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RESEARCH ARTICLE

Differential effects of specific carotenoids on oxidative damage and immune response of gull chicks

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ABSTRACT

Micronutrients are essential for normal metabolic processes during early development. Specifically, it has been suggested that diet-derived carotenoids can play a key role in physiological functions because of their antioxidant and immunostimulant properties. However, their role as antioxidants remains controversial. Additionally, it is also unclear whether oxidative stress mediates their immunostimulatory effects. In this field study, we separately supplemented yellow-legged gull (*Larus michahellis*) chicks with two carotenoids (lutein and β-carotene) with different molecular structures and different transformation pathways into other oxidative forms of carotenoids. We quantified their effect on the oxidative status and the immune response of chicks before and after an oxidative challenge with paraquat, a pro-oxidant molecule. Prior to oxidative challenge, none of the carotenoid treatments affected the oxidative status of chicks, but they enhanced the inflammatory response to an antigen compared with controls. The oxidative challenge enhanced plasma vitamin E levels (but not in β-carotene-supplemented chicks) and the antioxidant capacity in the short term. Interestingly, lutein-supplemented chicks showed lower oxidative damage to proteins than non-lutein-supplemented chicks. After the oxidative challenge, the positive effect of carotenoid supplementation on the immune response disappeared. Thus, these results suggest differential effects of two carotenoids with different molecular structures on the oxidative status. Lutein but not β-carotene helps to combat oxidative damage after a free-radical exposure. Additionally, the results indicate that the immunostimulatory effects of carotenoids are linked to oxidative status during early life.

KEY WORDS: Antioxidants, Beta-carotene, Early development, Inflammatory response, *Larus michahellis*, Lutein

INTRODUCTION

In animals, early development is a life stage characterized by elevated energy requirements. Malnutrition during this stage may permanently alter the adult phenotype (reviewed in Monaghan, 2008). Recent evidence suggests that, in addition to macronutrients, small amounts of certain non-energetic micronutrients are essential for normal metabolic and developmental processes (Ames, 2006; Christian and Stewart, 2010; Senar et al., 2010). Micronutrients, such as essential minerals, vitamin E and carotenoids, cannot be synthesized *de novo* by vertebrates, and must be obtained through the diet (Evans and Halliwell, 2001; Surai, 2002). Deficiencies in dietary micronutrients have been linked to an increased risk of many diseases (Ames, 2006; Christian and Stewart, 2010; Isaksson et al., 2011). During early life, variation in access to micronutrients or how they are allocated can have future implications for an organism's fitness (Evans and Halliwell, 2001; Ames, 2006; Catoni et al., 2008).

Carotenoids are micronutrients that are thought to play key physiological functions during early life, due to their immunostimulant and antioxidant properties (Bendich, 1989; Krinsky, 1993; Lozano, 1994; Møller et al., 2001; Surai, 2002). Diet-derived antioxidants are particularly important during early development because this life stage is characterized by high production of reactive oxygen species (ROS) due to elevated metabolic rate (reviewed by Monaghan et al., 2009; Metcalfe and Alonso-Álvarez, 2010). Thus, it has been suggested that carotenoids provided by parents to offspring could help to reduce oxidative stress (i.e. the imbalance that occurs when antioxidant defences cannot fully neutralize ROS) (Halliwell and Gutteridge, 2007) during early stages of offspring life (reviewed by Møller et al., 2001; Surai, 2002; Blount, 2004; Catoni et al., 2008; Metcalfe and Alonso-Álvarez, 2010; Alonso-Álvarez and Velando, 2012). However, in the past few years, the role of carotenoids as antioxidants has been questioned, especially in birds (Costantini and Møller, 2008; Cohen and McGraw, 2009).

The antioxidant role of carotenoids during early life has been extensively studied in birds, with experimental evidence either supporting it (Woodall et al., 1996; Surai and Speake, 1998; Blount et al., 2002a; Blount et al., 2002b) or not (e.g. Costantini et al., 2007; Pérez-Rodriguez et al., 2008; Larcombe et al., 2010). These contradictory results might be explained by different analytical approaches (reviewed in Pérez-Rodríguez, 2009; Monaghan et al., 2009; Hõrak and Cohen, 2010), but also because specific carotenoids may have different properties. On the one hand, carotenoids differ in their antioxidant potential according to their molecular structure. Thus, carotenes show higher antioxidant capacity *in vitro* than xanthophylls (Krinsky, 1993; Rice-Evans et al., 1997). Nevertheless, *in vivo*, the position and orientation of some xanthophylls (such as lutein and zeaxanthin) in the bilayer membrane is probably more adequate to protect membranes against oxidation than the carotenes' location (Britton, 1995; Woodall et al., 1997; Surai, 2002). On the other hand, specific carotenoids have different transformation pathways in other oxidative forms (reviewed by Møller et al., 2001), which may affect their antioxidant potential. Thus, the antioxidant function of carotenoids should be explored separately in carotenoids with different molecular structures and different routes of transformation. To date, there is limited information available on the comparative antioxidant or immunostimulant role of different types of carotenoids during early life (but see Woodall et al., 1996; Fitze et al., 2007).

Early life represents the most important period for immune system development, and carotenoids may play a key role in this

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List of abbreviations

ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate)
BMR	basal metabolic rate
DNPH	2,4-dinitrophenylhydrazine
HPLC	high-performance liquid chromatography
LSD	least significant difference
MDA	malondialdehyde
PHA	phytohemagglutinin
PQ	paraquat
ROM	reactive oxygen metabolite
ROS	reactive oxygen species

process (Bendich, 1989; Surai et al., 2001). In young birds, supplementation with different types of carotenoids has also yielded contradictory results on the immune response [a positive effect (Fenoglio et al., 2002; Saino et al., 2003; Cucco et al., 2006; Fitze et al., 2007); or no effect (Biard et al., 2006; Saino et al., 2008; Fitze et al., 2007)]. It has been suggested that the immunostimulatory effect of carotenoids could be mediated by their role as antioxidants (Bendich, 1989; Møller et al., 2001; Chew and Park, 2004). Carotenoids might alleviate the negative effects of large amounts of free radicals produced by some immune cells (such as macrophages and heterophils) in order to kill pathogens (Hampton et al., 1998; Sorci and Faivre, 2009). Indeed, mounting an effective cell-mediated immune response entails increased oxidative stress in nestlings (reviewed by Costantini and Møller, 2009; Sorci and Faivre, 2009). One way to test whether oxidative stress mediates the immunostimulatory effect of carotenoids is by simultaneously manipulating carotenoid availability and oxidative stress [e.g. by administration of pro-oxidant compounds such as paraquat (PQ) or diquat (see Isaksson and Andersson, 2008; Hõrak et al., 2010; Alonso-Álvarez and Galván, 2011)].

In the present study, we explored whether two carotenoids with different molecular structure play an antioxidant and immunostimulatory role during early life in yellow-legged gull (*Larus michahellis* Naumann 1840) chicks under free-living conditions. In this species, the first 2 weeks of age represent a life stage with high levels of oxidative stress [e.g. the study population (see Kim et al., 2011; Noguera et al., 2011)]. We manipulated the dietary availability of two carotenoid compounds and quantified their effect on oxidative status and the immune response of chicks before and after an oxidative challenge (Fig. 1). Chicks were supplemented with either lutein or β-carotene, which can be naturally acquired by animals from food (Goodwin, 1984) and are present in the diet of yellow-legged gull chicks (Czeczuga et al., 2000; Naczk et al., 2004; Moreno et al., 2010). Subsequently, we manipulated the oxidative status of chicks by oral administration of a low single dose of PQ, a pro-oxidant that generates ROS (see Bus and Gibson, 1984; Suntres, 2002). We quantified the effect of carotenoid supplementation on plasma antioxidant capacity, vitamin E and reactive oxygen metabolite (ROM) levels, as well as oxidative damage to lipids [malondialdehyde (MDA)] and proteins (carbonyls) before and after the oxidative challenge. Additionally, we induced an inflammatory immune response to phytohaemagglutinin (PHA) before and after the oxidative challenge with PQ.

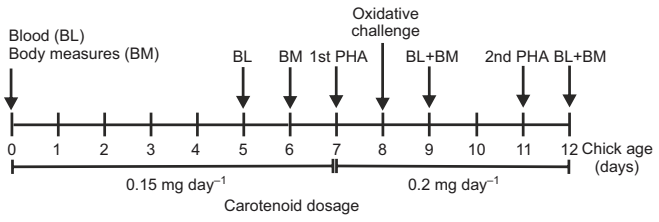

Fig. 1. Outline of the experimental design from hatching (day 0) to 12 days of age. BM: body measures (mass and tarsus length). BL: blood samples collected for biochemical assays [plasma lutein, β-carotene, vitamin E, plasma total antioxidant capacity, reactive oxygen species (ROS), malondialdehyde (MDA) and carbonyl). PHA: inflammatory response to phytohaemagglutinin. Carotenoid dosage (lutein and β-carotene): 0.15 mg day^{-1} from hatching to 6 days of age, and 0.2 mg day^{-1} from 7 to 12 days of age.

If carotenoids are used as antioxidant compounds in gull chicks, we predicted that carotenoid supplementation would ameliorate oxidative damage, especially after the oxidative challenge. Furthermore, carotenoid-supplemented chicks would mount stronger cell-mediated immune responses to PHA than non-supplemented chicks (Chew and Park, 2004), although after the challenge these effects would be probably attenuated if chicks prioritize carotenoids for facing the oxidative challenge over mounting an inflammatory response.

RESULTS

Effects of carotenoid treatment before PQ administration

At 5 days of age, carotenoid supplementation had a significant effect on plasma levels of lutein and β-carotene (Table 1). Thus, chicks supplemented with lutein had on average a 54% higher lutein concentration in plasma (0.70±0.03 μg ml^{-1}) than chicks not supplemented with lutein (no carotenoid chicks: 0.33±0.02 μg ml^{-1}; β-carotene chicks: 0.34±0.03 μg ml^{-1}). Lutein levels at 5 days of age were positively correlated with lutein levels at hatching (Table 1). Chicks supplemented with β-carotene showed on average a 90% higher β-carotene concentration (0.282±0.02 μg ml^{-1}) in plasma than chicks not supplemented with β-carotene (no carotenoid chicks: 0.07±0.02 μg ml^{-1}; lutein chicks: 0.07±0.02 μg ml^{-1}). The effects of sex, hatching date, brood size and the interaction between treatment and sex on lutein or β-carotene plasma levels were not statistically significant (supplementary material Table S1).

Carotenoid supplementation did not significantly affect the plasma concentration of vitamin E, total antioxidant capacity, ROMs, MDA or carbonyl group at 5 days of age (supplementary material Table S1). Chicks hatched earlier showed lower antioxidant capacity and higher plasma levels of carbonyl groups than chicks hatched later (Table 1). Age, sex, brood size, hatching date, MDA at hatching and the interaction between treatment and sex did not significantly affect oxidative stress markers in plasma (supplementary material Table S1).

Body mass and tarsus length at 6 days of age were not significantly affected by carotenoid supplementation (supplementary material Table S1), and were positively correlated with body mass and tarsus length at hatching (Table 1). Sex, brood size, hatching date and the interaction between treatment and sex did not significantly affect chick body mass or tarsus length (supplementary material Table S1).

The inflammatory immune response to PHA at 8 days of age was significantly affected by carotenoid supplementation (Table 1). Thus, the cellular immune response in lutein- and β-carotene-supplemented chicks was 42.12% and 56.43% higher, respectively, than unsupplemented chicks (Fig. 2). Lutein- and β-carotene-supplemented chicks did not significantly differ in their PHA response (LSD *post hoc* test, P=0.166). Brood size, sex, body mass at hatching, hatching date and the interaction between treatment and sex did not significantly affect the PHA response (supplementary material Table S1).

Until 8 days of age, 13 lutein-supplemented chicks (i.e. 43.3% of the initial sample size of this experimental group), 17 β-carotene-

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Table 1. Summary of minimum adequate general linear models of the effect of carotenoid supplementation on plasma lutein, β-carotene, total antioxidant capacity, carbonyl group, body mass, tarsus length and inflammatory immune response to PHA of yellow-legged gull chicks before paraquat administration

Dependent variable	Source of variation		d.f.	Estimate ± s.e.m.	*F*	*P*
Lutein (μg ml^{-1})	Intercept			0.61±0.05		
	Treatment	β-carotene	2,75	–0.37±0.04	55.72	<0.001
		No carotenoid		–0.38±0.04		
	Lutein at hatching		1,75	0.18±0.07	6.92	0.010
β-carotene (μg ml^{-1})	Intercept			0.07±0.02		
	Treatment	β-carotene	2,80	0.21±0.03	39.30	<0.001
		No carotenoid		–0.01±0.03		
Total antioxidant capacity (mmol Trolox equivalents l^{-1})	Intercept			–9.98±4.07		
	Hatching date		1,64	4.73±1.85	6.54	0.013
Protein damage (carbonyl) (nmol ml^{-1})	Intercept			14.37±6.59		
	Hatching date		1,71	–6.28±3.00	4.39	0.040
Body mass (g)	Intercept			19.46±29.94		
	Body mass at hatching		1,72	1.22±0.52	5.61	0.021
Tarsus length (mm)	Intercept			2.93±5.65		
	Tarsus length at hatching		1,72	1.07±0.22	23.34	<0.001
Inflammatory immune response to PHA (mm)	Intercept			0.54±0.09		
	Treatment	β-carotene	2,53	0.18±0.13	7.35	0.002
		No carotenoid		–0.23±0.12		

supplemented chicks (56.6%) and 26 no-carotenoid chicks (43.3%) survived, but these differences in survival were not significant (Wald's χ^2=1.39, d.f.=2, P=0.498). Sex (Wald's χ^2=0.38, d.f.=1, P=0.539), hatching date (Wald's χ^2=0.03, d.f.=1, P=0.857), brood size (Wald's χ^2=2.11, d.f.=1, P=0.146), body mass at hatching (Wald's χ^2=0.21, d.f.=1, P=0.647) and the interaction between treatment and sex (Wald's χ^2=5.14, d.f.=2, P=0.076) had no significant effect on chick survival.

Effects of carotenoid treatment after PQ administration

The effect of lutein and β-carotene supplementation on the plasma levels of, respectively, lutein (no carotenoid–no PQ chicks: 0.12±0.04 μg ml^{-1}; no carotenoid+PQ chicks: 0.20±0.04 μg ml^{-1}; lutein+PQ chicks: 0.66±0.04 μg ml^{-1}; β-carotene+PQ chicks: 0.18±0.04 μg ml^{-1}) and β-carotene (no carotenoid–no PQ chicks: 0.07±0.03 μg ml^{-1}; no carotenoid+PQ chicks: 0.11±0.03 μg ml^{-1}; lutein+PQ chicks: 0.09±0.03 μg ml^{-1}; β-carotene+PQ chicks: 0.25±0.03 μg ml^{-1}) remained statistically significant after PQ administration (Table 2).

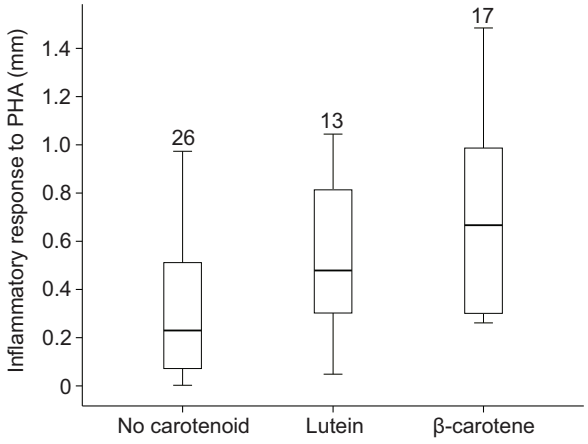

Fig. 2. Effect of carotenoid treatment (lutein or β-carotene) on the inflammatory immune response to PHA in yellow-legged gull chicks at 8 days of age (prior to paraquat administration). Values show the mean (horizontal bar), upper and lower quartiles (upper and lower edges of box) and maximum and minimum values (whiskers). Numbers indicate sample size.

Vitamin E levels decreased from 9 to 12 days of age and differed among experimental groups (Table 2, Fig. 3A). Lutein+PQ chicks showed higher vitamin E levels than no carotenoid–no PQ and β-carotene+PQ chicks (LSD test, P<0.02 in both cases), but levels similar to those of no carotenoid+PQ chicks (LSD test, P=0.22). Other variables did not significantly affect vitamin E levels (supplementary material Table S2). The effect of treatment on total plasma antioxidant capacity differed according to chick age (Table 2, Fig. 3B). At 9 days of age, lutein+PQ chicks showed higher antioxidant capacity than no carotenoid–no PQ chicks (LSD test, P=0.029) and β-carotene+PQ chicks (LSD test, P=0.046), but similar capacity to that of no carotenoid+PQ chicks (LSD test, P=0.189). This effect was not evident at 12 days of age (P>0.057 in all pairwise comparisons). Other variables or interactions did not significantly affect plasma antioxidant capacity (supplementary material Table S2).

Plasma levels of ROMs and MDA did not significantly differ among experimental groups and were not significantly affected by other variables (supplementary material Table S2). Carbonyl plasma levels differed significantly among experimental groups after PQ administration (Table 2). Interestingly, lutein+PQ chicks showed lower plasma levels of carbonyls than other chicks (LSD test, P<0.011 in all pairwise comparisons; Fig. 4). Other variables did not significantly affect plasma carbonyl levels (supplementary material Table S2).

After PQ administration, experimental groups did not significantly differ in either body mass or tarsus length (supplementary material Table S2). Body mass and tarsus length were positively correlated with body mass and tarsus length at hatching, and earlier-hatched chicks had higher body mass than late-hatched chicks (Table 2). Other variables did not significantly affect body mass or tarsus length (supplementary material Table S2).

At 12 days of age, experimental groups did not statistically differ in their response to the second PHA injection (supplementary material Table S3). Sex, hatching date, body mass at hatching and brood size did not significantly affect the inflammatory response to PHA at 12 days of age (supplementary material Table S3).

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Table 2. Summary of minimum adequate mixed models of the effect of carotenoid supplementation on plasma lutein, β-carotene, vitamin E, total antioxidant capacity, carbonyl group, body mass and tarsus length of yellow-legged gull chicks after paraquat (PQ) administration (at 9 and 12 days of age)

Dependent variable	Source of variation		d.f.	Estimate ± s.e.m.	*F*	*P*
Lutein (μg ml^{-1})	Intercept			11.41±3.70		
	Treatment	β-carotene+PQ	3,43.67	0.01±0.05	43.71	<0.001
		No carotenoid–no PQ		–0.06±0.06		
		Lutein+PQ		0.46±0.05		
	Age	Age 9	1,45.64	0.07±0.03	8.17	0.006
	Hatching date		1,49.21	–5.13±1.69	9.24	0.004
β-carotene (μg ml^{-1})	Intercept			0.11±0.03		
	Treatment	β-carotene+PQ	3,41.13	0.14±0.04	10.52	<0.001
		No carotenoid–no PQ		–0.04±0.04		
		Lutein+PQ		–0.02±0.04		
Vitamin E (μg ml^{-1})	Intercept			7.27±0.79		
	Treatment	β-carotene+PQ	3,41.73	–1.06±1.01	3.30	0.030
		No carotenoid–no PQ		–1.61±1.05		
		Lutein+PQ		1.27±1.01		
	Age	Age 9	1,40.26	1.28±0.55	5.53	0.024
Total antioxidant capacity (mmol Trolox equivalents l^{-1})	Intercept			0.39±0.06		
	Treatment	β-carotene+PQ	3,36.03	0.08±0.09	1.71	0.182
		No carotenoid–no PQ		–0.13±0.09		
		Lutein+PQ		–0.01±0.08		
	Age	Age 9	1,31.82	0.02±0.07	0.42	0.524
	Treatment × Age	Age 9, β-carotene+PQ	3,31.62	–0.15±0.10	2.92	0.049
		Age 9, no carotenoid–no PQ		0.04±0.11		
		Age 9, lutein+PQ		0.12±0.10		
Carbonyl group (nmol ml^{-1})	Intercept			0.54±0.05		
	Treatment	β-carotene+PQ	3,28.33	–0.01±0.06	6.69	0.001
		No carotenoid–no PQ		0.11±0.07		
		Lutein+PQ		–0.17±0.06		
Body mass (g)	Intercept			2055.4±868.45		
	Age	Age 9	1,34.94	–34.13±6.55	27.12	<0.001
	Hatching date		1,44.58	–921.77±398.05	5.36	0.025
	Body mass at hatching		1,40.76	2.28±0.85	7.12	0.011
Tarsus length (mm)	Intercept			6.76±10.55		
	Age	Age 9	1,36.24	–3.13±0.29	115.879	<0.001
	Tarsus length at hatching		1,42.30	1.18±0.41	8.14	0.007

Chick survival from 8 to 12 days of age did not significantly differ among treatments (Wald's χ^2=1.41, d.f.=3, *P*=0.704). At the end of the experiment, 11 lutein+PQ chicks (i.e. 36.7% of the initial sample size in this group), nine β-carotene+PQ chicks (30%), nine no carotenoid+PQ chicks (30%) and seven no carotenoid–no PQ chicks (23.3%) survived. Chicks with higher body mass at 6 days of age had higher survival probability (Wald's χ^2=7.25, d.f.=1, *P*=0.007). Sex (Wald's χ^2=0.01, d.f.=1, *P*=967), brood size (Wald's χ^2=0.23, d.f.=1, *P*=0.633) and hatching date (Wald's χ^2=0.71, d.f.=1, *P*=0.400) did not have a significant effect on chick survival.

DISCUSSION

Prior to the oxidative challenge, we found that both carotenoid treatments enhanced the cell-mediated immune response to PHA. Oxidative challenge by PQ administration enhanced plasma vitamin E concentration, except in chicks supplemented with β-carotene, and it also increased antioxidant capacity in the short term (i.e. after 24 h) in chicks supplemented with lutein. Interestingly, lutein-supplemented chicks showed the lowest oxidative damage in proteins compared with the other treatments. Overall, these results suggest an upregulation of antioxidant defenses after oxidative challenge, which was especially strong in lutein-supplemented chicks. The positive effect of carotenoid supplementation on the cell-mediated immune response disappeared after the challenge, suggesting that the immunostimulatory effects of carotenoids are linked to oxidative status.

Effects of oxidative challenge

Our results suggest that chicks upregulated their antioxidant defences (plasma vitamin E and plasma antioxidants) after PQ administration, probably to counteract the increase of free-radicals produced by PQ exposure (see Bus and Gibson, 1984; Suntres, 2002). Our results are consistent with previous findings in the same species, as adult yellow-legged gulls exposed to oil pollution [i.e. an oxidative challenge (Leighton, 1993)] upregulated plasma antioxidants, especially plasma vitamin E levels (Pérez et al., 2010). Interestingly, in our study, ROM levels did not differ among treatments. Importantly, our assay detects those ROM levels resulting from the interaction between ROS levels and antioxidant defenses. Thus, the increase of antioxidant defenses after PQ administration was probably enough to prevent oxidative damage. Indeed, MDA and carbonyl levels did not statistically differ between PQ-exposed and non-exposed chicks (non-carotenoid groups).

These results reveal high plasticity in antioxidant defenses, which are especially mobilized under situations of high oxidative stress, such as PQ exposure. In our experiment, we did not detect any adverse effect of PQ administration. Nevertheless, we cannot discard the possibility that the mobilization of antioxidants after PQ

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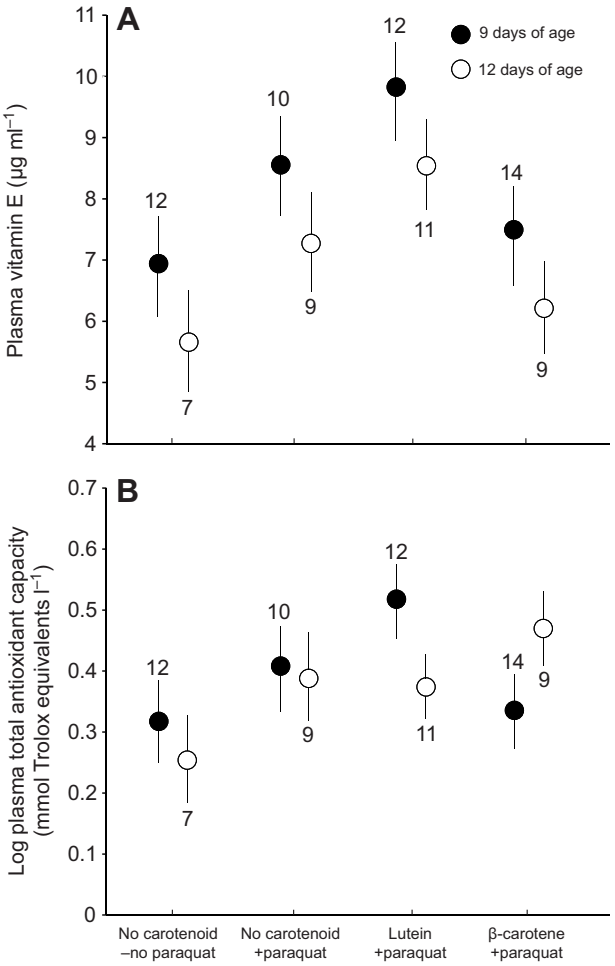

Fig. 3. Effect of carotenoid treatment (lutein or β-carotene) on (A) plasma vitamin E and (B) total plasma antioxidant capacity in yellow-legged gull chicks at 9 (filled circles) and 12 days of age (open circles) (i.e. after paraquat administration). Values shown are means ± s.e.m. Numbers indicate sample size.

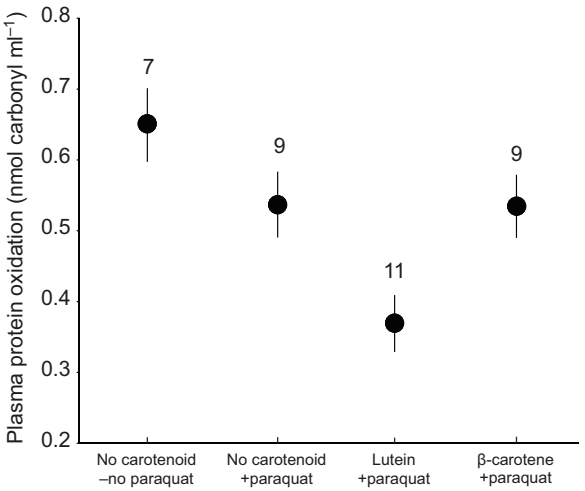

Fig. 4. Effect of carotenoid treatment (lutein or β-carotene) on plasma carbonyl group in yellow-legged gull chicks after paraquat administration. Values are least square means ± s.e.m. Numbers indicate sample size.

administration has a delayed negative effect. If antioxidant resources are limited, maintaining high antioxidant levels for a long period of time can be costly, because antioxidant reserves can be depleted (Cohen et al., 2007) or because antioxidants are needed to maintain other physiological functions (Monaghan et al., 2009). Accordingly, we found that the antioxidant capacity decreased 4 days after the oxidative challenge.

Carotenoids and oxidative status

Prior to oxidative challenge, our results support previous findings in other bird species (e.g. Biard et al., 2006; Costantini et al., 2007; Larcombe et al., 2010), as we found no clear evidence of an effect of carotenoid supplementation on the overall oxidative status in gull chicks. Nevertheless, soon after the oxidative challenge, we found that chicks supplemented with lutein (but not with β-carotene) showed the highest increase in total plasma antioxidant capacity and vitamin E levels. To our knowledge, this is the first study that has measured the total antioxidant capacity of both hydrophilic (e.g. –SH group of proteins, uric acid) and lipophilic (e.g. vitamin E and carotenoids) components of plasma in wild birds. Additionally, chicks supplemented with lutein showed lower protein oxidation than chicks not supplemented with lutein. Thus, the role of lutein as an antioxidant was only evident after an oxidative challenge, suggesting that its role in decreasing oxidative damage is context dependent (Costantini and Møller, 2008). Interestingly, daily ingestion of lutein alleviated the oxidative damage in proteins, but had no effect on lipid peroxidation, underlining that oxidative damage should be assessed among different biomolecules (Monaghan et al., 2009; Hõrak and Cohen, 2010).

Carotenoids and immune function

Carotenoid supplementation enhanced the immune response to PHA of gull chicks at an early age, as previously found in young of other avian species [e.g. the barn swallow, *Hirundo rustica* (Saino et al., 2003); grey partridge, *Perdix perdix* (Cucco et al., 2006); and great tits, *Parus major* (Fitze et al., 2007)]. T-cells and phagocytes that are activated by PHA injection (Martin et al., 2006) can kill pathogens by releasing free radicals (Hampton et al., 1998), leading to ROS-induced oxidative damage (reviewed by Costantini and Møller, 2009; Sorci and Faivre, 2009). Carotenoids have been suggested to play an immunostimulatory role through alleviating these pro-oxidant side-effects of the immune response (Bendich, 1989; Chew and Park, 2004). In our study, the immunostimulatory benefits of carotenoids disappeared after the oxidative challenge with PQ, indicating that gull chicks were not able to prioritize the immune response over antioxidant-demanding functions (Monaghan et al., 2009; Mougeot et al., 2012). Interestingly, plasma carotenoid levels in carotenoid-supplemented chicks remained high after the oxidative challenge. This suggests that the amount of carotenoids available in plasma may not reflect those available for the immune response (Alonso-Álvarez et al., 2004). It is also possible that high carotenoid concentration in plasma reflected the mobilization of carotenoids to tissues in order to combat oxidative stress.

Additionally, carotenoids can directly stimulate the immune response by inducing lymphocyte proliferation, immunoglobulin and cytokine production, and gene regulation (Bendich, 1989), and by favoring intercellular communication (Chew and Park, 2004). Thus, we cannot discard that carotenoid supplementation had a direct effect on the inflammatory response to PHA. Regardless of the exact underlying mechanism (direct stimulation of the immune response versus alleviating pro-oxidant side-effects of the immune response), our results suggest that the link between carotenoids and the immune system is mediated by oxidative stress.

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Differences between carotenoids

Lutein-supplemented chicks showed higher plasma antioxidant capacity and vitamin E levels than β-carotene-supplemented chicks. In our experiment, plasma concentration of both carotenoids increased according with the dosage, suggesting that both were similarly absorbed and transported from the intestinal mucosal epithelium. Thus, our results support previous findings indicating that lutein and zeaxanthin, two major carotenoids in the plasma of avian species, have higher antioxidant potential *in vivo* than β-carotene [e.g. in poultry chicks (Woodall et al., 1996; Surai and Speake, 1998; for a review, see also Surai, 2002)], in contrast with *in vitro* studies (Krinsky, 1993; Rice-Evans et al., 1997). It should be taken into account that supplementation with Lutecol contained lutein with minor amounts of zeaxanthin (see Materials and methods). The chemical structures of lutein and zeaxanthin are very similar (Surai, 2002), with similar radical scavenging abilities *in vitro* (Miller et al., 1996; Rice-Evans et al., 1997; Sujak et al., 1999), although it has been suggested that zeaxanthin has a greater antioxidant potential than lutein (Stahl et al., 1998; Cantrell et al., 2003). Thus, the effect of lutein could be also due to traces of zeaxanthin present in our treatment.

We also found that chicks supplemented with lutein reduced protein oxidative damage compared with chicks supplemented with β-carotene. Lutein is almost exclusively associated with high-density lipoproteins in plasma, the major lipoprotein during the first weeks after hatching, whereas β-carotene is exclusively associated with low-density lipoproteins (Rice-Evans et al., 1997; Surai, 2002). Thus, one possible explanation for the low carbonyl levels observed in lutein-supplemented chicks is that lutein protected plasma-circulating high-density lipoproteins after PQ administration, while these molecules were more exposed to ROS attack in no-carotenoid and β-carotene-supplemented chicks. Additionally, lutein has membrane-spanning orientation and can trap radicals almost throughout the whole bilayer, providing protection to membrane proteins (e.g. glycoproteins), whereas carotenes are located entirely within the hydrophobic membrane bilayer and thus they only protect proteins allocated in the inner core of the membrane (Britton, 1995; Surai, 2002). Thus, membrane cells in no-carotenoid and β-carotene chicks were probably more exposed to ROS attack.

Results suggest that carotenoids differ in their plasma antioxidant potential and ability to prevent protein oxidative damage according to their molecular structure (Surai, 2002) or different transformation pathways into other oxidative forms (Møller et al., 2001). Additionally, differences in the electron affinities and ionization energies among carotenoid types may determine their capacity to scavenge free radicals (rather than to prevent oxidation of the molecular machinery; Martínez et al., 2008).

We did not find differences in lipid peroxidation among carotenoid treatments. However, previous work shows that lipid peroxidation is reduced when birds are supplemented with β-carotene together with other carotenoids [adult gulls (see Blount et al., 2002a; Blount et al., 2002b); domestic chicks (see Woodall et al., 1996; Surai and Speake, 1998)]. Thus, it may be that β-carotene protects lipids against oxidation only in the presence of other types of carotenoids, probably through cooperative or synergistic effects (Surai, 2002).

Conclusions

Our findings suggest that only after facing an oxidative challenge does lutein supplementation enhance antioxidant capacity and reduce protein oxidative damage during early life. Additionally, our results indicate that the immunostimulatory effect of carotenoids depends on oxidative status. Finally, we have shown that there are clear differences between lutein and β-carotene in their effects on the oxidative status, indicating that the antioxidant function of different types of carotenoids should be explored separately.

MATERIALS AND METHODS

The study was carried out between May and June 2011 in a large colony of yellow-legged gulls on Sálvora Island, Parque Nacional das Ilas Atlánticas, Galicia, Spain (42°28′N, 09°00′W). Yellow-legged gulls are ground-nesting colonial breeders, and the care of chicks is shared by both parents. In the study population, clutches typically contain three eggs (Kim et al., 2011), and the first and second eggs are laid at intervals of 1–5 days (mean ± s.e.m.: 2.12±0.14 days). Laying and hatching order are highly positively correlated (Kendall's tau-b correlation, τ=0.73, P<0.001, n=130; authors' unpublished data) and hatching is asynchronous (Kim et al., 2011).

At the end of May, the colony was checked daily to locate nests (n=120) with a clutch of three eggs in which only one of the eggs was pipping (i.e. A-chick). In large gulls, hatching asynchrony leads to marked stable, within-nest hierarchies (Boncoraglio et al., 2006). By selecting nests with only one pipping egg, we studied A-chicks with reduced sibling competition (i.e. their siblings are expected to hatch at least 1 day later). A-chicks have larger body mass, higher antioxidant levels (Royle et al., 2001) and higher survival prospects (Hillström et al., 2000) than chicks hatched from later laid eggs. To recognize the A-chick after hatching, the tip of the chick's bill was marked through the pipped egg with non-toxic acrylic paint (ArtCreation, Royal Talens, The Netherlands). After hatching, chicks were individually marked with a strip of Velcro on the right leg. Before hatching, we installed a semitransparent mesh (height 30 cm) surrounding an area of ~1.5 m^2 around each nest to prevent the semiprecocial gull chicks from moving to nearby nests. These enclosures allow normal parental breeding and chick feeding (see Noguera et al., 2011).

Our experiment was designed to study the effect of carotenoid supplementation before and after an oxidative challenge induced by PQ administration (Fig. 1). Thus, we randomly assigned hatched chicks to one of the following treatment groups (n=30 per group): (1) lutein+PQ, (2) β-carotene+PQ, (3) no carotenoid+PQ and (4) no carotenoid–no PQ. During the first 6 days of life, chicks from groups 1 and 2 received a daily dose of 0.15 mg of lutein (together with small amounts of zeaxanthin, see below) and β-carotene, respectively. Thereafter, they received a daily dose of 0.2 mg lutein or β-carotene. Lutein and β-carotene were orally administered by mixing Lutecol (50 mg g^{-1} of lutein and 6 mg g^{-1} of zeaxanthin) or Betacol (100 mg g^{-1} of β-carotene), respectively, provided by CaroTech (Carotenoid Technologies S.A., IQF group, Tarragona, Spain) in 0.5 ml of water. Solutions were freshly prepared each day in opaque tubes in order to avoid oxidation. Chicks in non-carotenoid treatments (groups 3 and 4) received 0.5 ml of water daily. In the yellow-legged gull, lutein, zeaxanthin and β-carotene are present in the diet (Czeczuga et al., 2000; Naczk et al., 2004; Moreno et al., 2010), yolk (Romano et al., 2008) and plasma (Saino et al., 2008; Pérez et al., 2008; Rubolini et al., 2011; authors' unpublished data). The estimated daily amount of carotenoid consumed by gull chicks is 0.04–0.13 mg day^{-1} during the first 6 days of life and 0.05–0.18 mg day^{-1} from 7 to 12 days of life, depending on the amount of crabs consumed. These values were calculated according to: (1) the energy requirements of gull chicks during the first 6 days of life [mean ± s.e.m. body mass=88.86±1.29 g, n=271; basal metabolic rate (BMR)=166.25 kJ day^{-1}] and from 7 to 12 days of life (body mass=140.71±3.19 g, n=222; BMR=223.73 kJ day^{-1}) [warm-water seabirds (see Nagy et al., 1999)], (2) the estimated ingested chick diet in our population by stable isotopes [~95% fishes, mainly *Micromesistius* sp. and *Trachurus* sp., and 5% crustaceans, mainly *Polybius* sp. (Moreno et al., 2010)], (3) the energy of prey items [energy per gram of wet mass, 3.22 kJ g^{-1} in fishes and 5.20 kJ g^{-1} in crustaceans; Spanish Food Composition Database, www.bedca.net; for conversion to dry mass units, the water content is ~72% in fishes and 74% in crustaceans (Holmes and Donaldson, 1969; Ricciardi and Bourget, 1998)], and (4) the amount of carotenoids in the prey items [~0.71 μg g^{-1} dry mass in fishes and 44–192 μg g^{-1} dry mass in crustaceans (Czeczuga et al., 2000; Naczk et al., 2004)]. Thus, our dosage was probably within the

natural range of carotenoids consumed by gull chicks, and lower than doses found to produce detrimental effects on birds (Costantini et al., 2007; Huggins et al., 2010).

At 8 days of age, we exposed chicks to an oxidative challenge by PQ administration. Only 56 out of 118 hatched chicks reached 8 days of age (2011 was one of the worst breeding seasons recorded at Sálvora Island). Chicks in PQ groups (no carotenoid+PQ, n=13; lutein+PQ, n=13; β-carotene+PQ, n=17) received a single dose of 0.3 mg of PQ (1,1′-dimethyl-4,4′-bipyridinium dichloride, Sigma-Aldrich, Barcelona, Spain) in 0.5 ml of water by oral administration with the aid of a syringe. Chicks in the no carotenoid–no PQ group (n=13) received 0.5 ml of water. PQ was prepared each day to avoid degradation. To avoid provoking long-term adverse effects, the dosage selected [2.3 mg kg^{-1} body mass (BM)] was based on a pilot study previously performed in 2010 in yellow-egged gull chicks (J. C. Noguera and A.V., unpublished) and was lower than that used in previous studies [from 5 to 60 mg kg^{-1} BM (Hoffman et al., 1987; Galvani et al., 2000; Isaksson and Andersson, 2008)]. A higher concentration of PQ (5–10 mg kg^{-1} BM) in juvenile great tits did not significantly affect chick survival (Isaksson and Andersson, 2008). Moreover, our PQ dosage was 86-fold lower than the sub-lethal dosage reported in aquatic birds [estimated at 199 mg kg^{-1} BM (reviewed by Eisler, 1990)].

Chicks were weighed (±1 g) and their tarsus length was measured (±0.01 mm) at hatching (day 0 of age) and at 6, 9 and 12 days of age (Fig. 1). Chick survival was recorded until 12 days of age. For oxidative status assays, we took a blood sample from the brachial vein with heparinized capillary tubes at 0, 5, 9 and 12 days of age (Fig. 1). The samples were kept cool until the plasma was separated from blood cells by centrifugation (6 min×3824 ***g***) within a few hours of collection and then stored in liquid nitrogen. We identified chick sex from blood cell DNA (Fridolfsson and Ellegren, 1999). Plasma antioxidant capacity, ROMs and oxidative damage to proteins could not be measured for all samples, as these were the last analyses performed and the volume of plasma was not always sufficient.

Inflammatory response

The inflammatory response was assessed by the dermal reaction to a subcutaneous injection of PHA (PHA test) in the wing web, a well-validated technique traditionally used in ecological studies (Smits et al., 1999; Martin et al., 2006). We performed two PHA tests, one at 7 days of age (primary inflammatory response) and another at 11 days of age (secondary inflammatory response; i.e. before and after PQ administration; Fig. 1). Prior to the subcutaneous injection with PHA, the right wing patagium thickness was measured (±0.01 mm) three times with a micrometer (Micrometer Series 293, Mitutoyo). Then, 0.2 mg of PHA (Sigma-Aldrich) dissolved in 0.05 ml of phosphate-buffered saline was injected. Approximately 24 h after injection (±1 h), we measured thickness three times at the injection site. The average difference in thickness was assumed to represent a reliable index of inflammatory response (Smits et al., 1999). The second inflammatory test was performed in the left wing patagium. The values of thickness were highly repeatable within individuals [r>0.96 in all four measurements, calculated following Lessells and Boag (Lessells and Boag, 1987)].

Plasma carotenoid and vitamin E levels

We quantified the concentration in plasma of lutein, β-carotene and vitamin E (because of potential physiological interactions between carotenoids and vitamin E) (Woodall et al., 1996; Surai, 2002) by high-performance liquid chromatography (HPLC; JASCO Comparison Proven, Madrid, Spain; model 1500) following the protocol described by Pérez et al. (Pérez et al., 2008). Extractions were performed by diluting 20 µl of plasma in 100 µl of absolute ethanol, centrifuged for 10 min at 10,621 ***g***, dried under nitrogen flow and diluted again in 80 µl of methanol. Carotenoids were determined at λ=445 nm with a UV detector (JASCO Comparison Proven; model UV-1570) and quantified in relation to an external standard of lutein (r^2=0.99; VWR, Barcelona, Spain) (Pérez et al., 2008; Pérez et al., 2010). Vitamin E (α-tocopherol) was simultaneously determined from the same extract but with a fluorescence detector (JASCO Comparison Proven; model FP-1520). The excitation and emission wavelengths used were λ=295 and 330 nm, respectively. Concentrations were calculated in relation to the vitamin E standard (α-tocopherol; Sigma-Aldrich, Madrid, Spain; r^2=0.99). Samples were injected per duplicate except in five cases where the fluorescence detector failed when determining vitamin E (repeatability; vitamin E: r=0.92, $F_{279,280}$=23.49, P<0.001; lutein: r=0.94, $F_{284,285}$=31.22, P<0.001; β-carotene: r=0.92, $F_{284,285}$=23.32, P<0.001). Plasma concentrations are reported as micrograms per milliliter of plasma.

Total antioxidant capacity of plasma

In ecological studies, only the hydrophilic fraction of plasma antioxidants is usually estimated [but see Alan et al. (Alan et al., 2013) for a study in wild fruits]. However, this measure only quantifies the reaction of antioxidants present in the aqueous phase of the plasma but not the reaction of antioxidants present in the lipophilic phase (Prior et al., 2005). In this study, the antioxidant capacity of plasma was estimated as the sum of both the hydrophilic and the lipophilic antioxidant capacity (Prior et al., 2005).

We determined the hydrophilic plasma antioxidant capacity by the method described by Erel (Erel, 2004). Briefly, 5 µl of plasma reacts with 20 µl of $ABTS^{\bullet+}$ (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonate], 10 mmol l^{-1}) radical, which is decolorized by plasma antioxidants according to their concentration and antioxidant capacity. The $ABTS^{\bullet+}$ radical was generated from the ABTS form using hydrogen peroxide (H_2O_2, 2 mmol l^{-1}) alone in acidic medium (acetate buffer 30 mmol l^{-1}, pH 3.6) and incubated in darkness at room temperature for 1 h. The change in color was measured as the change in absorbance at λ=415 nm before and after addition of $ABTS^{\bullet+}$ to plasma samples in a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek, Madrid, Spain). Samples were assayed in duplicate (repeatability; r=0.97, $F_{185,186}$=67.15, P<0.001) as Trolox equivalents.

We quantified the lipophilic plasma antioxidant capacity following the method described by Re et al. (Re et al., 1999) and the modifications of Silva et al. (Silva et al., 2007). Extractions of the lipophilic phase of plasma were performed as described for carotenoids analysis (see above). The $ABTS^{\bullet+}$ radical was generated by chemical reaction of ABTS with potassium persulfate ($K_2S_2O_8$) (see Re et al., 1999). The $ABTS^{\bullet+}$:ethanol solution was prepared daily by diluting with ethanol to an absorbance A=0.7±0.02 at λ=734 nm and equilibrated at 30°C (Synergy HT Multi-Mode Microplate Reader, BioTek). In the microplate, 5 µl of plasma was mixed with 200 µl of $ABTS^{\bullet+}$:ethanol solution and measured at λ=734 nm in a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek) at 1 and 6 min at a constant temperature of 30°C. Levels of plasma antioxidant capacity were quantified in duplicate (repeatability; r=0.96, $F_{137,138}$=45.79, P<0.001) relative to Trolox calibration curves included in each microplate (r^2>0.91, in all cases). Trolox was dissolved in Milli-Q water for hydrophilic assays, and in absolute ethanol for lipophilic assays. Total plasma antioxidant capacity was expressed as the sum of millimoles of Trolox equivalents per liter in both assays.

Reactive oxygen metabolites

We measured ROM levels in plasma using the method described by Brambilla et al. (Brambilla et al., 2001). Briefly, ROMs in 5 µl volume of plasma sample were reacted with 5 µl of DEPPD (*N*,*N*-diethyl-*p*-phenylenediamine, 0.37 mol l^{-1}) in acidic medium (200 µl of 0.1 mol l^{-1} acetate buffer, pH 4.8). To produce a colored complex, each microplate was incubated at 37°C for 75 min and then absorbance was measured spectrophotometrically at λ=490 nm (Synergy HT Multi-Mode Microplate Reader, Biotech). ROMs were quantified in duplicate (repeatability; r=0.91, $F_{173,174}$=20.10, P<0.001) relative to external hydrogen peroxide calibration curve (r^2>0.98 in all cases). ROM levels were expressed as millimoles of hydrogen peroxide equivalents per liter.

Lipid peroxidation (MDA)

Lipid peroxidation in plasma (a measure of oxidative damage in lipids) was assessed in triplicate by quantifying MDA by HPLC, according to Karatas et al. (Karatas et al., 2002). Briefly, a 10 µl volume of plasma was added to 50 µl of perchloric acid (in order to precipitate proteins and release the MDA bonded to amino compounds) and 140 µl of distilled water. Samples were centrifuged at 2655 ***g*** for 5 min and supernatants were used for HPLC analysis (for details, see Noguera et al., 2011). The absorbance of the sample was obtained at λ=254 nm and quantified relative to external MDA

standards (calibration curves, r^2=0.99; repeatability, r=0.99, $F_{267,536}$=720.46, P<0.001). Lipid peroxidation was expressed as micrograms of MDA per milliliter of plasma.

Because MDA levels may be affected by total plasma lipids, we also quantified plasma lipids in a microplate (Synergy HT Multi-Mode Microplate Reader, Biotech) by the colorimetric sulfo-phospho-vanillin method according to Cheng et al. (Cheng et al., 2011). Lipids were quantified relative to a cholesterol standard calibration curve (Calbiochem, Germany; r^2>0.99 in all cases). Plasma lipid levels were analyzed by duplicate (repeatability; r=0.87, $F_{101,102}$=14.87, P<0.001) and expressed as millimoles of cholesterol equivalents per liter.

Plasma carbonyl groups

The levels of protein carbonyl groups in plasma (20 µl) were quantified in duplicate by reaction of 20 µl volume of plasma samples with DNPH (2,4-dinitrophenylhydrazine) as described by Levine et al. (Levine et al., 1990) with minor modifications. Briefly, plasmatic proteins were reacted with 0.2% DNPH in 2 mol l^{-1} hydrochloric acid for 15 min at 25°C, precipitated with 20% trichloroacetic acid and washed three times by resuspension in ethanol-ethyl acetate (1:1 v/v). Proteins were solubilized in 6 mol l^{-1} guanidine hydrochloride and centrifuged at 2655 ***g*** for 1 min. Carbonyl groups were measured spectrophotometrically at λ=370 nm in duplicate (repeatability; r=0.98, $F_{151,152}$=107.43, P<0.001) in quartz cuvettes (Biomate 3; Thermo Fisher Scientific, Rochester NY, USA). Carbonyl groups in plasma were calculated relative to the molar extinction coefficient for DNPH-hydrazone [i.e. carbonyl (Levine et al., 1990)] and were expressed as nanomoles per milliliter of plasma.

Because carbonyl levels may be affected by total plasma proteins, we estimated plasma protein concentration according to absorbance at λ=276 nm from the same extract (by duplicate, repeatability; r=0.95, $F_{149,150}$=39.80, P<0.001). Absorbance was corrected from hydrazone peak absorbance (see Levine et al., 1990). Protein concentration was estimated relative to the glutamine synthetase molar extinction coefficient (see Levine et al., 1990). Total protein concentration was expressed as micrograms of protein per milliliter of plasma.

Statistical analyses

At hatching (i.e. prior to carotenoid supplementation), chicks did not differ in their body mass ($F_{2,115}$=1.20, P=0.305), tarsus length ($F_{2,115}$=1.45, P=0.238) or plasma levels of lutein ($F_{2,109}$=0.10, P=0.905), β-carotene ($F_{2,109}$=0.03, P=0.971), vitamin E ($F_{2,106}$=1.58, P=0.211) and MDA ($F_{2,104}$=0.76, P=0.472) between treatment groups. The effects of treatment on chick morphological and physiological variables were analyzed separately before (until 8 days of age) and after (9 to 12 days of age) PQ administration. Treatment included three categories before PQ administration (lutein, β-carotene and no carotenoid chicks) and four after PQ administration (lutein+PQ, β-carotene+PQ, no carotenoid+PQ and no carotenoid–no PQ chicks; see above). All statistical analyses were performed with IBM SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

Because only one measurement of each variable was recorded before the oxidative challenge with PQ, the effects of carotenoid treatment on levels of lutein, β-carotene, vitamin E, total plasma antioxidant capacity, ROMs, MDA and carbonyls at 5 days of age (n=83) and on body mass and tarsus length at 6 days of age (n=74) were analyzed by general linear models (GLMs). We included treatment, sex and their interaction as categorical factors and hatching date and brood size (recorded at day 3) as covariates. Values at hatching of lutein, β-carotene and vitamin E, as well as body mass and tarsus length, were included as covariates in their respective models. Plasma protein and plasma lipid levels at 5 days of age were used as covariates for carbonyl and MDA models, respectively. MDA at hatching was also included as a covariate in total antioxidant capacity, ROMs, MDA and carbonyl group models to control for any possible influence of initial oxidative status.

The effect of carotenoid supplementation on PHA response at 8 days of age was analyzed by a GLM with treatment, sex and their interaction as factors. Body mass at hatching [given the well-known effect of body mass on PHA response (see Alonso-Álvarez and Tella, 2001)], brood size and hatching date were also included as covariates. The effect of carotenoid supplementation on chick survival until 8 days of age (n=118) was analyzed by a generalized linear model with binomial error and logit link, including treatment and sex and their interaction as fixed factors. Hatching date, brood size and body mass at hatching were also included as covariates in the model.

After oxidative challenge with PQ, the effect of treatment on lutein, β-carotene, vitamin E, total plasma antioxidant capacity, ROMs, MDA, carbonyls, body mass and tarsus length was analyzed by linear mixed models. Age (9 and 12 days; n=48 and 36, respectively) was included as a repeated measure (within chicks), with chick identity as the subject term, treatment and sex as fixed factors and hatching date and brood size as covariates. The interactions between treatment and age, treatment and sex, and sex and age were also included. Values at hatching of body mass, tarsus length, lutein, β-carotene and vitamin E were included as covariates in their respective analyses. Plasma protein and plasma lipid levels at 9 and 12 days of age were also used as covariates for carbonyl and MDA analysis, respectively. When we analyzed oxidative stress variables, MDA at hatching was also included as a covariate. Variance components were estimated using a restricted maximum likelihood function.

The effect of treatment on chick PHA test at 12 days of age was analyzed by a GLM with treatment and sex as factors. Hatching date, body mass at hatching and brood size were included as covariates. The interaction between treatment and sex was not included in the model because we noticed that only one male chick in the no carotenoid–no PQ group survived until 12 days of age. The effect of experimental treatment on chick survival between 8 and 12 days of age (n=56) was analyzed by a generalized linear model with binomial error and logit link, including treatment and sex as fixed factors. Hatching date, brood size and body mass at 6 days of age were also included as covariates in the model.

Initial models were simplified by removing non-significant terms (α=0.05) in a backward deletion procedure, starting with the interactions. The full models are reported, with all significant and non-significant variables (see supplementary material Tables S1–S3). When the variables were analyzed with linear mixed models, Satterthwaite approximation was used for the estimation of denominator degrees of freedom. *Post hoc* comparisons were carried out using Fisher's least significant difference (LSD) test. Differences in sample sizes in some analyses reflect missing values because of death or loss of chicks and insufficient volume of blood sample. Values of total plasma antioxidant capacity, lutein, β-carotene and hatching date were log transformed to achieve a normal distribution. Data are expressed as means ± s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

The study was conceived and designed by all three authors. A.L. and A.V. performed the experiment. A.L. performed laboratory work. Data were analyzed by all three authors, who co-wrote the manuscript.

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Supplementary material

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