the ones in the field, perhaps the benefits from enhanced carotenoids could not be observed in such ideal conditions. Nevertheless, our survival rates (0.867±0.04) are very close to the ones observed in the wild species, the Island canary, *Serinus canaria* [44].

beta-carotene does however have a positive effect on chicks' growth. Our results indicate that from days 7 to 17, chicks hatched from high carotenoid concentration eggs and fed with β-carotene enriched food, show a faster growth than the ones fed with regular food after hatching, independent of the carotenoid contents of their eggs. However, chicks fed with supplemental β-carotene only after hatching (B_NA_C group), do not seem to show a regular better growth rate during the testing period than the other groups. Thus, when mothers lay eggs with more carotenoids, their chicks grow better, but it is only true if the β-carotene supply is continued after hatching. Chicks' mouth and gape color is a signal for parental care in Canaries and other precocious species [45, 46, 47, 48]. Parents tend to feed chicks with redder mouth more often [45, 34]. This coloration can be due to blood circulation [45] and to carotenoid coloration of the mouth integument [8, 34]. When chicks are fed with carotenoid enriched food their mouth gets brighter [34]. A possible explanation for our chicks' growth enhancement could be due to an increase in maternal care consequent to the brighter mouth coloration of their chicks. It could also explain why their growth rates are always higher than those of the other groups (even if not significant during the first few days). We can hypothesize that these chicks with the fastest growth will be favored later on, since heavier chicks in the nest present a better long term survival rate [49].

To conclude, our results show a positive influence of β-carotene on several aspects of a chicks' physiology, such as growth and cellular immunity when it is assimilated after hatching but none of them show a clear effect of higher yolk carotenoid levels.

Acknowledgments

We would like to thank Myrl Venter and two anonymous referees for their helpful comments on an earlier version of the manuscript, Francesco Logozzo for his colorimetry analysis software, André Lacroix and Sabrina Henaud for the HPLC, Fanny Maddalena for her help during the first experiment, and Colette Trouvé who cared for the birds.

References

1. Mousseau, T.A.; Fox, C.W. Maternal effects as adaptations. New York: Oxford University Press, 1998.

2. Price, T. Maternal and paternal effects in birds. Effects on offspring fitness. In: Maternal effects as adaptations, T.A. Mousseau; C.W. Fox, editors, New York: Oxford University Press. 1998. 202–226.

3. Schwabl, H. Yolk is a source of maternal testosterone for developing birds. Proc Natl Acad Sci U S A 1993. 90:11446–11450.

4. Gil, D. Golden eggs: maternal manipulation of offspring phenotype by egg androgen in birds. Ardeola 2003. 50:281–294.

5. Blount, J.D.; Houston, D.C.; Møller, A.P. Why egg yolk is yellow. Trends Ecol Evol 2000. 15:47–49.

6. Blount, J.D.; Surai, P.F.; Nager, R.G.; Houston, D.C.; Moller, A.P.; Trewby, M.L.; Kennedy, M.W. Carotenoids and egg quality in the lesser blackbacked gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc Biol Sci 2002. 269:29–36.

7. Blount, J.D. Carotenoids and life-history evolution in animals. Arch Biochem Biophys 2004. 430:10–15.

8. Godwin, T.W. The biochemistry of carotenoids. Volume 2, Animals, 2nd edn. New York: Chapman & Hall, 1984.

9. Griffith, S.C.; Parker, T.H.; Olson, V.A. Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? Anim Behav 2006. 71:749–763.

10. Godwin, T.W. The biochemistry of carotenoids. Volume 1, plants, 2nd edn. New York: Chapman & Hall, 1980.

11. Lozano, G.A. Carotenoids, parasites, and sexual selection. Oikos 1994. 70:309–311.

12. Lozano, G.A. Carotenoids, immunity, and sexual selection: comparing apples and oranges. Am Nat 2001. 158:200–203.

13. Fenoglio, S.; Cucco, M.; Malacarne, G. The effect of a carotenoid-rich diet on immunocompetence and behavioural performance in Moorhen chicks. Ethol Ecol Evol 2002. 14:149–156.

14. Blount, J.D.; Metcalfe, N.B.; Birkhead, T.R.; Surai, P.F. Carotenoid modulation of immune function and sexual attractiveness in Zebra finches. Science 2003. 300:125–127.

15. McGraw, K.J.; Ardia, D.R. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 2003. 162:704–712.

16. Faivre, B.; Préault, M.; Salvadori, F.; Théry, M.; Gaillard, M.; Cézilly, F. Bill colour and immunocompetence in the European blackbird. Anim Behav 2003. 65:1125–1131.

17. Saks, L.; Ots, I.; Horak, P. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. Oecologia 2003. 134:301–307.

18. Aguilera, E.; Amat, J.A. Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). Naturwissenschaften 2007. in press.

19. von Schantz, T.; Bensch, S.; Grahn, M.; Hasselquist, D.; Wittzell, H. Good genes, oxidative stress and condition-dependant sexual signals. Proc Biol Sci 1999. 266:1–12.

20. Royle, N.J.; Surai, P.F.; McCartney, R.J.; Speake, B.K. Parental investment and egg yolk lipid composition in gulls. Funct Ecol 1999. 13:298–306.

21. Surai, P.F.; Noble, R.C.; Speake, B.K. Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidantion during development of the chick embryo. Biochim Biophys Acta 1996. 1304:1–10.

22. Royle, N.J.; Surai, P.F.; Hartley, I.R. Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects. Behav Ecol 2000. 12:381–385.

23. Burton, G.W.; Ingold, K.U. Beta-carotene: an unusual type of lipid antioxidant. Science 1984. 11:569–573.

24. Surai, P.F.; Speake, B.K. Distribution of carotenoids from the yolk to the tissues of the chick embryo. J Nutr Biochem 1998. 9:645–651.

25. Hartley, R.C.; Kennedy, M.W. Are carotenoids a red herring in sexual display? Trends Ecol Evol 2004. 19:353–354.

26. Haq, A.U.; Bailey, C.A.; Chinnah, A. Effect of beta-Carotene, Canthaxanthin, Lutein and Vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. Poult Sci 1996. 75:1092–1097.

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

28. Hill, G.E. A red bird in a brown bag. The function and evolution of colourful plumage in the House Finch. New York: Oxford University press, 2002.

29. Nys, Y. Dietary carotenoids and egg yolk coloration. Arch Geflügelk 2000:45–54.

30. Pohl, H. Entrainment properties of the circadian system changing with reproductive state and molt in the canary. Physiol Behav 1994. 55:803–810.

31. Dawson, A. Control of molt in birds: Association with prolactin and gonadal regression in starlings. Gen Comp Endocr 2006. 147:314–322.

32. Pomarède, M. Le canari. Précis de canariculture. Maisons-Alfort: Editions du Point Vétérinaire, 2nd edition ed., 1992.

33. Hoi-Leitner, M.; Romero-Pujante, M.; Hoi, H.; Pavlova, A. Food availability and immune capacity in serin (*Serinus canaria*) nestlings. Behav Ecol Sociobiol 2001. 49:333–339.

34. Saino, N.; Ninni, P.; Calza, S.; Martinelli, R.; De Bernardi, F.; Møller, A.P. Better red than dead: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. Proc Biol Sci 2000. 267:57–61.

35. Blount, J.D.; Surai, P.F.; Houston, D.C.; Møller, A.P. Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. Functional Ecology 2002. 16:445–453.

36. Koutsos, E.A.; Clifford, A.J.; Calvert, C.; Klasing, K.C. Maternal Carotenoid Status Modifies the Incorporation of Dietary Carotenoids into Immune Tissues of Growing Chickens (*Gallus gallus domesticus*). J Nutr 2003. 133:1132–1138.

37. Koutsos, E.A.; Garcia-Lopez, J.C.; Klasing, K.C. Carotenoids from In Ovo or Dietary Sources Blunt Systemic Indices of the Inflammatory Response in Growing Chicks (*Gallus gallus domesticus*). J Nutr 2006. 136:1127–1131.

38. Surai, P.F.; Speake, B.K.; Wood, N.A.; Blount, J.D.; Bortolotti, G.R.; Sparks, N.H. Carotenoid discrimination by the avian embryo: a lesson from wild birds. Comp Biochem Physiol B Biochem Mol Biol 2001. 128B:743–750.

39. Hencken, H. Chemical and physilogical behavior of feed carotenoids and their effects on pigmentation. Poult Sci 1992. 71:711–717.

40. Saino, N.; Ferrari, R.; Romano, M.; Martinelli, R.; Møller, A.P. Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. Proc. R. Soc. Lond. B 2003. 270:2485–2489.

41. Cucco, M.; Guasco, B.; Malacarne, G.; Ottonelli, R. Effects of ß-carotene on adult immune condition and antibacterial activity in the eggs of the Grey Partridge, *Perdix perdix*. Comp Biochem Physiol A Mol Integr Physiol 2007. 147:1038–1046.

42. Olson, V.A.; Owens, I.P.F. Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol 1998. 13:510–514.

43. Cucco, M.; Guasco, B.; Malacarne, G.; Ottonelli, R. Effects of ß-carotene supplementation on chick growth, immune status and behaviour in the Grey Partridge, *Perdix perdix*. Behav Process 2006. 73:325–332.

44. Voigt, C.; Leitner, S. Breeding biology of the island canary *Serinus canaria* (aves: fringillidae) on the desertas island Ilhéu Chao. Boletin do museu municipal di Funchal (Historia Natural) 1998. 50:117–124.

45. Kilner, R. Mouth color is a reliable signal of need in begging canary nestling. Proc Biol Sci 1997. 264:963–968.

46. Kilner, R.; Davies, N.B. Nestling mouth colour ecological correlates of a begging signal. Anim Behav 1998. 56:705–712.

47. Kilner, R. Family conflicts and the evolution of nestling mouth colour. Behaviour 1999. 136:779–804.

48. Kilner, R. The evolution of complex begging displays. In: The evolution of begging. Competition, cooperation and communication, J. Wright; M.L. Leonard, editors, Dordecht, Netherlands: Kluwer Academic Publishers. 2002. 87–106.

49. Magrath, R.D. Nestling weight and juvenile survival in the Blackbird, *Turdus merula*. J Anim Ecol 1991. 60:335–351.

1 # Figures

Table 1: Yolk colorimetry results (two way RM ANOVAs and Tukey tests for *Post hoc* analyses, N=108)

	B_C m±se	B_N m±se	Experimental Group	Laying order	B_C vs B_N
Hue	44.602±1.053	53.156±0.371	$F_{(1)}$=49.693, p<0.001	$F_{(6)}$=0.749, p=0.612	p<0.001
Saturation	76.391±1.220	62.293±1.721	$F_{(1)}$=44.896, p<0.001	$F_{(6)}$=0.916, p=0.487	p<0.001
Tone	77.286±0.442	80.597±0.395	$F_{(1)}$=33.523, p<0.001	$F_{(6)}$=1.643, p=0.143	p=0.001

Figure captions

Figure 1:

Chronological representation of the feeding periods. The first line represents the experimental periods. Lines 2-5 represent the variations of the diets according to the experimental group, each line corresponding to an experimental group. Plain bars represent the period when pairs were giving access to $\beta-$carotene enriched food. Grey bars represent the period when birds were fed with regular diet. n indicates the number of pairs of each group.

Figure 2:

Chicks weight depending on their age (means±S.E.M.). Significant differences (Tukey test, $p<0.05$) between groups B_CA_C and B_NA_C, B_CA_C and B_NA_N, B_CA_C and B_CA_N are indicated on the figure, * represents a significant difference between groups B_NA_C and B_CA_N.

Figure 3:

Chicks responses to PHA-P depending on their experimental group (means+SEM).

* indicates a significant difference (Tukey test, $p<0.05$).

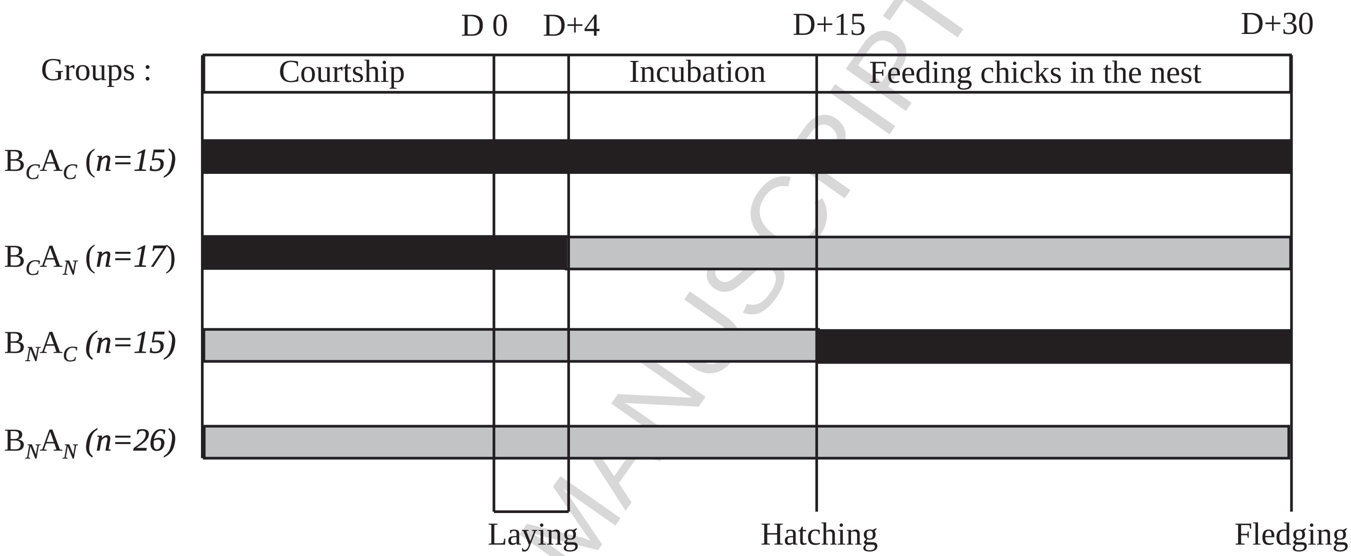
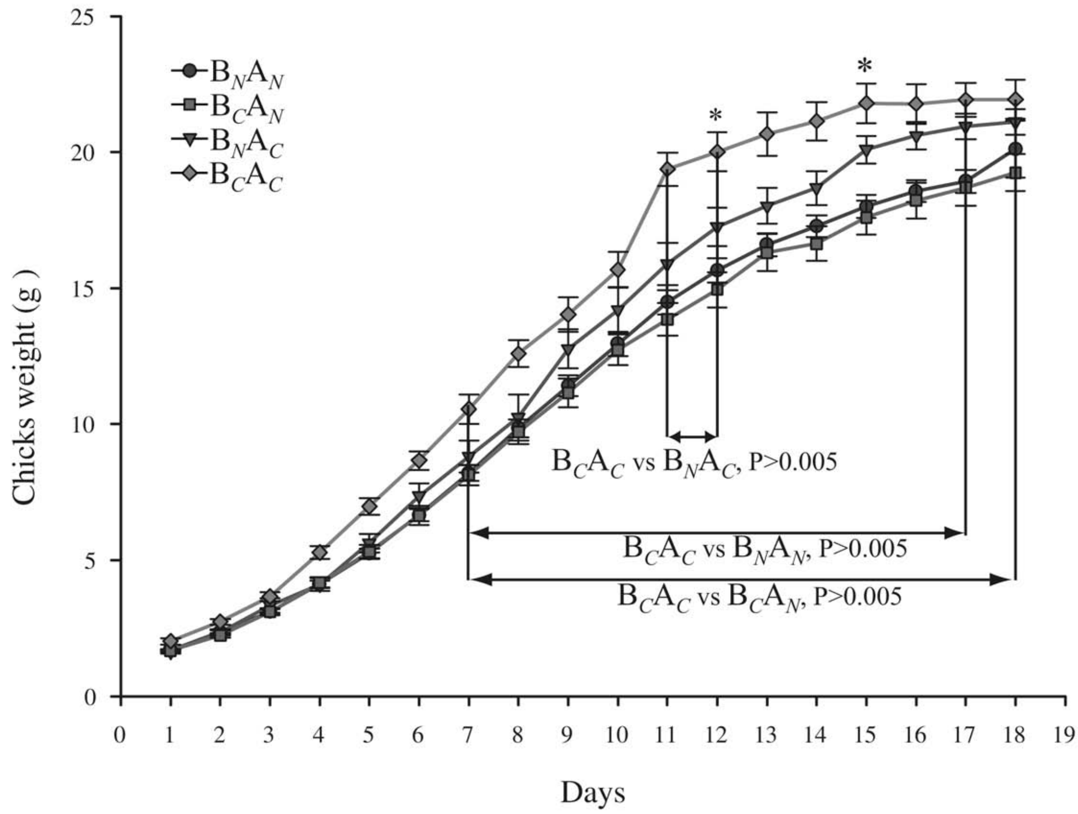

D 0
D+4
D+15
D+30
Groups :
Courtship
Incubation
Feeding chicks in the nest
B_CA_C (n=15)
B_CA_N (n=17)
B_NA_C (n=15)
B_NA_N (n=26)
Laying
Hatching
Fledging

Figure 1:



Chicks weight (g)
Days
B_NA_N
B_CA_N
B_NA_C
B_CA_C
*
*
B_CA_C vs B_NA_C, P>0.005
B_CA_C vs B_NA_N, P>0.005
B_CA_C vs B_CA_N, P>0.005
0
5
10
15
20
25
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 2:

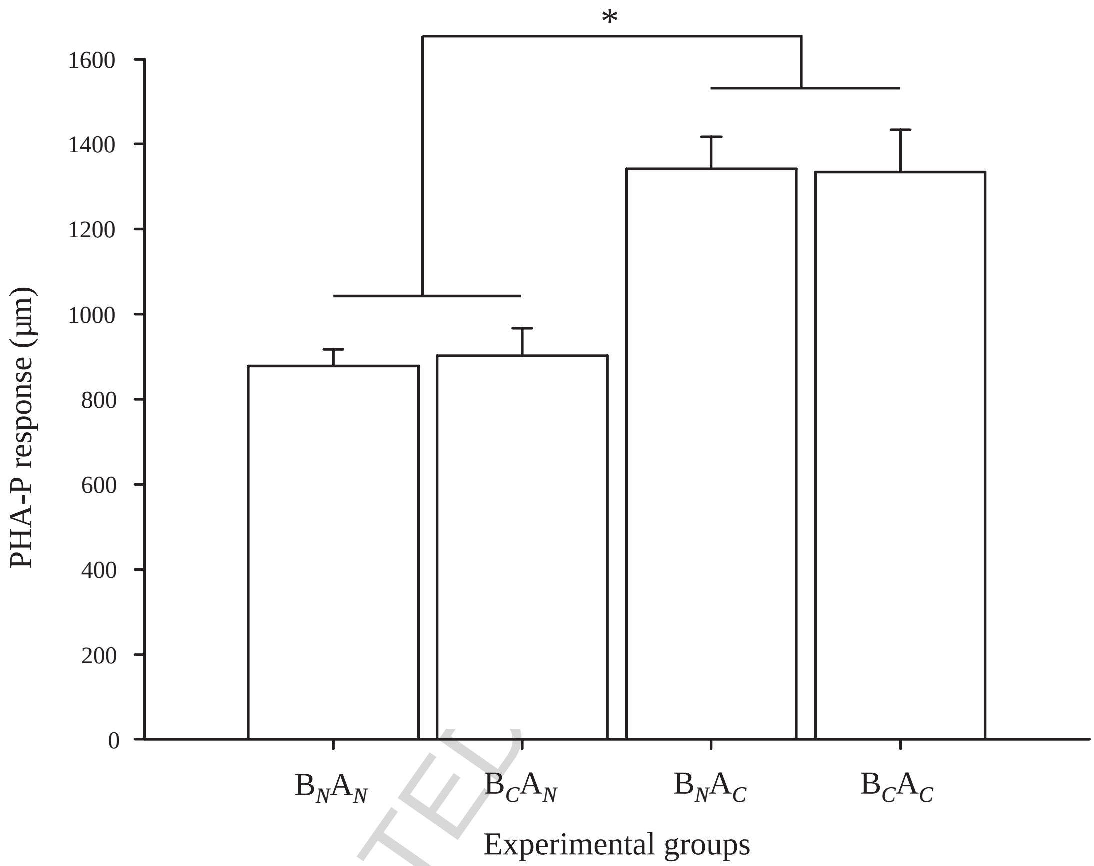

*
1600
1400
1200
1000
800
600
400
200
0
PHA-P response (µm)
BNAN
BCAN
BNAC
BCAC
Experimental groups

Figure 3: