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RESEARCH ARTICLE

Antioxidants and embryo phenotype: is there experimental evidence for strong integration of the antioxidant system?

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ABSTRACT

Organisms have evolved complex defense systems against oxidative stress. Bird eggs contain maternally derived antioxidants that protect embryos from oxidative damage. The antioxidant system components are thought to be integrated, but few studies have analyzed the covariation between antioxidant concentrations, embryo 'oxidative status' and morphology. In addition, no study has tested the effects of experimental change in yolk antioxidant concentration on other antioxidants, on their reciprocal relationships and on their relationships with embryo oxidative status or growth, which are expected if antioxidants defenses are integrated. In yellow-legged gull (*Larus michahellis*) embryos, we analyzed the covariation between several antioxidants, markers of 'oxidative status' [total antioxidant capacity (TAC), concentration of pro-oxidants (TOS), lipid peroxidation (LPO) and protein carbonylation (PC)] in the yolk, liver and brain, and morphology. Yolk and liver antioxidant concentrations were positively correlated reciprocally and with embryo size, and positively predicted TAC but not oxidative status. TOS and LPO were positively correlated in the liver, while TAC and LPO were negatively correlated in the brain. Weak relationships existed between antioxidants and TOS, PC and LPO. The effects of antioxidants on oxidative status and morphology were non-synergistic. An experimental physiological increase in yolk vitamin E had very weak effects on the relationships between other antioxidants or oxidative status and vitamin E concentration, the concentration of other antioxidants or oxidative status; the covariation between other antioxidants and oxidative status, and relationships between morphology or oxidative status and other antioxidants, challenging the common wisdom of strong functional relationships among antioxidants, at least for embryos in the wild.

KEY WORDS: Bivariate mixed models, *Larus michahellis*, Maternal effects, Morphological traits, Oxidative status, Vitamin E

INTRODUCTION

Organisms are exposed to oxidizing agents originating from the external environment and also from their internal physiological milieu (Halliwell and Gutteridge, 1999). Because oxidation of biological molecules can result in loss of biological function, selection has promoted the evolution of complex physiological adaptations to prevent or reduce propagation, or repair oxidative damage (Costantini, 2014).

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Antioxidant defenses of vertebrates consist of two major classes of mechanisms and the associated effector molecules. First, enzymatic defense pathways, mainly mediated by endogenous substances, remove reactive molecular species or their intermediate derivatives, or catalyze their transformation into less active compounds (Halliwell and Gutteridge, 2007; Surai, 2000). Second, non-enzymatic antioxidants act as cofactors of antioxidant enzymes, remove metal ions, or undergo oxidation to quench free radicals and other reactive species. Several non-enzymatic antioxidants cannot be synthesized by animals and are therefore acquired either via the food or, before hatching, from the maternal egg materials (Møller et al., 2000; Surai, 2002).

While antioxidant defense is thought to be important throughout an organism's life, this is especially the case during embryo development and growth because intense embryonic metabolism entails massive production of oxidizing molecules, and inefficient defense from oxidative damage can have long-lasting, negative fitness consequences (Surai, 2002). The eggs of vertebrates contain large amounts of antioxidants of maternal origin (Surai, 2002). Mothers are expected to tend to optimally equip their eggs with exogenous antioxidants, under the constraints set by trade-offs with their self-maintenance requirements, dietary limitation of antioxidants and other environmental effects (Mousseau and Fox, 1998; Müller et al., 2012; Surai, 2002). Transfer of antioxidants to the eggs is therefore part of complex epigenetic 'maternal effects' whereby mothers modulate offspring performance and phenotype.

The developmental and growth consequences of variation in the concentration of egg yolk antioxidants have been at the focus of increasing interest in ecological evolutionary studies and in animal production disciplines (Ebrahimi et al., 2012; Müller et al., 2012; Romano et al., 2008; Saino et al., 2002, 2003; Selim et al., 2012; Surai, 2002). Some studies have investigated the consequences of variation in maternal dietary antioxidants on egg composition and subsequent offspring performance (Blount et al., 2002). Other studies have manipulated the concentration of specific antioxidants in the yolk and recorded the behavioral, growth or physiological consequences in the offspring (de Ayala et al., 2006; Gao et al., 2013; Romano et al., 2008; Saino et al., 2003).

The antioxidant system is thought to operate in a highly integrated way, meaning that functional relationships occur among antioxidants and their physiological pathways (Surai, 2002). For example, vitamin E can be recycled to its non-oxidized form by other antioxidants (e.g. ascorbic acid, carotenoids; Palozza and Krinsky, 1992; Surai, 2002). Functional integration also implies that different antioxidant pathways may operate in a synergistic way, if the effect of one antioxidant depends on the concentration of other antioxidants.

Functional relationships among exogenous antioxidants lead to the expectation that mothers should tune not only the absolute amount of antioxidants that they allocate to the eggs, but also their

Journal of Experimental Biology (2017) 220, 615-624 doi:10.1242/jeb.146498

List of abbreviations

BLMM	bivariate linear mixed model
LMM	linear mixed model
LPO	lipid peroxidation
PC	protein carbonylation
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances (method)
TOS	total concentration of pro-oxidant molecules

relative concentrations, so as to achieve an optimal balance. A corollary expectation is therefore that variation in the concentration of individual antioxidants alters the functional relationships between other interacting antioxidants. Moreover, the patterns of covariation among the concentrations of different antioxidants can vary according to embryo sex (Berthouly et al., 2008; Martínez-Padilla and Fargallo, 2007; McGraw et al., 2005) and laying order (Rubolini et al., 2011). This is expected because maternal physiology limits the ability to transfer antioxidants to eggs that are laid in rapid sequence, and/or because mothers adopt adaptive strategies of allocation of critical resources to eggs with different expected reproductive value (see Rubolini et al., 2011). Finally, variation in the concentration of a specific antioxidant can affect the distribution of other antioxidants across bodily districts, and also their covariation with embryo traits (such as growth) and oxidative status.

Despite evidence that optimal functioning of antioxidant defenses depends on the relative concentration of the individual antioxidants, studies of the patterns of correlation among functionally related egg components are rare (Rubolini et al., 2011). Moreover, the consequences of experimental supplementation of antioxidants on the distribution of other antioxidants and on their effects on markers of oxidative status are largely unknown. Indeed, notwithstanding considerable interest in the analysis of variation in pre-natal exposure to antioxidants and oxidative status, several important issues still need to be tackled.

Here, we therefore strived to answer the general questions that are detailed below and are graphically, qualitatively illustrated in Fig. 1. We capitalize on an experiment on the yellow-legged gull (*Larus michahellis* Naumann 1840) where we injected the egg yolk with physiological vitamin E (α- and γ-tocopherol) doses. We collected tocopherol-supplemented and control eggs shortly before hatching and dissected them to measure embryo morphology. The residual yolk in the yolk sac (ca. 70% of the estimated original yolk mass), the liver and brain were dissected to measure the concentration of vitamin E (α- and γ-tocopherols and the corresponding tocotrienols), carotenoids (mainly lutein, zeaxanthin and β-carotene; see Rubolini et al., 2011), retinol (vitamin A), coenzyme Q10 and ascorbic acid (vitamin C) were measured only in yolk and brain, respectively. Oxidative status was assessed by measuring total antioxidant capacity (TAC), the concentration of total pro-oxidant molecules (TOS, according to the terminology by Erel, 2005), protein carbonylation (PC) and lipid peroxidation (LPO), in the liver and in the brain. TAC was also measured in residual yolk. We focused on liver because it is the main organ where antioxidants are stored, and on brain, because it is believed to be particularly sensitive to lipid peroxidation (Surai, 2002).

The following questions were addressed.

(Q1) Do embryo morphological traits covary with the concentration of egg antioxidants or oxidative status? We expected embryo growth to be positively predicted by the antioxidant concentrations and TAC, and negatively predicted by TOS and markers of oxidative damage (Fig. 1A; see also Rubolini et al., 2011).

(Q2) Do antioxidants and oxidative status markers covary within or between organs? We predicted that antioxidant concentrations and oxidative status markers would positively reciprocally correlate within and between organs (or the yolk) (Fig. 1B,C). This was expected because antioxidants are believed to be most often limiting in the diet, thereby causing mothers with larger access to dietary antioxidants to allocate more of them to all bodily districts.

(Q3) Does the concentration of antioxidants predict oxidative status? Higher concentrations of antioxidants were expected to be associated with better oxidative status (Fig. 1D), i.e. lower concentration of total pro-oxidant molecules and oxidative damage.

(Q4) Do antioxidants synergistically affect embryo growth and oxidative status? Because of limited information on the combined effects of different antioxidants on other embryo traits, we had no specific prediction on synergistic (i.e. interaction) effects of antioxidants (Fig. 1E).

(Q5) Does the concentration of one antioxidant affect the relationship between that particular antioxidant and other antioxidants? The increase in the concentration of one antioxidant could affect the distribution and use of other antioxidants, and thus the relationship of embryo traits with other antioxidants. However, we have no directional expectation on these relationships (Fig. 1F).

(Q6) Does egg supplementation with one antioxidant affect the concentration of other antioxidants or oxidative status, and do the effects depend on sex or laying order?

(Q7) Does egg supplementation with one antioxidant affect the covariation between other embryo traits?

(Q8) Does egg supplementation with one antioxidant affect the relationship between morphology or oxidative status and other antioxidants? An increase in the concentration of a focal antioxidant may differentially affect the use of other antioxidants (Fig. 1G,H), variation in other antioxidant or oxidative status (Fig. 1I), and thus the relationships between embryo traits and other antioxidants (Fig. 1J).

As for the consequences of experimental manipulation of egg vitamin E levels (Q6–Q8), because of limited information on interactions among antioxidants, we had no explicit directional predictions. However, the paradigm of the integration of the antioxidant system led us to expect functional interactions between components. We therefore tested for any such effects and decided to interpret any emerging pattern *a posteriori*.

We emphasize that the present study is conceived as an exploratory exercise of the relationships between antioxidants, oxidative status markers and morphology of the embryos also after manipulation of antioxidant concentrations to contribute filling a remarkable gap of studies asking the very general questions listed above.

The analyses that specifically refer to antioxidants in the yolk rest on the assumption that the antioxidant concentrations in the residual yolk at the stage when the eggs were collected, which is on average 70% of the original yolk mass, are proportional to the concentrations in the yolk at earlier times of embryo development. Because we are aware of no study where this has been tested and we have no reason to speculate that differential absorption of antioxidants from the yolk produced the relationships that we observed, this will be considered as an assumption.

MATERIALS AND METHODS

Field and experimental procedures

We studied a large colony of yellow-legged gull (*Larus michahellis*) in the Comacchio lagoon (NE Italy; 44°20′ N–12°11′ E) during March–May 2014. The colony was visited every second day and when a new egg was found, it was temporarily removed from the

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 615-624 doi:10.1242/jeb.146498

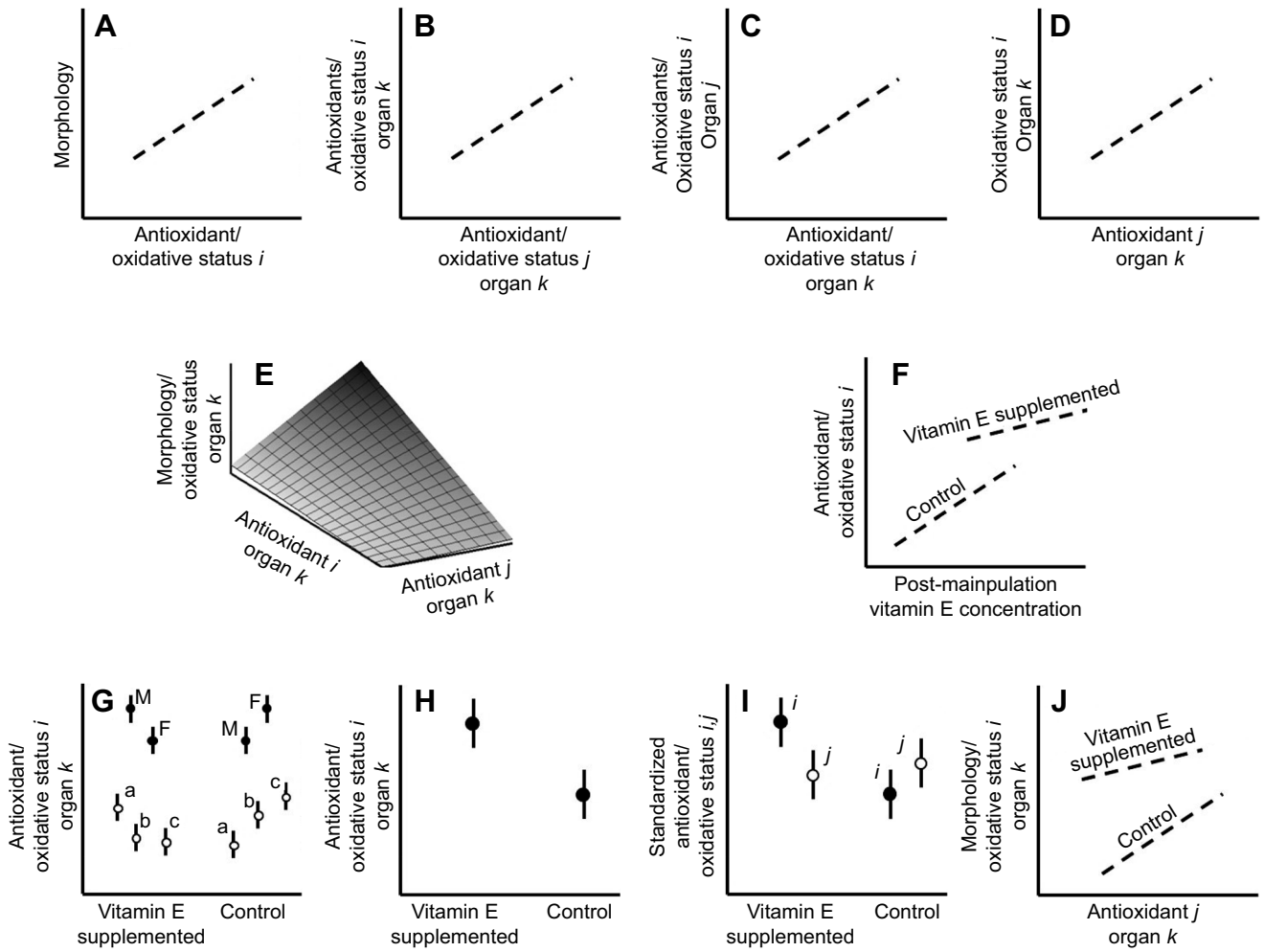

Fig. 1. Investigated relationships among antioxidant concentrations in the yolk, embryo morphological traits and oxidative status. The graphs are merely illustrative examples. M: males; F: females; a, b, c: first-, second-, third-laid eggs.

nest for experimental manipulation. The experiment was performed as described in Parolini et al. (2015). We aimed at increasing the concentration of vitamin E (α- and γ-tocopherol; 93:7 ratio) by 1 standard deviation of that measured in the eggs from the same colony (Rubolini et al., 2011), by *in ovo* injection, so that the final concentration of vitamin E was within the natural range of variation. The dose due to be injected was scaled depending on egg mass and laying order (Parolini et al., 2015) (Table S1).

We adopted a within-clutch design, whereby both control and vitamin E-injected groups 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 (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.

Egg collection and embryo dissection and measurement

When eggshell fractures appeared (ca. 24 days after laying), the eggs were collected and stored frozen (−20°C) until dissection. In the laboratory, we removed the eggshell and the residual yolk sac was detached from the embryo. Before dissection, the embryo was weighed and tarsus and skull lengths were measured using calipers. The liver and brain were explanted from the embryo, weighed and frozen at −80°C until biochemical analyses. All measurements were performed blind of embryo treatment, sex and laying order by a single operator to ensure consistency. Embryo sex was determined molecularly (Saino et al., 2008).

The study was performed under permission of the Parco Regionale del Delta del Po (no. 657, 4 February 2014). Although the Guideline on The Use and Euthanasia Procedures of Chicken/Avian Embryos draft by the Animal Care and Use Committee discourages hypothermia for euthanasia of avian embryos, we had to perform this procedure, placing the eggs into a −20°C freezer within 2 h of collection owing to facility constraints. As confirmed by 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. This was the case here because we performed a field experiment in which we could not euthanize embryos by methods such as carbon dioxide (CO_2), anesthetic agents or decapitation. Dissection occurred within 1 month of collection.

Antioxidant concentrations

As antioxidants, we measured vitamin E (α- and γ-tocopherol and -tocotrienols), retinol and carotenoid concentrations in liver, brain and residual yolk sac, while coenzyme Q10 and ascorbic acid were measured only in yolk and in brain, respectively. All the analyses were performed using high-performance liquid chromatography (HPLC), as described by Karadas et al. (2016) and Mitić et al. (2011; for ascorbic acid).

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 615-624 doi:10.1242/jeb.146498

Markers of oxidative status

As markers of oxidative status, we measured TAC, TOS, PC and LPO. These assays were performed on liver and brain homogenates, while only TAC was measured in residual yolk sac. Briefly, TAC and TOS were measured according to colorimetric methods developed by Erel (2004, 2005, respectively), with slight modifications. Carbonylated proteins were measured as described by Parolini et al. (2016), while lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979). However, it should be noted that the TBARS method may not measure oxidative damage to lipids accurately because TBA reacts with other compounds, apart from the main LPO byproduct malondialdehyde. Thus, TBARS results should be interpreted with caution because they may overestimate LPO (Halliwell and Gutteridge, 2007).

Statistical analyses

Given the complexity of the statistical analyses performed to answer the focal questions of the study (see Introduction), we present the analyses for each question separately. However, as we run the same correlation analyses to answer Q1, Q2 and Q3 (see above), these were grouped under a single heading.

Questions 1–3

The correlation between morphological traits, antioxidant concentrations and oxidative status markers (Fig. 1A–D) was analyzed using bivariate linear mixed models (BLMMs) where egg treatment, sex, laying order (factors) and egg mass (covariate) were included as independent effects and brood was included as a grouping factor, according to the procedure outlined in Dingemanse and Dochtermann (2013). Restricted maximum likelihood was adopted to estimate model parameters. The within-brood correlations were computed using the variance and covariance estimates according to eqn 7d in Dingemanse and Dochtermann (2013). The significance of the within-brood correlation coefficients was estimated by likelihood ratio tests (maximum likelihood estimation) (Dingemanse and Dochtermann, 2013). When BLMMs failed to converge (23% of the cases), we relied on correlation analyses.

We did not test all the possible 780 bivariate relationships and focused on the relationships between embryo morphology and antioxidants or oxidative status markers in all 'organs' (including the yolk); antioxidants and oxidative status markers within organs; and individual antioxidants or oxidative status markers between organs, yielding a total of 497 relationships (Fig. S1A–F). However, in calculating the number of relationships that were consistent or, conversely, opposite to the expectation, we did not consider total tocopherols or total tocotrienols but only the α and γ isoforms of these compounds separately, in order to avoid pseudo-replication of the information.

For easier visualization of the results of BLMM analyses, we graphically represented the relationships as ellipses in synoptic graphs included in Fig. S1. We refrained from estimating the between-brood correlations from BLMMs (Dingemanse and Dochtermann, 2013) because of the small size of the clutches (maximum three eggs) and the unbalanced sample of either sex or egg treatment within broods.

Question 4

Individual embryo morphological traits or oxidative status markers were analyzed in linear mixed models (LMMs) including vitamin E egg treatment, sex and laying order as factors and egg mass as a covariate. In the model we also included one pair at a time of antioxidant concentration variables together with their interaction (Fig. 1E). Clutch was included as a random effect. In these analyses we only considered total tocotrienols or tocopherols, because we assume that the effects of α and γ isoforms of either class of compounds are additive.

Question 5

We tested whether an experimental increase in yolk vitamin E concentration affected the relationship between embryo traits and (post-manipulation) vitamin E concentration (i.e. if the slope of the relationship between embryo traits and post-manipulation vitamin E concentration differed between control and vitamin E supplemented eggs; Fig. 1F) in LMMs where we included the effects of sex, laying order (factors), original egg mass (covariate) as well as vitamin E treatment, vitamin E post-manipulation concentration (covariate; total tocopherols only) and their two-way interaction. In the model we also included the random effect of clutch.

Question 6

We tested whether vitamin E treatment differentially affected the concentration of the focal substances and oxidative status markers depending on sex and laying order (Fig. 1G,H) in LMMs where we included the effects of vitamin E egg treatment, sex, laying order (factors), original egg mass (covariate) as well as the two-way interaction effects between factors. Clutch was included as a random effect. We also ran these models excluding the two-way interaction between factors.

Question 7

To test whether vitamin E treatment had a differential effect on the concentration of antioxidants or markers of oxidative status (Fig. 1I), we designed LMMs with treatment, sex, laying order (factors) and original egg mass (covariate) as predictors. In addition, we included a trait (factor) and its two-way interaction between treatment and trait. This analysis posed the problem that different traits can be incommensurable (being measured in different units) or have different means and/or variances. The values of each of the two focal traits were therefore standardized to a mean of 0 and variance of 1. These analyses were performed considering each pair of variables within organs. Thus, in these models, the trait-by-vitamin E treatment term tests whether an increase in vitamin E concentration caused a differential variation, expressed in standard deviation units, in the concentration of different antioxidants or oxidative status markers.

Question 8

We tested whether the relationship between morphological traits or oxidative status markers and antioxidants differed between vitamin E treatment groups (Fig. 1J) in LMMs where vitamin E egg treatment, sex, laying order (factors) and original egg mass (covariate) were included as predictors. In addition, in the model we included the effect of the specific antioxidant under scrutiny as well as its interaction with vitamin E treatment.

Multiple testing issues

Throughout this study, we performed a large number of tests. This was the case because this was admittedly an exploratory exercise where we described the relationships among many variables. Performing multiple tests can inflate the risk of incurring type I statistical errors. In contrast, lowering of the α-level of the tests according to commonly used procedures (e.g. Bonferroni correction) would cause excessive reduction of statistical power. We therefore adopted the approach taken, for example, by Cohen

Journal of Experimental Biology (2017) 220, 615-624 doi:10.1242/jeb.146498

et al. (2008): we present the results of the tests and qualify those with *P*-values <0.05 as 'significant'. However, we warn the readers that part of these 'significant' tests could be due to type I statistical errors. In addition, we focus on the general patterns of association among the variables and qualitatively check whether the relationships are consistent in sign with the expectation. To qualitatively summarize the information from so many tests, in analyses relevant to Q1–Q4 we report the number of tests that were statistically significant (see above), whose associated r was >0.15, or was such that $-0.15 \leq r \leq 0.15$, while distinguishing between the relationships that were in the direction predicted or, respectively, opposite to the expectation. Because very limited evidence for significant relationships emerged from the analyses used to answer Q5–Q8, the results of these analyses are only briefly summarized in the Results. All analyses were run in SAS 9.3 (see Dingemanse and Dochtermann, 2013 and references therein).

Meta-analyses of the relationships among morphological traits, antioxidants and oxidative status markers

We computed unsigned Fisher z-transformed correlation coefficients (Zr) for each relationship. When the direction of the observed correlation was consistent with the expectation, we assigned Zr a positive sign, whereas we assigned a negative sign when the direction was opposite to the expectation. We then tested whether mean Zr, weighted by n–3 (n=number of individuals in the correlation analysis), significantly deviated from 0 (Borenstein et al., 2009) within each set of correlations (see Results). Significance was implied when the confidence interval (CI) of the estimated mean Zr did not encompass 0.

RESULTS

Overall, the sample included 66 late-stage embryos from the eggs of 26 clutches (30 controls, 36 vitamin E-treated; 29 males, 37 females; 21 a-, 26 b-, 19 c-eggs). In some analyses, information for up to 10 eggs was not available; sample size thus ranged between 56 and 66 eggs depending on the analysis.

Question 1

Embryo morphological measures were generally positively correlated (relationship in Fig. 1A and Fig. S1A), as expected (n=10 relationships; positive: 90%; significantly positive: 60%; null: 10%), with mean Zr (0.372; CI: 0.249–0.495) being significantly larger than 0 (Fig. 2).

The concentration of carotenoids, tocopherols and γ-tocotrienols in the yolk were generally positively correlated with the morphological measures (relationship in Fig. 1A and Fig. S1B), consistent with expectations (n=45; expected positive direction: 58%; expected and significant: 33%; null: 36%; opposite: 7%; significantly opposite: 0%). Morphological measures also positively covaried with the concentration of retinol (body size and tarsus length only) and α- and γ-tocopherol in the liver. Also for the liver, the pattern of association of the morphological measures with the concentrations of antioxidants was mostly consistent with expectations (n=40; expected positive direction: 53%; expected and significant: 20%; null: 45%; opposite: 3%; significantly opposite: 0%). However, there were generally weak, inconsistent relationships between morphological traits and antioxidant concentrations in the brain (n=45; expected positive direction: 24%; expected and significant: 0%; null: 64%; opposite: 11%; significantly opposite: 4%). The mean Zr (0.141; CI: 0.105–0.177) for the relationships between antioxidants and morphological traits was significantly larger than 0 and thus consistent with the expectation (Fig. 2).

The correlations between embryo morphology and markers of oxidative status are shown in Fig. S1C (relationship in Fig. 1A). Embryo morphological traits showed a weak positive relationship with yolk TAC. As expected, TOS in the liver significantly negatively covaried with body mass, skull length and liver mass, and non-significantly negatively covaried with tarsus length, whereas the association with brain size was 'null'. LPO and PC in the liver did not show any clear pattern of association with morphological traits. TOS and LPO in the brain were negatively associated with brain size and also with liver size, as expected. Finally, body mass was also negatively associated with LPO in the brain. The mean Zr (0.070; CI: 0.010–0.131) for the relationships between morphological traits and markers of oxidative status was significantly larger than 0, again consistent with the expectation (Fig. 2).

Question 2

The correlations that we observed between pairs of antioxidants are summarized in Fig. S1D. Within (relationship in Fig. 1B) the yolk or the liver, the correlations between the concentrations of carotenoids, retinol, and α- or γ-tocopherols or -tocotrienols were mostly positive and consistent with expectations (yolk: n=15; expected direction: 60%; expected and significant: 53%; null: 40%; opposite: 0%; liver: n=15; expected direction: 60%; expected and significant: 20%; null: 40%; opposite: 0%) (total tocopherols and tocotrienols are not considered here as they are the sum of α and γ isoforms). In addition, the concentration of coenzyme Q10 in the yolk was positively correlated with yolk antioxidants (n=6; expected direction: 83%; expected and significant: 67%; null: 17%; opposite: 0%). Within the brain, the relationships were also mostly positive, as expected (n=15; expected direction: 60%; expected and significant: 27%; null: 20%), while some were in the direction opposite to the expectation (opposite: 20%; significantly opposite: 13%).

Between (relationship in Fig. 1C) yolk and liver or brain, the correlations of antioxidants (we considered concentrations of carotenoids, retinol and α- and γ-tocopherols or -tocotrienols isoforms separately) were also generally positive and consistent with the expectation, or null, but were never negative and in the direction opposite to the expectation (yolk–liver: n=18; expected direction: 44%; expected and significant: 22%; null: 56%; opposite: 0%; yolk–brain: n=18; expected direction: 39%; expected and significant: 17%; null: 61%; opposite: 0%). The correlations between liver and brain were generally weak (n=18; expected direction: 28%; expected and significant: 6%; null: 61%; opposite: 11%; opposite and significant: 0%). The mean Zr (0.266; CI: 0.189–0.343) for the relationships between pairs of antioxidants traits was significantly larger than 0, as expected (Fig. 2).

The correlations that we observed between pairs of oxidative status markers are summarized in Fig. S1F. There were generally weak relationships within organs between markers of oxidative status, with the exception of the expected positive relationship between TOS and LPO in the liver and the negative relationship between TAC and LPO in the brain. However, the relationship between TAC and TOS in the brain was statistically significantly positive, contrary to our expectation. No correlations for oxidative status markers emerged between organs. The mean Zr (0.003; CI: –0.092 to 0.099) for the relationships between oxidative status markers did not significantly deviate from 0, contrary to the expectation (Fig. 2).

Question 3

The observed correlations (relationship in Fig. 1D) are summarized in Fig. S1E. Antioxidant capacity in the yolk

Journal of Experimental Biology (2017) 220, 615-624 doi:10.1242/jeb.146498

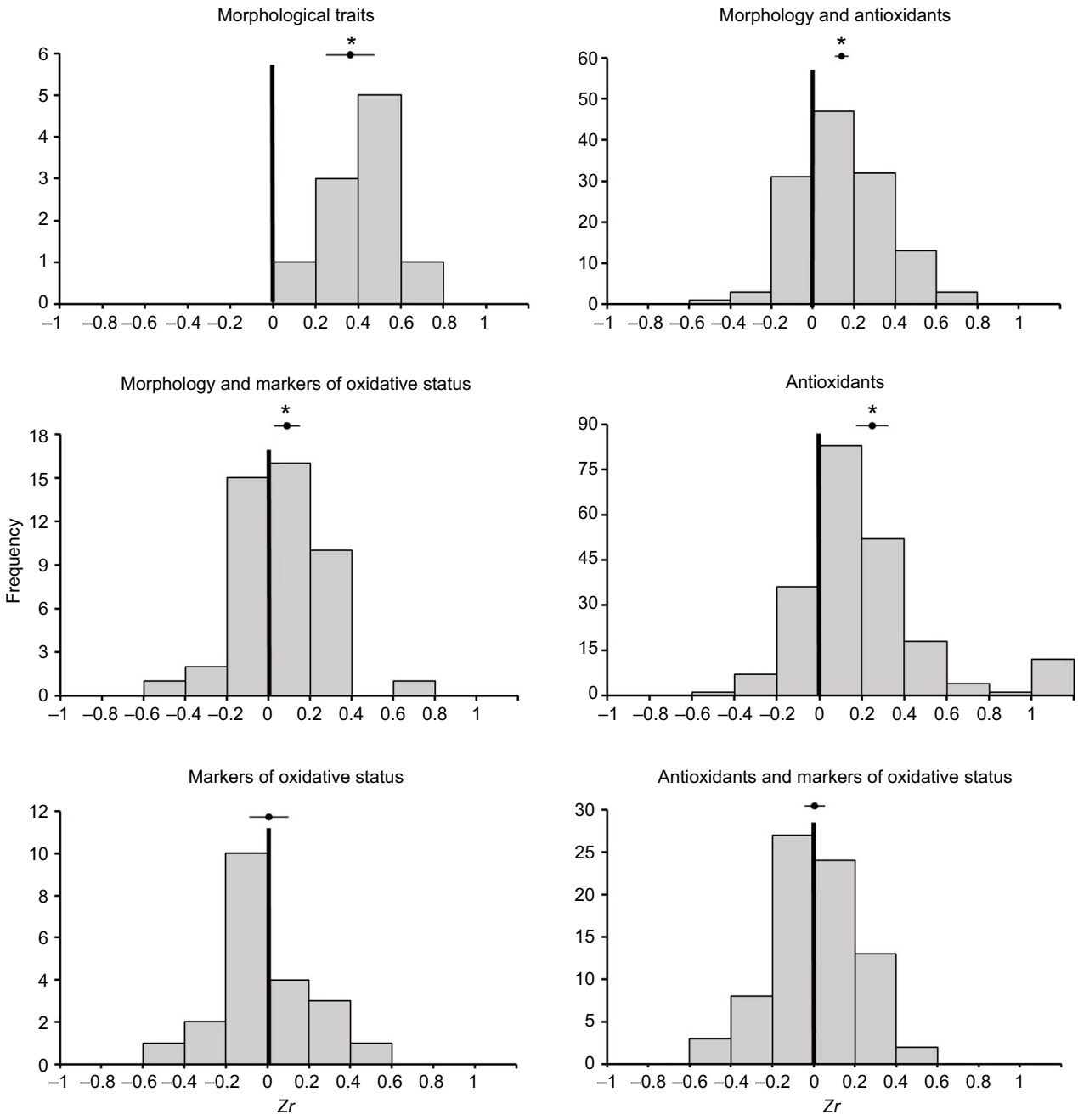

Fig. 2. Frequency of Fisher *z*-transformed correlation coefficients (*Zr*) calculated for the relationships among morphological traits, between morphology and antioxidants, between morphology and markers of oxidative status, among antioxidants, among markers of oxidative status, and between antioxidants and markers of oxidative status. For the relationship among antioxidants, we pooled *Zr* values >1 into a single class. When the direction of the observed correlation was consistent with the expectation (see Introduction), we assigned *Zr* a positive sign, whereas we assigned a negative sign when direction was opposite to the expectation. The mean *Zr* value (black circle) is reported with its 95% confidence interval (CI). Significance (*) is implied when the estimated CI did not encompass zero.

positively covaried with the concentration of most of the focal antioxidants (total tocopherols and tocotrienols are not considered here as they are the sum of α and γ isoforms), although statistical significance was attained in just one case (n=7; expected direction: 57%; expected and significant: 14%; null: 43%). Similarly, in the liver, TAC was positively predicted by the concentration of all antioxidants but in no test was statistical significance attained (n=6; expected direction: 100%; expected and significant: 0%). A positive, expected association between TAC in the brain and antioxidants was observed for half of the relationships (n=7; expected direction: 43%; expected and significant: 14%; null: 29%; opposite: 29%; significantly opposite: 0%).

There were generally weak, inconsistent relationships between antioxidants and markers of oxidative status in the liver (n=18; expected direction: 6%; expected and significant: 0%; null: 67%; opposite: 28%; significantly opposite: 17%) or in the brain (n=21; expected direction: 5%; expected and significant: 0%; null: 57%; opposite: 38%; significantly opposite: 5%; Fig. S1E). The mean z (0.015; CI: –0.033 to 0.063) for the relationships between

antioxidants and markers of oxidative status did not significantly differ from 0, contrary to the expectation (Fig. 2F).

Question 4

There was only very weak evidence that the statistical effects of antioxidants on embryo traits were synergistic (relationship in Fig. 1E). In fact, out of 135 LMMs testing the interaction between pairs of antioxidants on morphological traits or oxidative status markers, only in three cases did the interaction effect between antioxidants attain statistical significance (see Table S2).

Question 5

Experimental increase in vitamin E yolk concentration could affect the relationship between post-manipulation vitamin E concentration and the concentration of the other antioxidants or oxidative status markers (relationship in Fig. 1F). In LMMs, we found generally no statistically significant effects of treatment by vitamin E concentration in the yolk, with the exception of a significant interaction effect on α-tocotrienol or total tocotrienol concentration in the yolk (Table S3). The relationships were marginally non-significantly positive in control eggs and non-significantly negative in vitamin E eggs (Table S3).

Question 6

We tested whether egg treatment differentially affected the concentration of antioxidants and the oxidative status markers depending on sex and position in the laying sequence (relationship in Fig. 1G). In no case did we find a significant effect of the egg treatment by sex or the egg treatment by laying order interactions in LMMs ($P>0.05$).

LMMs testing the main effect of egg vitamin E treatment on the concentrations of the focal substances in the yolk (excluding tocopherols) and in the organs (relationship in Fig. 1H) generally did not disclose significant effects ($P>0.05$), with the exception of the concentration of α-tocotrienol ($F_{1,35}=4.83$, $P=0.035$) and total tocotrienols ($F_{1,35}=4.46$, $P=0.042$) in the liver, which was significantly larger in vitamin E eggs.

Hence, vitamin E treatment did not generally affect the relationship between vitamin E concentration post-treatment and the concentration of the focal antioxidants or oxidative status markers in the yolk and organs. In addition, vitamin E treatment did not affect the concentration of the other compounds in the yolk or in the liver and brain.

Question 7

In general, there was only weak evidence for a differential variation of pairs of antioxidants/oxidative status markers (values standardized to a mean of 0 and variance of 1) between the control and the vitamin E supplemented eggs (relationship in Fig. 1I). γ-tocotrienol concentration and LPO in the liver declined after vitamin E supplementation, while concentration of α-tocotrienol increased. In addition, the total antioxidant capacity in the brain declined in vitamin E-treated embryos, while TAC increased in the yolk. It must be emphasized that these differential patterns of variation among the endpoints following vitamin E treatment with respect to controls emerged out of a large number ($n=497$) of tests and should therefore be considered with caution.

Question 8

Out of 122 LMMs of morphological or oxidative status traits, the two-way interaction between vitamin E treatment and antioxidant concentrations (the interactions were tested for all the possible pairs of antioxidants; relationship in Fig. 1J), had a significant effect in only 12 cases (Table S4). Thus, there was weak overall evidence for differential effects of individual antioxidants depending on the experimental manipulation of vitamin E in the egg.

DISCUSSION

In this study of the yellow-legged gull, we explored the patterns of covariation among late-stage embryo morphological traits, concentration of antioxidants and oxidative status markers in focal embryo organs and in the yolk. In addition, we manipulated the concentration of yolk vitamin E to test for the consequences of variation in a major antioxidant on the distribution and use of the other antioxidants and on oxidative status markers in the yolk and focal organs. We found evidence that the embryo growth was positively associated with the antioxidant concentrations, and weaker evidence that it was negatively predicted by markers of oxidative status (Q1). Consistent with the expectations, the antioxidant concentrations were positively correlated both within organs and between the yolk and the other organs (Q2). However, markers of oxidative status were only partly correlated within organs and not correlated between organs (Q2). TAC was positively associated with the concentration of antioxidants in the yolk, liver and also in the brain, but there was no clear pattern of association between TOS, LPO or PC and antioxidant concentration (Q3). Antioxidants did not synergistically predict embryo growth or oxidative status (Q4). Finally, experimental increase in egg vitamin E level did not affect the relationship between other antioxidants or markers of oxidative status and vitamin E concentration (Q5); the concentration of other antioxidants or the markers of oxidative status, also depending on sex and laying order (Q6); the covariation between other antioxidants or oxidative status markers (Q7); or the relationship between morphological traits or oxidative status and other antioxidants (Q8).

The main methodological novelty of our study is that we coupled information on traits (morphology, antioxidant concentration and oxidative status markers) of late-stage embryos with information on the concentration of antioxidants in the residual yolk, thereby investigating how late-embryo traits covary with the quality of the original egg environment. Below, we discuss the general findings of our study, but we do not go into the specific details of the individual relationships because part of the statistically significant correlations could have arisen because of an inflation of type I statistical errors.

Question 1

Consistent with the general expectation that antioxidants promote condition and thus embryonic growth (Bhanja et al., 2012; de Ayala et al., 2006; Noguera et al., 2011; Parolini et al., 2015; Selim et al., 2012), there were generally positive bivariate relationships between antioxidant (carotenoids, tocopherols) concentrations in the yolk and in the liver (retinol, tocopherols) and embryo morphological traits, while controlling for the effects of sex, laying order and egg mass. This relationship could be causal, as suggested by experimental studies (Deeming and Pike, 2013; Marri and Richner, 2014; Parolini et al., 2015; Romano et al., 2008; Saino et al., 2011). Alternatively, the size of an embryo may also reflect its degree of maturation, with larger/more mature embryos showing stronger antioxidant defenses. The observations that embryo size was negatively associated with TOS in the liver and that markers of oxidative status (PC in the liver, TOS and LPO in the brain) negatively predicted brain size are also consistent with expectations, because overproduction of pro-oxidants and the consequent oxidative imbalance should be detrimental to developmental and growth processes (Smith et al., 2016).

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Question 2

Eggs with relatively large concentrations of one antioxidant also tended to have relatively high concentrations of other antioxidants. In addition, the concentrations of antioxidants in the yolk tended to be positively correlated with those in the liver and brain. These results are consistent with those of a previous study where we considered yolk but not liver and brain composition because we relied on eggs at a very early incubation stage (Rubolini et al., 2011). Present findings suggest that some mothers have access to relatively large amounts of all antioxidants, and this results in large concentrations in the yolk and, concomitantly, in embryo organs (Costantini and Verhulst, 2009). Alternatively, in order to optimally accomplish their functions, the amounts of antioxidants that mothers allocate to the eggs must be balanced. Thus, dietary antioxidants may not be limiting, and mothers may decide to allocate different antioxidants to the eggs in amounts that are reciprocally positively correlated.

The relationships between markers of oxidative status were consistent with expectations, with, for example, markers of oxidative damage being reciprocally positively correlated within the brain and negatively correlated with TAC. Thus, particularly in the brain, embryos with large TAC have smaller oxidative damage to lipids. In addition, TOS in the liver was associated with more severe lipid peroxidation.

Importantly, the correlations between markers of oxidative status in the brain and liver were generally weak, implying that oxidative damage in a particular organ does not allow inference on oxidative status in other organs. This weak relationship may also suggest a sort of 'hierarchy' of protection and/or differential sensitivity of the organs to oxidative stress. The brain contains the highest concentration of double bonds, especially C_{20} and C_{22} polyunsaturated fatty acids, which exposes the brain to the risk of oxidative damage (Surai, 2002). Embryos may prioritize antioxidant protection of the brain, thereby uncoupling oxidative damage to the brain from oxidative damage to other organs.

Question 3

Notably, the correlation between antioxidants and markers of oxidative status was consistent with the expectations for TAC, but not for TOS, LPO and PC. This result implies that oxidative damage cannot be inferred by the concentration of antioxidants. Hence, large amounts of dietary antioxidants that mothers allocate to the eggs do not necessarily result in lower oxidative damage, possibly because oxidation of lipids and proteins also largely depends on antioxidant defense afforded by other physiological pathways, mediated by enzymatic activity (Costantini and Verhulst, 2009).

Question 4

By measuring several antioxidants in the yolk and organs, we could test whether the statistical effect of the concentration of one antioxidant on morphological traits or oxidative status depended on the concentration of other antioxidants. Assuming that the relationships between embryo morphology or oxidative status markers and antioxidants considered individually at least partly reflect causation (see above), the present findings of no statistically significant interaction effects between antioxidants suggest that the effects of individual antioxidants on embryo traits do not depend on the concentration of other antioxidants, i.e. there are no measurable synergistic effects between antioxidants on embryo traits. This result is further corroborated by the results of the egg vitamin E supplementation experiment (see below), implying small integration of the components of the antioxidant system, at least under the experimental conditions of the present study.

Questions 5–8

As the increase of egg vitamin E level did not affect embryonic traits, we collectively discuss the results relevant to Q5–Q8. To the best of our knowledge, this is the first study in which the effects of a direct manipulation of egg concentration of one major antioxidant on the destination of other antioxidants and their relationships with morphological traits and oxidative status have been investigated in any species in the wild. The main outcome of the experiments is that there is no evidence for major functional interactions among maternal egg antioxidants of dietary origin. A major strength of the present experiment is that manipulation of the concentration of vitamin E in the egg occurred within the physiological range and directly into the eggs, rather than via the mother.

Several experimental studies have suggested that interactions exist among different antioxidants as well as between these and other components of the antioxidant system (Surai, 2002). These interactions occur, for example, in the form of reciprocal modulation of absorption or retention of antioxidants in specific tissues; modulation of the effects of other dietary or endogenous antioxidants; and/or in recycling of oxidized to non-oxidized forms or protection from auto-oxidation (Catoni et al., 2008; Surai, 2000). As a result of these interactions, the effects of individual antioxidants on physiological or morphological endpoints should depend on the combined effects of individual antioxidants. These experiments have typically been performed on domestic and/or artificially selected strains (e.g. mice, poultry; Jacob, 1995; Surai, 2000) and under captive conditions, and have very seldom concerned the effects of pre-natal exposure to (egg) antioxidants on pre-natal phenotype. In addition, experimental design, in terms of dosage of dietary antioxidants, has seldom been framed in terms of natural variation, possibly partly owing to the lack of natural reference conditions for domestic strains. The lack of measurable interaction effects between antioxidants on embryo phenotype (morphology or oxidative status) or effects of an experimental increase of vitamin E on distribution and effects of other antioxidants (i.e. no evidence for synergist effects) challenges the common wisdom that major functional interactions occur between exogenous egg antioxidants in determining embryo phenotype, at least in this species in the wild, and within physiological limits of variation of antioxidant concentrations.

Previous studies of birds led to partly inconsistent results on the interactions among components of the antioxidant system. A correlative study demonstrated that the correlations among some antioxidants (uric acid, carotenoids and vitamin E) and TAC varied across species. Overall, TAC strongly covaried with uric acid levels, both across species and within 23 of 30 studied species, while carotenoid concentrations positively covaried both among and within species. In contrast to our findings, vitamin E concentration did not strongly correlate with other antioxidants or with TAC (Cohen et al., 2008). Studies of the Leach's storm petrel (*Oceanodroma leucorhoa*), a long-lived seabird, and of the Savannah sparrow (*Passerculus sandwichensis*), a short-lived migratory passerine, showed a significant correlation between vitamin E and total antioxidant capacity in the former but not in the latter species (Cohen et al., 2009a,b). All these findings, in combination with those from the present study, confirm the complexity of the relationships among antioxidants and phenotypic traits in birds.

Because vitamin E has a well-established role in antioxidant defense (Surai, 2002), we expected that vitamin E supplementation interfered with the relationships among embryo phenotypic traits and the concentration of other antioxidants or markers of oxidative status (see Introduction). One potential cause for the lack of such

interaction effects is the low dose of vitamin E that we administered. We emphasize that we deliberately used a dose that did not result in post-manipulation concentrations exceeding the natural range of variation because we aimed at investigating the effects of variation in egg composition in a natural ecological and evolutionary setting. An additional possibility is that exogenous maternal egg antioxidants are contained in the eggs at or above their maximal effective dose, and any increase in their concentration is therefore ineffective. This interpretation is contradicted by the general tenet that dietary antioxidants are limiting in maternal diet (Møller et al., 2000). While this may obviously not apply to the particular population we studied, this interpretation is further contradicted by the positive effect that vitamin E egg supplementation had on post-natal growth in the same population (Parolini et al., 2015). In addition, the positive relationships between embryo morphological traits and antioxidant concentrations that we observed may be causal. If that is the case, the concentrations of individual antioxidants cannot be considered to be (at least) the maximal effective ones and interactions between the effects of individual compounds should be expected. Moreover, antioxidant concentrations have been shown to decline with laying order (Rubolini et al., 2011). This pattern can be interpreted as evidence that dietary limitation and/or maternal physiological constraints result in suboptimal composition of at least the last laid eggs. Hence, the availability of antioxidants to the average embryo does not seem to correspond to the maximal effective concentrations. Finally, an additional possibility is that statistical power of the tests was too low to detect significant effects. We deem the size of the sample as large, also given the experimental nature of the study. Thus, if 'real' effects existed that went undetected, these must be of small intensity and therefore they do not alter the main message of the study, that no marked combined effects among antioxidants on morphological or oxidative status endpoints occur.

In conclusion, for the first time in any experimental study in the wild, we explored the patterns of covariation between different components of the embryonic antioxidant defense system in the yolk and specific organs. Residual egg yolk shortly before hatching and embryo organs consistently differed in their concentration of the different exogenous antioxidants that we considered, and the antioxidant concentrations positively covaried among the yolk and the liver or brain. Eggs with larger antioxidant concentrations hosted larger embryos and had larger antioxidant capacity but not relatively low values at markers of oxidative damage, suggesting that other components of the antioxidant system intervene, with overwhelming effects, in protecting the egg from oxidation.

Vitamin E is among the main exogenous antioxidants, with well-documented effects on functional interactions between the branches of the antioxidant system and on oxidative status. The lack of consequences of increased vitamin E concentration on the other antioxidants and oxidative status markers therefore obviously do not completely dismiss this role of vitamin E. Rather, they suggest that under a natural selection regime in the wild and in a non-artificially selected population, physiological variation in the concentration of one major antioxidant has minor, if any, effects on other components of the antioxidant system and on their consequences for embryo growth and oxidative status.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.S., D.R., M.P.; Methodology: C.D.P., M.P., M.C., F.K., G.C., A.M., I.D.D.; Resources: N.S.; I.D.D. and A.M.; Investigation: C.D.P., M.C., M.P., G.C., D.R. and N.S.; Statistical analyses: N.S., C.D.P., M.P. Writing–Original Draft: N.S.; Writing–Review and Editing: N.S., M.P. and C.D.P.; Supervision: N.S.

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Figure S1

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Table S1. Amount (µg) of vitamin E (α : γ – tocopherol ratio *per* egg) injected into the yolk of yellow-legged gull eggs depending on egg mass at the time of deposition and laying order (first, second or third egg is a-, b-, or c-egg, respectively). The doses were designed to increase the post-manipulation vitamin E concentration of 1 standard deviation compared to that previously recorded in the same population for each class of egg mass and position in the laying sequence.

Laying order	***Egg mass (g)***	***Vitamin E (µg)*** ***(α : γ - tocopherol)***
a-egg	84-91	670 (623:47)
	92-95	748 (696:52)
	96-108	697 (648:49)
b-egg	80-88	509 (473:36)
	89-92	699 (650:49)
	93-99	688 (640:48)
c-egg	75-82	305 (283:22)
	82-87	616 (573:43)
	88-98	643 (596:45)

Table S2. Summary of the results of linear mixed models of morphological or oxidative status traits on two-way interactions between antioxidant concentrations. In the models we also included the effect of vitamin E treatment, sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between antioxidants were significant are reported. Overall, the effects of the interaction were tested in 135 models for all the possible pairs of antioxidants.

	F	d.f.	*P*	Coefficients (SE)
Skull Length				
Treatment	1.48	1,32	0.23	
Sex	1.68	1,32	0.20	
Laying Order	1.67	2,32	0.20	
Egg Mass	14.78	1,32	<0.001	
Carotenoids Yolk	8.85	1,32	0.006	-2.890 (0.971)
Tocopherols Yolk	0.61	1,32	0.44	-0.132 (0.169)
Carotenoids Yolk*Tocopherols Yolk	7.56	1,32	0.0097	0.023 (0.008)
Brain Mass				
Treatment	0.60	1,32	0.44	
Sex	2.38	1,32	0.13	
Laying Order	0.06	2,32	0.94	
Egg Mass	0.21	1,32	0.65	
Carotenoids Yolk	0.83	1,32	0.37	-0.004 (0.005)
Coenzyme Q10 Yolk	7.50	1,32	0.010	-0.059 (0.022)
Carotenoids Yolk*Coenzyme Q10	5.94	1,32	0.021	0.002 (0.001)
TOS Brain				
Treatment	0.32	1,30	0.58	
Sex	1.66	1,30	0.21	
Laying Order	0.19	2,30	0.83	
Egg Mass	2.70	1,30	0.11	
Retinol Brain	5.11	1,30	0.031	-259.99 (115.02)
Tocotrienols Brain	3.50	1,30	0.071	-10.081 (5.391)
Retinol Brain*Tocotrienols Brain	9.08	1,30	0.005	61.075 (20.265)

Table S3. Summary of the results of linear mixed models of antioxidant concentrations or oxidative status markers on two-way interactions between vitamin E treatment and post-manipulation vitamin E concentration. In the models we also included the effect of sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between vitamin treatment and post-manipulation vitamin E concentration were significant are reported. Overall, the effects of the interaction were tested in 111 models for all the possible pairs of antioxidants. C: control; E: vitamin E treated.

	F	d.f.	*P*	Coefficients (SE)
α-tocotrienol Yolk				
Treatment	6.00	1,33	0.020	
Sex	0.80	1,33	0.38	
Laying Order	0.22	2,33	0.80	
Egg Mass	0.01	1,33	0.92	
Tocopherols Yolk	0.14	1,33	0.71	
Treatment*Tocopherols Yolk	5.99	1,33	0.020	C: 0.019 (0.010) E: -0.014 (0.009)
Tocotrienols Yolk				
Treatment	6.00	1,33	0.020	
Sex	0.85	1,33	0.36	
Laying Order	0.19	2,33	0.82	
Egg Mass	0.00	1,33	0.99	
Tocopherols Yolk	0.20	1,33	0.66	
Treatment*Tocopherols Yolk	5.99	1,33	0.020	C: 0.020 (0.010) E: -0.014 (0.009)

Table S4. Summary of the results of linear mixed models of morphological or oxidative status traits on two-way interactions between treatment and antioxidant concentrations. In the models we also included the effect of sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between treatment and antioxidants were significant are reported. Overall, the effects of the interaction were tested in 122 models for all the possible pairs of antioxidants. C: control; E: vitamin E treated.

	F	d.f.	*P*	Coefficients (SE)
Liver Mass				
Treatment	2.46	1,30	0.13	
Sex	3.13	1,30	0.09	
Laying Order	0.66	2,30	0.52	
Egg Mass	10.31	1,30	0.003	
γ-tocotrienol Yolk	0.61	1,30	0.44	
Treatment*γ-tocotrienol Yolk	5.21	1,30	0.030	C: 2.145 (0.976) E: 1.105 (1.105)
Liver Mass				
Treatment	4.29	1,32	0.047	
Sex	3.91	1,32	0.057	
Laying Order	1.58	2,32	0.22	
Egg Mass	7.43	1,32	0.010	
Coenzyme Q10	0.65	1,32	0.43	
Treatment*Coenzyme Q10	6.22	1,32	0.018	C: 0.03235 (0.016) E:-0.01594 (0.012)
TOS Liver				
Treatment	1.77	1,31	0.19	
Sex	0.01	1,31	0.90	
Laying Order	7.71	2,31	0.002	
Egg Mass	10.35	1,31	0.003	
Retinol Liver	6.11	1,31	0.019	
Treatment*Retinol Liver	5.82	1,31	0.022	C: 176.47 (299.87) E:1044.80 (311.48)
PC Liver				
Treatment	1.57	1,28	0.22	
Sex	0.41	1,28	0.53	
Laying Order	0.25	2,28	0.78	
Egg Mass	0.19	1,28	0.67	
α-tocotrienol Liver	0.52	1,28	0.48	
Treatment*α-tocotrienol Liver	4.66	1,28	0.040	C: 0.123 (0.068) E: -0.060 (0.054)
PC Liver				
Treatment	2.25	1,28	0.14	
Sex	0.67	1,28	0.42	
Laying Order	0.32	2,28	0.73	
Egg Mass	0.201	1,28	0.66	
Tocotrienols Liver	0.39	1,28	0.54	
Treatment*Tocotrienols Liver	4.55	1,28	0.042	C: 0.118 (0.068) E: -0.064 (0.053)

	F	d.f.	*P*	Coefficients (SE)
Brain Mass				
Treatment	4.21	1,33	0.048	
Sex	0.50	1,33	0.48	
Laying Order	0.04	2,33	0.96	
Egg Mass	0.59	1,33	0.45	
α-tocotrienol Yolk	0.03	1,33	0.86	
Treatment*α-tocotrienol Yolk	5.04	1,33	0.032	C: 0.027 (0.018) E: -0.032 (0.018)
Brain Mass				
Treatment	4.39	1,33	0.044	
Sex	0.53	1,33	0.47	
Laying Order	0.05	2,33	0.95	
Egg Mass	0.64	1,33	0.43	
Tocotrienols Yolk	0.03	1,33	0.85	
Treatment*Tocotrienols Yolk	5.24	1,33	0.029	C: 0.027 (0.018) E: -0.032 (0.018)
TOS Brain				
Treatment	4.03	1,32	0.053	
Sex	6.27	1,32	0.018	
Laying Order	0.29	2,32	0.75	
Egg Mass	1.53	1,32	0.23	
Retinol Brain	0.35	1,32	0.56	
Treatment*Retinol Brain	5.23	1,32	0.029	C: -225.72 (136.95) E: 126.64 (85.260)
TOS Brain				
Treatment	6.05	1,31	0.020	
Sex	5.05	1,31	0.03	
Laying Order	0.15	2,31	0.86	
Egg Mass	5.23	1,31	0.029	
Tocotrienols Brain	0.30	1,31	0.59	
Treatment*Tocotrienols Brain	8.74	1,31	0.006	C: -5.574 (4.013) E: 8.089 (2.248)
PC Brain				
Treatment	4.80	1,27	0.037	
Sex	1.01	1,27	0.32	
Laying Order	0.45	2,27	0.64	
Egg Mass	0.11	1,27	0.74	
α-tocopherol Brain	0.58	1,27	0.45	
Treatment*α-tocopherol Brain	6.87	1,27	0.014	C: -0.008 (0.006) E: 0.014 (0.006)
PC Brain				
Treatment	1.31	1,25	0.26	
Sex	1.20	1,25	0.28	
Laying Order	1.01	2,25	0.38	
Egg Mass	0.91	1,25	0.35	
α-tocotrienol Brain	0.11	1,25	0.74	
Treatment*α-tocotrienol Brain	4.85	1,25	0.037	C: -0.014 (0.011) E: 0.019 (0.009)

	F	d.f.	*P*	Coefficients (SE)
PC Brain				
Treatment	4.73	1,27	0.039	
Sex	0.97	1,27	0.33	
Laying Order	0.56	2,27	0.58	
Egg Mass	0.11	1,27	0.75	
Tocopherols Brain	0.56	1,27	0.46	
Treatment*Tocopherols Brain	6.79	1,27	0.015	C: -0.007 (0.006) E: 0.013 (0.005)