

## Article

# Yolk vitamin E positively affects prenatal growth but not oxidative status in yellow-legged gull embryos

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## Abstract

Parental effects occur whenever the phenotype of parents or the environment that they experience influences the phenotype and fitness of their offspring. In birds, parental effects are often mediated by the size and biochemical quality of the eggs in terms of maternally transferred components. Exogenous antioxidants are key egg components that accomplish crucial physiological functions during early life. Among these, vitamin E plays a vital role during prenatal development when the intense metabolism accompanying rapid embryo growth results in overproduction of pro-oxidant molecules. Studies of captive birds have demonstrated the positive effect of vitamin E supplementation on diverse phenotypic traits of hatchling and adult individuals, but its effects on embryo phenotype has never been investigated neither in captivity nor under a natural selection regime. In the present study, we experimentally tested the effect of the *in ovo* supplementation of vitamin E on morphological traits and oxidative status of yellow-legged gull (*Larus michahellis*) embryos. The supplementation of vitamin E promoted somatic growth in embryos soon before hatching, but did not affect their oxidative status. Our results suggest that maternally transferred vitamin E concentrations are optimized to prevent imbalances of oxidative status and the consequent raise of oxidative damage in yellow-legged gull embryos during prenatal development.

**Key words:** *Larus michahellis*, maternal effects, morphological traits, oxidative status, prenatal period, vitamin E.

Parents can maximize their Darwinian fitness by modulating the allocation of care to individual offspring according to their reproductive value. In oviparous organisms, mothers can adjust offspring phenotype via the modulation of the size and biochemical quality of their eggs, which can widely vary not only among mothers but also among sibling eggs (Mousseau and Fox 1998; Saino et al. 2002; Groothuis et al. 2005). In fact, the size and the concentration of quantitatively major (e.g., lipids, albumen) and minor (e.g., steroid hormones, vitamins) maternally transferred components often vary within-clutch according to laying order (Royle et al. 2001; Badyaev et al. 2006;

Groothuis et al. 2006; Rubolini et al. 2011; von Engelhardt and Groothuis 2011). Mothers may adaptively tune their investment, including prenatal maternal effects *via* eggs to individual offspring.

Exogenous antioxidants (e.g., vitamins and carotenoids) are acquired with food, and may be available in limiting amounts, implying that mothers may tune the amount of antioxidants they transfer to their eggs according to the reproductive value of their individual offspring as determined, for example, by hatching order (Grether et al. 2001; Catoni et al. 2008). The existence of reproductive trade-offs and the major role that antioxidants have in early-life

offspring physiology have attracted considerable attention to the study of ecology and evolution of maternal effects mediated by egg antioxidants. Egg antioxidants of maternal origin provide protection to the developing embryo against the detrimental effects of free radicals produced during early-life growth (Surai et al. 1996). Low levels of maternally transferred yolk antioxidants impair embryonic development (Wilson 1997), suggesting that they play a crucial role in counteracting oxidative stress (Surai and Speake 1998; Blount et al. 2000; McGraw et al. 2005). In fact, intense metabolic activity during early developmental stages exposes the organism to oxidative stress, resulting from the breakdown of the equilibrium between the production of pro-oxidants (reactive oxygen and nitrogen species, ROS and RNS, respectively), and antioxidant defense and repair mechanisms in favor of the former (Finkel and Holbrook 2000). The prenatal period is crucial to redox homeostasis because high metabolic rates during rapid growth stages can induce ROS overproduction (Rollo 2002), leading to oxidative damage to cellular macromolecules (i.e., DNA, lipids, and proteins) and providing a potential mechanism for negative effects on fitness-related traits (Costantini 2014). Because of the adverse consequences of oxidative stress on phenotype, selection is expected to favor the evolution of mechanisms for antioxidant defense and repair of oxidative damage (Costantini et al. 2010; Metcalfe and Alonso-Alvarez 2010; Isaksson et al. 2011; Metcalfe and Monaghan 2013; Costantini 2014). Variation in oxidative stress (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010) and in maternal transfer of antioxidants depending on environmental conditions experienced by the mother (Blount et al. 2002; Royle et al. 2003) suggests that the response to oxidative stress may be modulated by maternal effects. Therefore, maternal allocation of exogenous antioxidants to egg yolk may constitute a strategy to minimize oxidative damage to developing embryos (Blount et al. 2002).

In birds, vitamin E is one of the most important yolk antioxidants (Surai et al. 2016). Vitamin E is transported from the yolk to the embryonic tissues during development (Surai et al. 1996; Cherian and Sim 2003) and protects embryos against the toxicity of free radicals (Khan et al. 2011). Vitamin E acts as chain-breaking lipid antioxidant and free radical scavenger in the membranes of cells and subcellular organelles (Young et al. 2003), maintaining the integrity and functioning of the reproductive, muscular, circulatory, nervous, and immune systems of vertebrates (Leshchinsky and Klasing 2001). The effects of egg vitamin E have been mostly investigated by means of maternal dietary supplementation in captivity. These studies have shown that vitamin E supplementation positively affects growth, immune function, performance, and antioxidant capacity of poultry (Gore and Qureshi 1997; Surai et al. 2001; Bhanja et al. 2012; Selim et al. 2012; Goel et al. 2013), as well as the transcription and the expression of specific genes involved in diverse metabolic pathways (Surai 2002). Experiments in captivity where egg vitamin E has been manipulated by injection have partly clarified its direct effects on offspring phenotype. Direct manipulation of yolk vitamin E levels improved hatchability, immune status, and both embryonic and post-hatch growth of Muscovy ducks *Cairina moschata* (Selim et al. 2012), and reduced the production of ROS in tissues of hen chicks (Cherian and Sim 1997; Surai et al. 1999a). Although these experiments are valuable to identify the effects and mechanisms behind the allocation of antioxidants to eggs, the most insightful perspective for the interpretation of the evolution of maternal effects rests on the experimental analysis of the consequences of egg quality manipulation under a natural selection regime in the wild. However, information on yolk vitamin E effects derived from yolk manipulation in free-ranging populations under

natural selection regimes is scanty and to date no study has investigated the effects on embryonic growth or oxidative status in important organs that are likely to be the target of the antioxidant activity of vitamin E.

In a recent study, we have shown that a physiological increase of vitamin E concentration in yellow-legged gull *Larus michahellis* eggs enhanced postnatal body size of chicks from the last-laid eggs in a clutch (Parolini et al. 2015). However, information on the effects of vitamin E during the prenatal period in free-living species is largely unavailable. For this reason, here we investigate the effect of a physiological increase in yolk vitamin E concentration on phenotypic traits of embryos shortly before hatching. We expected that the supplementation of vitamin E would promote growth, positively affect oxidative status, and reduce embryo oxidative damage. In addition, because vitamin E concentration declines with laying order (Rubolini et al. 2011) and in our previous study we showed that it limits postnatal growth of chicks from third-laid eggs, we expected a decrease of pro-oxidant molecules accompanied by an increase of total antioxidant capacity (TAC) mainly in embryos from last-laid vitamin E-injected eggs. Lastly, although the concentration of vitamin E in the yolks of yellow-legged gull eggs does not vary according to the sex of developing embryos (Rubolini et al. 2011), we also tested if the effect of egg treatment depended on the sex of the embryo because embryos of either sex may differ in their susceptibility to yolk antioxidants (Romano et al. 2008). Thus, we studied the effects of vitamin E on embryo morphology (body mass and tarsus length) and oxidative status by measuring TAC, amount of pro-oxidant molecules (called as 'TOS' 128 according to the terminology by Erel 2005) lipid peroxidation (LPO) and protein carbonylation (PCO) in brain and liver explanted from the embryos. We focused on brain for 3 reasons; it is particularly sensitive to LPO because the phospholipids of the neuronal membranes contain large amounts of highly polyunsaturated fatty acids, it generates free radicals at a greater extent than other tissues as a consequence of high rates of energy metabolism and oxygen consumption, and the amount of many exogenous antioxidants is lower compared to other tissues (Surai et al. 1999b). Liver was chosen because it is the main repository of antioxidants, including vitamin E (Surai 2002), and it has a crucial role in antioxidant defense.

## Materials and Methods

### Field and experimental procedures

The yellow-legged gull is a monogamous species that breeds mostly colonially (Cramp 1998). Clutch size ranges between 1 and 3 eggs (modal size = 3), which are laid at 1–4 (most frequently 2) days intervals and hatch 27–31 days after laying. Hatching is asynchronous and spans over 1–4 days. The chicks are semi-precocial and are fed by both parents and fledge at 35–40 days of age (Cramp 1998). We studied a large colony (>400 pairs) breeding on an island in the Comacchio lagoon (NE Italy, 44°20' N–12°11' E) in March–May 2014. The colony was monitored every other day and when a new nest was found the newly laid egg was temporarily removed and replaced with an egg collected from a nest outside of the study colony (i.e., "dummy" egg) to avoid interference with parental incubation behavior. Nests that were found with more than 1 egg were considered, but egg order was estimated based on previously described differences in egg mass for the species. The removed egg was marked and taken to a nearby tent for experimental manipulation.

The experimental design has been described in details by Parolini et al. (2015) and in the Supplementary material, therefore it is only

briefly summarized here. Our objective was to increase the concentration of vitamin E by 1 standard deviation (SD) of the concentrations measured in the egg yolk of individuals from the same colony in a previous study (Rubolini et al. 2011). Since the concentration of vitamin E in the yolk of yellow-legged gull eggs varied according to egg size and position in the laying sequence (Rubolini et al. 2011), we adjusted the injection dose according to these factors. We estimated yolk mass based on total egg mass for each of the eggs in laying sequence based on a Linear Mixed Model from previously collected yellow-legged gull eggs (yolk mass =  $0.227 (0.039 \text{ SE})$  egg mass +  $1.815 (3.461 \text{ SE})$ ;  $F_{1,88} = 34.38$ ,  $P < 0.001$ ). Then, we grouped first (a-), second (b-), or third (c-) laid eggs into 3 classes (tertiles) of size according to egg mass and calculated the standard deviation of vitamin E concentration in the yolk for each tertile. The injection amount of vitamin E was computed as the product of the SD (in  $\mu\text{g g}^{-1}$ ) of vitamin E concentration for each tertile and position in the laying sequence and the estimated yolk mass (see Supplementary material). Corn oil was used as the carrier solvent of vitamin E and it was used as a control treatment in the control group of eggs. We adopted a within-clutch design, whereby both sham-(control) and vitamin E-injected eggs were established within each clutch to minimize the confounding effects of environmental and parental effects. The following treatment schemes were assigned sequentially to the clutches as follows: (nest, a-, b-, c-egg): nest 1, vitamin E injection (E), control injection (C), E; nest 2, C-E-C; nest 3, E-C-C; nest 4, C-E-E and so forth with the following nests. The injection procedure was performed according to a previously validated method on eggs from the same species (Romano et al. 2008).

After the *in ovo* vitamin E supplementation and 5 days before the earliest expected hatching date, all the nests were visited daily to check for any sign of imminent hatching such as eggshell fractures (i.e., “cracking stage”). When eggshells were fractured, eggs were weighed (to the nearest g), collected and frozen at  $-20^\circ\text{C}$  within 3 h from sampling.

Field collected eggs ( $n = 76$  eggs) were transferred to the lab where they were dissected. We focused on 26 clutches, 15 of which had 3 eggs, while the remaining 11 clutches had 2 eggs only. We first removed and weighed the residual yolk sac from each egg, which was frozen at  $-80^\circ\text{C}$  until the analysis of total vitamin E concentration and TAC that we performed as a validation of the experimental treatment. We expected that vitamin E injection would result in a measurable increase in the yolk concentrations into late developmental stages, as well as in an increase in TAC. Then, the embryos were weighed (to the nearest g) and tarsus length was measured by calipers before the dissection of liver and brain, which were immediately weighed (to the nearest mg) and frozen at  $-80^\circ\text{C}$  until biochemical analyses. All the measurements were taken by the same person to ensure consistency. Molecular sexing of embryos and chicks was performed according to Saino et al. (2008).

The study was carried out under permission of the Parco Regionale del Delta del Po (#657, 4 February 2014), which allowed both the manipulation and the collection of eggs when the eggshell showed signs of imminent hatch (eggshell fractures). According to the Guidelines for the Euthanasia of Animals by the American Veterinary Medical Association, physical methods of euthanasia may be necessary in some field situations if other methods are impractical or impossible to implement. We performed a field experiment in which we could not euthanize embryos by methods such as carbon dioxide ( $\text{CO}_2$ ), anesthetic agents, or decapitation. Thus, we euthanized embryos by placing eggs into a  $-20^\circ\text{C}$  freezer within 3 h from the collection.

## Analysis of vitamin E content in residual yolk sac, brain, and liver of embryos

The concentration of vitamin E in residual yolk sac, brain, and liver from embryos was determined according to Karadas et al. (2006) using a high-performance liquid chromatography system (Shimadzu Liquid Chromatography, LC-10AD, Japan Spectroscopic Co. Ltd.). Briefly, 100–150 mg of yolk and organs were homogenized with 1 mL of ethanol plus 0.7 mL NaCl 5% and extracted twice by centrifugation with 2 mL of hexane each. Then, hexane extracts were pooled and evaporated at  $60\text{--}65^\circ\text{C}$  under nitrogen flow and the residual was dissolved in 500  $\mu\text{L}$  of a dichloromethane:methanol mixture (50:50 v/v). Vitamin E ( $\alpha$ -, and  $\gamma$ -tocopherol) concentrations were detected with a Hypersil GOLD type 3  $\mu\text{m}$  C18 reverse-phase column ( $150 \times 4.6 \text{ mm}$  Phase Separation, Thermo Fisher Scientific 81, Wyman, Street Waltham, MA USA) with a mobile phase of methanol:distilled water (97:3 v/v) at a flow rate of  $1.05 \text{ mL min}^{-1}$  using fluorescence detection by excitation and emission wavelength of 295 nm and 330 nm, respectively. Peaks of  $\alpha$ -, and  $\gamma$ -tocopherol were identified and quantified by comparison with the retention time of standards of tocopherols at renowned concentration (Sigma, Poole, UK). According to Karadas et al. (2006), standard solutions  $\alpha$ -tocopherol in methanol were used for instrument calibration, while tocol was used as an internal standard to check for the reliability of analytical process.

## Oxidative stress methods

TAC, TOS, PCO and LPO were measured in liver and brain homogenates. In addition, TAC was also measured in the residual yolk from sampled eggs.

An appropriate amount of yolk ( $\sim 0.15 \text{ g}$ ), brain, and liver ( $\sim 0.1 \text{ g}$ ) was homogenized in 100 mM phosphate buffer pH 7.4, with 1 mM EDTA and 100 mM KCl, by an automatic homogenizer. After 10 min centrifugation at 13,000 rpm, an aliquot of the supernatant was immediately processed for the determination of protein content according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard, while the remainder was used for oxidative stress assays. A detailed description of applied methods is reported in Supplementary material. Briefly, TAC was measured according to a colorimetric method based on the discoloration of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS $^{*+}$ ), adapted from Erel (2004). TOS was measured according to a colorimetric method developed by Erel (2005), adapted to tissue homogenates. Carbonylated proteins were measured with 2,4-dinitrophenylhydrazine (DNPH). Protein carbonylation (PCO) was measured by Western immunoblotting and immunostained protein bands were visualized with enhanced chemiluminescence detection. Carbonylated proteins were quantified by densitometric analysis using Image J 1.40d software (Schneider et al. 2012). LPO was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al. 1979), adapted to tissue homogenates of embryos and were expressed as nmol TBARS  $\text{g}^{-1}$  wet weight.

## Statistical analyses

The effect of vitamin E treatment on its concentration in residual yolk sac and embryo focal organs, embryo morphological traits and oxidative status markers, was analyzed in Linear Mixed Models (LMM; Normal as the distribution and Identity as the link function), including clutch identity as a random intercept effect. Egg mass at the time of laying was included as a covariate in all the models.

Egg treatment (vitamin E versus sham-injection), embryo sex, and egg-laying order were included as fixed-effect factors along with their two-way interactions. All non-significant ( $P > 0.05$ ) interaction terms were removed from the model in a single step. In all the models, the effect of clutch identity was tested by a likelihood ratio test, by comparing the log-likelihood value of the model including or excluding the random effect of clutch identity. Mixed models with the same design, but assuming a binomial error distribution, were run to investigate the effects of vitamin E treatment on the proportion of eggs that reached the “cracking stage”, as well as on the sex ratio of embryos. A single embryo could not be dissected because of sample deterioration. All the statistical analyses were performed by using SAS 9.3 PROC MIXED and PROC GLIMMIX. Group statistics are presented as estimated marginal means ( $\pm$  SE).

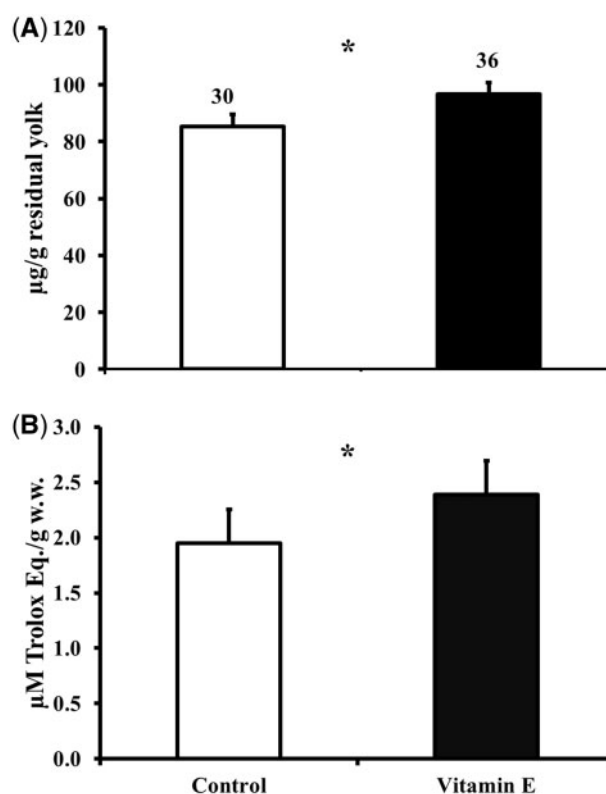
## Results

### Vitamin E concentration in residual yolk sac, embryo brain, and liver

To assess the reliability of the injection procedure in causing an increase in vitamin E yolk concentration, and consequently on yolk TAC, we first analyzed whether the concentration of vitamin E and TAC in the residual yolk sac differed between sham- and vitamin E-injected eggs. We used the yolk sac samples from 66 embryos ( $n = 26$  nests). As expected, vitamin E concentration was significantly higher in vitamin E treated eggs compared to controls ( $F_{1,44.5} = 4.314$ ;  $P = 0.044$ ) (Figure 1A). Neither sex ( $F_{1,38} = 1.162$ ;  $P = 0.286$ ) nor laying sequence ( $F_{1,36} = 0.841$ ;  $P = 0.438$ ) affected yolk sac vitamin E concentrations. In addition, we estimated the total amount of vitamin E in the yolk as the product of vitamin E concentration (expressed in  $\mu\text{g/g}$ ) and the yolk mass estimated according to the relationship described above (see Materials and Methods Section). The total amount of yolk vitamin E was significantly higher in vitamin E treated eggs compared to controls ( $F_{1,44.3} = 4.623$ ;  $P = 0.037$ ), while neither sex ( $F_{1,50.7} = 0.905$ ;  $P = 0.346$ ) nor laying sequence ( $F_{1,48.7} = 0.899$ ;  $P = 0.413$ ) affected mass of vitamin E in the yolk. Accordingly, vitamin E supplementation caused a significant increase of TAC in residual yolk sac (Figure 1B;  $F_{1,36} = 4.298$ ;  $P = 0.045$ ), while no significant effect of embryo sex ( $F_{1,38} = 0.01$ ;  $P = 0.929$ ) or laying order ( $F_{1,36} = 1.25$ ;  $P = 0.298$ ) was found. Since vitamin E is efficiently transferred from yolk to developing embryos, we also measured its concentration in brain and liver. The concentrations of vitamin E measured in focal organs from vitamin E-treated embryos soon before hatching were not significantly higher than controls in the brain ( $F_{1,59} = 1.707$ ;  $P = 0.196$ ) or in the liver ( $F_{1,35} = 0.305$ ;  $P = 0.584$ ), and did not vary according to sex (brain:  $F_{1,59} = 0.083$ ;  $P = 0.775$ ; liver:  $F_{1,38} = 0.107$ ;  $P = 0.746$  for liver), laying order (brain:  $F_{1,35} = 0.066$ ;  $P = 0.936$ ; liver:  $F_{2,42} = 2.561$ ;  $P = 0.089$ ) or their interactions (all  $P > 0.05$ ), which were removed from the LMM.

### Effect of vitamin E on embryo morphology and oxidative status

The sample included 26 clutches, 15 of which had 3 eggs while the remaining 11 clutches had 2 eggs. The proportion of eggs that reached the cracking stage did not differ significantly between the control (proportion of eggs at cracking =  $30/66 = 0.455$ ; 95% confidence interval =  $0.335\text{--}0.575$ ) and the vitamin E-injected eggs ( $36/66 = 0.545$ ; 95% confidence interval =  $0.424\text{--}0.575$ ;  $\chi^2_1 = 0.76$ ,  $P = 0.384$ ). In a LMM where clutch identity was included as a random effect, egg mass did not differ between the experimental groups



**Figure 1.** Marginal means ( $\pm$  SE) of (A) concentration of total vitamin E ( $\mu\text{g g}^{-1}$  wet weight) and (B) total antioxidant capacity (TAC -  $\mu\text{M}$  Trolox Eq.  $\text{g}^{-1}$  wet weight) in the residual yolk sac from the embryos at the cracking stage. Sample sizes are reported. Significant differences between vitamin E and control embryos are indicated by the asterisk (\* $P < 0.05$ ).

( $F_{1,36} = 0.40$ ,  $P = 0.530$ ) but significantly declined with laying order ( $F_{2,36} = 36.41$ ,  $P < 0.001$ ; estimated marginal means (SE): a-eggs:  $91.8 (1.25)$ ; b-eggs:  $90.1 (1.21)$ ; c-eggs:  $83.8 (1.28)$ ). The sex ratio (proportion of males) did not differ significantly between the experimental groups (controls:  $10/30 = 0.333$ ; 95% confidence interval =  $0.164\text{--}0.502$  and vitamin E:  $19/36 = 0.528$ ; 95% confidence interval =  $0.365\text{--}0.691$ ;  $\chi^2_1 = 1.78$ ,  $P = 0.182$ ).

A LMM of embryo body mass showed no significant effect of the interactions among fixed-effect factors (Table 1; Figure 2). The reduced model, retaining the main effects of sex, treatment and laying order, showed a statistically significant difference in body mass of embryos between the experimental groups ( $F_{1,38} = 4.19$ ,  $P = 0.048$ ; control embryos:  $43.2 (1.03)$  and vitamin E embryos  $45.0 (0.99)$ ). There was large among-clutch variation in body mass (Likelihood ratio test;  $\chi^2_1 = 21.00$ ,  $P < 0.001$ ). No significant effect on tarsus length was found (Table 1). LMM of embryo liver and brain mass revealed no significant effect of vitamin E treatment (liver:  $F_{1,38} = 0.17$ ;  $P = 0.678$ ; brain:  $F_{1,39} = 0.08$ ;  $P = 0.784$ ).

No significant effect of vitamin E treatment, embryo sex, laying order, and their interactions was found for all the considered oxidative stress endpoints in both the target organs, with the exception for a significant effect of laying order on TOS in the liver, and of sex on TOS in the brain (Table 2).

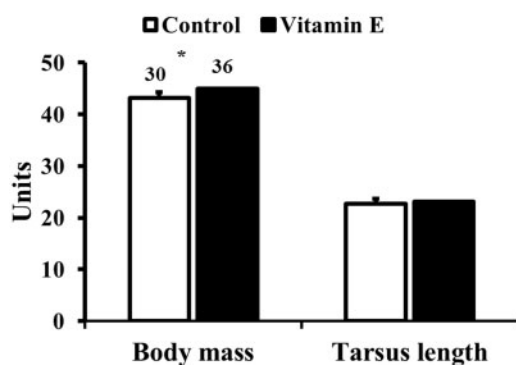
## Discussion

We experimentally increased vitamin E concentrations within physiological limits in yolks of yellow-legged gull eggs and found



**Table 1.** LMM of morphological traits of embryos at the cracking stage in relation to vitamin E treatment, sex of the embryo, and laying order. Clutch identity was included in the model as a random intercept effect. We controlled for egg mass at the time of laying by including it as covariate in the models. The non-significant effects of the 2-way interactions were excluded from the final model. Significant effects are reported in bold

Morphological traits	Body mass			Tarsus length		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Final model						
Treatment	4.19	1, 38	0.048	1.54	1, 38	0.222
Sex	0.34	1, 41	0.565	0.64	1, 42	0.430
Laying order	1.68	2, 47	0.198	2.01	2, 46	0.145
Excluded terms						
Treatment × sex	0.08	1, 48	0.777	0.37	1, 47	0.545
Treatment × laying order	1.14	2, 51	0.329	0.49	2, 50	0.613
Sex × laying order	2.83	2, 49	0.069	1.61	2, 50	0.210



**Figure 2.** Marginal means ( $\pm$  SE) of body mass (g) and tarsus length (mm) at the cracking stage of embryos from control or vitamin E injected eggs. Sample sizes are reported. Significant differences between vitamin E and control embryos are indicated by the asterisk (\* $P < 0.05$ ).

that the supplementation of this exogenous antioxidant promoted growth of embryos at late prenatal stages, while it did not affect oxidative status of their brains or livers.

A number of studies of captive and free-living birds have shown that vitamin E supplementation via the maternal diet increased the concentration of this antioxidant in the egg yolk and in embryonic tissues, promoting somatic growth at hatching (Surai et al. 1999b; Larcombe et al. 2010; Noguera et al. 2011; Surai and Fisinin 2013; Surai et al. 2016). However, these studies manipulated vitamin E availability to mothers and tested the effect of dietary vitamin E supplementation on the offspring. This approach integrates information on the direct effect of maternal vitamin E on progeny with indirect effects mediated by the consequences of increased availability of dietary vitamin E on maternal physiology. In contrast, our *in ovo* injection approach reveals the direct effects of vitamin E on the offspring, independently of maternal physiology (Surai et al. 1998; Blount 2004). In addition, several experiments (e.g., Cherian and Sim 1997; Surai et al. 1999a; Selim et al. 2012; Goel et al. 2013), mainly in captivity, applied supra-physiological vitamin E doses, hampering the ecological and evolutionary interpretation of maternal effects mediated by egg vitamin E content. In designing our experiment we, therefore, paid special attention to scale the injection amount of vitamin E to natural variation, as well as according to

estimated yolk size and to position in the laying sequence. Thus, we are confident that our vitamin E supplementation caused a post-manipulation concentration that did not exceed the upper limit of the natural range of variation, at least in the vast majority of the eggs.

The injection of a physiological dose of vitamin E into the yolk caused an increase in embryo body mass around hatching, independently of egg laying order and mass of the original egg. Since the concentration of vitamin E in yellow-legged gull eggs from the same colony declines with laying order, showing a 1.6-fold difference between the second- and third-laid eggs (Rubolini et al. 2011), a more marked positive effect of vitamin E supplementation on somatic growth of embryos from third-laid eggs was expected. In fact, previous evidence showed that chicks hatched from third-laid eggs injected with vitamin E were heavier and had significantly longer tarsi than controls, whereas vitamin E treatment had no effect on the size of chicks from first- or second-laid eggs (Parolini et al. 2015). These positive effects on morphological traits of chicks from the third-laid eggs suggest that the concentration of vitamin E in first- and second-laid eggs at hatching is close to optimal, whereas in the third-laid eggs is sub-optimal. In contrast, the results from embryos suggest that during pre-hatching development the concentration of vitamin E in the yolk might be sub-optimal for somatic growth, and the administration of an additional dose is beneficial to growth independent of position in the laying sequence.

From a functional perspective, maternal allocation of vitamin E to the eggs may serve to increase body size in late prenatal stages and to enhance post-hatching growth. Yet, the mechanisms underlying the positive effect of vitamin E supplementation on embryo (and chick) body size remains to be elucidated. Although no information is available for embryos of any bird species, vitamin E may increase the efficiency of conversion of egg materials into somatic tissues, as suggested for commercial Muscovy ducks during the first 2 weeks after hatching (Selim et al. 2012). Alternatively, vitamin E supplementation may reduce the production of pro-oxidant molecules, preventing oxidative stress. During early developmental stages, embryos and chicks are particularly prone to suffering oxidative stress because of high metabolic rates and the onset of aerobic respiration at hatching, implying that they need efficient antioxidant protection particularly during the late embryo and the early post-hatching stages (Panda and Cherian 2014). The overproduction of pro-oxidants and the consequent oxidative imbalance should be detrimental to developmental and growth processes (Smith et al. 2016). The latter hypothesis is supported by a number of studies showing that vitamin E supplementation improves antioxidant defense increasing superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity, preventing negative effects of LPO in broiler chicks (Sodhi et al. 2008; Tsai et al. 2008). Since oxidative stress has been suggested to limit growth rates (Alonso-Alvarez et al. 2007), enhanced body mass in vitamin E-treated embryos (and hatchlings) likely reflects the antioxidant properties of tocopherols (Marri and Richner 2015). Thus, vitamin E may protect lipid membranes from the harmful effects of ROS, allowing increased lipid utilization for energy production (Schaal 2008) to be used in somatic growth. However, the present results on oxidative status markers do not support this interpretation. While TAC was found to be larger in residual yolk from vitamin E-injected eggs (Figure 1B), TAC, TOS, and oxidative damage to lipids and proteins in the brain and in the liver were not affected by vitamin E supplementation (Table 2). The lack of significant effects on oxidative status markers may depend on the amount of residual yolk in eggs at the cracking stage. Thus,

**Table 2.** LMM of oxidative status markers in the liver and brain of embryos at the cracking stage in relation to vitamin E treatment, sex of the embryo, and laying order. Clutch identity was included in the model as a random intercept effect. We controlled for egg mass at the time of laying by including it as covariate in the models. The non-significant effects of the 2-way interactions were excluded from the final model. Significant effects are reported in bold

Oxidative status markers	TAC			TOS			PCO			LPO		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
<b>Liver</b>												
Final model												
Treatment	1.33	1, 37	0.257	0.16	1, 40	0.694	0.10	1, 38	0.750	1.45	1, 31	0.237
Sex	0.03	1, 40	0.861	0.15	1, 45	0.701	0.29	1, 41	0.595	0.68	1, 34	0.417
Laying order	1.07	2, 46	0.350	3.98	2, 48	<b>0.025</b>	0.25	2, 43	0.780	0.20	2, 39	0.816
Excluded terms												
Treatment × sex	0.01	1, 45	0.938	0.75	1, 49	0.391	0.22	1, 42	0.642	0.62	1, 39	0.435
Treatment × laying order	0.13	2, 49	0.880	0.14	2, 51	0.872	2.98	2, 46	0.061	0.36	2, 43	0.697
Sex × laying order	0.65	2, 46	0.526	1.88	2, 52	0.163	1.98	2, 46	0.150	0.32	2, 38	0.729
<b>Brain</b>												
Final model												
Treatment	1.03	1, 37	0.316	0.26	1, 40	0.616	1.65	1, 41	0.206	2.30	1, 38	0.138
Sex	0.20	1, 41	0.657	6.24	1, 44	<b>0.016</b>	2.13	1, 42	0.152	0.20	1, 41	0.655
Laying order	0.01	2, 46	0.987	0.07	2, 48	0.932	0.73	2, 44	0.486	0.46	2, 47	0.636
Excluded terms												
Treatment × sex	1.79	1, 47	0.187	0.96	1, 50	0.332	0.02	1, 49	0.902	0.32	1, 46	0.576
Treatment × laying order	1.81	2, 50	0.174	1.91	2, 52	0.158	0.77	2, 46	0.468	0.12	2, 50	0.889
Sex × laying order	2.11	2, 48	0.133	0.96	2, 53	0.390	0.24	2, 50	0.790	0.43	2, 48	0.650

the small amount of yolk adsorbed by the embryos up to the cracking stage may have limited the transfer of an “effective” dose of vitamin E, able to affect the oxidative status and to reduce oxidative damage of developing embryos. In fact, although the amount of vitamin E was higher in the residual yolk sac of injected eggs compared to controls (Figure 1), no significant differences were measured in brains or livers dissected from embryos. Indeed, our results may suggest that maternally transferred vitamin E concentration up to late prenatal stages seems to be optimal in preventing the occurrence of oxidative damage, and embryos may use the supplemental vitamin E dose to promote somatic growth rather than to limit the detrimental consequences of oxidative stress. These findings are consistent with those reported in a study of red-winged blackbird *Agelaius phoeniceus* nestlings treated with an antioxidant-enriched diet (Hall et al. 2010). The lack of positive consequences of increased vitamin E concentration on oxidative status markers do not lessen the role of vitamin E in protecting embryo by oxidative stress during prenatal development. It simply suggests that vitamin E concentrations transferred from yolk to embryo tissues during pre-hatching development could show its beneficial effects after hatching. In fact, yolk vitamin E is effectively transferred to the embryo and its initial concentration determines the reserve of the chick at least for the first week post-hatch (Surai et al. 1997). For instance, the highest concentrations of vitamin E in the liver occur at hatching and protect chicks from the adverse effects of oxidative stress for up to 2 weeks post-hatching (Surai et al. 1998). Thus, since newly hatched chicks are not able to effectively assimilate vitamin E from the diet and are dependent on their reserve built during embryonic development (Surai 2002), its accumulation in the embryo tissues, mainly in liver, is considered an adaptive mechanism providing antioxidant defense in the critical time of hatching (Surai et al. 1996).

In conclusion, our findings suggest that physiological variation in maternally transmitted vitamin E has no major effect on embryo oxidative status in two major target organs, that is, the liver and brain. In addition, they show that a physiological increase in yolk vitamin E

concentration boosts embryonic somatic growth, consistent with previous findings on hatchlings. The conspicuous differences in the effects of maternal vitamin E on offspring phenotype occurring between the prenatal and the early postnatal life stages, which may differ according to hatching order, should suggest that vitamin E is of primary importance mainly during post-hatching periods. However, the partial inconsistency of the present results compared to some previous experimental studies of birds suggests that further studies are required to assess the role of this maternally transferred antioxidant during early life periods under a natural selection regime.

## Supplementary material

Supplementary material can be found at <http://www.cz.oxfordjournals.org/>.

## Author contribution

M. P. participated in field activity, performed biochemical analyses and wrote the article; C. D. P. and G. C. performed biochemical analyses; F. K. performed analyses to assess vitamin E concentration in yolk eggs; M. R. and M. C. performed the field experiment; I. D. D. and A. M. supervised biochemical analyses and contributed writing the article; D. R. participated in field activity and helped to write the article; N. S. designed the experiment, helped to perform statistical analyses, and to write the article and supervised both field and laboratory work.

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