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RESEARCH ARTICLE

Independent and combined effects of egg pro- and anti-oxidants on gull chick phenotype

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ABSTRACT

Oviparous mothers transfer to their eggs components that have both independent and combined effects on offspring phenotype. The functional interaction between egg components, such as antioxidants and hormones, suggests that a change in the concentration of one component will have effects on offspring traits that depend on the concentration of other interacting components. However, the combined effects of variation in different egg components are virtually unknown. Bird eggs contain vitamin E, a major antioxidant, and also maternal corticosterone. The independent consequences of variation in the egg concentrations of these compounds for offspring phenotype are largely unknown and no study has investigated their combined effects. We manipulated the concentration of vitamin E and corticosterone in the eggs of the yellow-legged gull (*Larus michahellis*) by administering a physiological (2 s.d.) dose both independently and in combination. We tested for an effect on chick post-natal growth, plasma antioxidant capacity (TAC) and oxidative compounds (TOS). Separate administration of vitamin E or corticosterone caused a reduction in body mass relative to controls, whereas the combined administration of the two compounds reversed their negative effects. These results suggest that maternal egg components, such as antioxidants and steroid hormones, interact and mothers must balance their concentrations in order to achieve optimal offspring phenotype. The functional relationship between vitamin E and corticosterone is corroborated by the observation of positive covariation between these compounds.

KEY WORDS: Corticosterone, Growth, *Larus michahellis*, Oxidative stress, Vitamin E

INTRODUCTION

In oviparous reproductive systems, the egg is the major source of environmental stimuli for the developing embryo from the zygote to the hatching stage (Mousseau and Fox, 1998; Surai, 2002). The effects of the egg milieu on physiological, morphological and behavioural offspring traits largely depend on the egg chemical composition in terms of the content of major components (e.g. proteins, lipids and water) as well as the concentration of minor constituents like hormones, antioxidants and immune factors (Mousseau and Fox, 1998; Surai, 2002). In fact, these effects occur during the pre-hatching stage but they also extend well into the juvenile and adult life stages, as shown by studies where the content/concentration of any individual egg component has been experimentally altered separately (i.e. one component at a time), either directly by egg manipulation or via the mother by, for example, dietary supplementation (e.g. Blount et al., 2002; Rubolini et al., 2006; Selim et al., 2012; Surai et al., 2002).

However, egg components are not expected to act independently in exerting their short- and long-term effects, as individual egg compounds are thought to act in concert in shaping offspring phenotype. For example, multiple antioxidants of maternal origin intervene in antioxidant defence processes and in regeneration of the antioxidant potential of individual antioxidant compounds, although their interactions are far from being fully understood (Surai, 2002; see also Possenti et al., 2017). Similarly, egg hormones are thought to participate in developmental and growth processes with independent as well as combined effects (e.g. Eising et al., 2001; Schwabl, 1996; von Engelhardt et al., 2011; reviewed by Groothuis et al., 2005). The existence of functional interactions between egg components suggests that mothers should allocate compounds that are involved in interacting offspring physiological pathways in concentrations that reciprocally co-vary. A corollary expectation is that variation in the egg concentration of a specific compound should have effects that depend on the concentration of the other interacting molecules (Giraudeau and Ducatez, 2016; Possenti et al., 2017). While several studies have been carried out on the effect of individual egg components on offspring phenotype (e.g. Haussmann et al., 2012; Noguera et al., 2011; Parolini et al., 2015, 2017; Saino et al., 2003; Selim et al., 2012), surprisingly little experimental work has been done on the functional interactions between egg compounds, by manipulating the egg concentrations of multiple components and testing these combined effects (Giraudeau et al., 2016).

Antioxidants are fundamental egg components that mainly serve to prevent oxidative damage of the biomolecules of the developing offspring during pre- and early post-natal growth (Surai, 2002; Costantini, 2014). Among these, vitamin E, including tocopherols and tocotrienols, is the most abundant and functionally important antioxidant in several vertebrate model species, and particularly in birds, whose eggs have been studied most intensively (Costantini, 2014; Surai, 2002). Vitamin E is an exogenous antioxidant that can be acquired by animals only through their diet. Whether allocation of maternal exogenous antioxidants to the egg is limited by dietary availability is still in question and probably depends on the trophic level of the focal species, on the species-specific diet and also on contingent, local environmental conditions (de Ayala et al., 2006; Ninni, 2003). Experimental manipulation of vitamin E concentration via direct egg manipulation in the yellow-legged gull (*Larus michahellis*), which is the focal organism of the present study, has shown that chicks from last-laid, vitamin E-depleted eggs benefit from vitamin E supplementation in terms of enhanced body growth (Parolini et al., 2015). These findings suggest

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that dietary vitamin E is limiting to laying mothers and supplementation of the eggs with a physiological dose (1 s.d. of the yolk vitamin E concentration) boosts offspring growth. Experimental manipulation of the egg via maternal dietary supplementation of vitamin E, in addition, has demonstrated a positive effect on growth, immune function and antioxidant capacity of chicks (Blount et al., 2002; Koutsos et al., 2003; Leshchinsky and Klasing, 2001; Lin et al., 2005). However, maternal supplementation studies are not immune from potential confounding effects, owing to effects of dietary supplementation on general maternal physiology and thus on multiple egg traits that can affect offspring phenotype.

Excess vitamin E can be toxic (Surai, 2002). While the threshold concentrations for vitamin E toxicity are not firmly established, and toxicity may be expressed only at very high concentrations, circumstantial evidence suggests that, under natural conditions, a vitamin E excess, even within the physiological range of variation, can be detrimental to growth. For example, supplementation of dietary vitamin E that increased the total intake by 2 s.d. of the normal intake by barn swallow (*Hirundo rustica*) nestlings was found to reverse the positive effect of supplementation by 1 s.d. on somatic growth (de Ayala et al., 2006).

Maternal steroid hormones are also a functionally major group of maternal substances transferred into the egg with pervasive, multi-faceted actions on offspring phenotype that can extend well beyond the pre- and early post-natal stages into adulthood, via their organizational effects on morphological, physiological and behavioural traits (Eising et al., 2003; reviewed by Groothuis et al., 2005). Maternal corticosterone in particular is transferred to the eggs in concentrations that can depend on extrinsic ecological conditions (Groothuis et al., 2005; Love and Williams, 2008; Love et al., 2008; Saino et al., 2005). Increased corticosterone levels in the eggs have been shown to impair the development of brain asymmetry, suppress somatic growth and immune functions and, ultimately, reduce survival of chicks (Eriksen et al., 2003; Janczak et al., 2006; Rogers and Deng, 2005; Rubolini et al., 2005; Saino et al., 2005; see review by Henriksen et al., 2011). Like other maternal egg steroid hormones (e.g. androgens), corticosterone can have pro-oxidant effects, thus increasing oxidative stress and damage (Costantini, 2014; Costantini et al., 2008, 2011; Haussmann et al., 2012; Lin et al., 2009; Monaghan, 2014; Monaghan and Haussmann, 2015; Stier et al., 2009). Because of functional interactions between corticosterone acting as a pro-oxidant and the antioxidant defence system, it is to be expected that the consequences of increased corticosterone egg concentrations depend on the concentration of antioxidants. Reciprocally, it is also to be expected that any effect of antioxidants, including negative effects of elevated concentration (although within physiological limits), depend on the concentration of pro-oxidants (see Giraudeau et al., 2016). However, to the best of our knowledge, no study has experimentally manipulated both corticosterone and antioxidant concentrations in the eggs and tested for the independent and combined effect of the hormone and the antioxidants on post-natal growth and oxidative status, in terms of antioxidant capacity and concentration of pro-oxidant compounds, in any species. In the only previous study we are aware of where egg maternal steroid hormones (testosterone) and antioxidants (carotenoids) were manipulated, high levels of egg testosterone or carotenoids increased the amount of reactive metabolites in chick plasma (only a tendency in chicks from the testosterone group), but when the two egg compounds were manipulated simultaneously, their combined effect suppressed the independent detrimental effects of both testosterone and carotenoids, probably because the ratio between the two substances was balanced (Giraudeau et al., 2016).

In the present experimental study, we manipulated the concentrations of vitamin E and corticosterone in the yolk of yellow-legged gulls, both independently and in combination, and tested for an effect on body growth and oxidative status markers, namely plasma total antioxidant capacity (TAC) and concentration of total oxidative compounds (total oxidation status, TOS; according to the terminology by Erel, 2005), soon after hatching. The dose of both vitamin E and corticosterone that we decided to inject while taking egg mass into account was such that the final concentrations of these substances in the yolk were both increased by 2 s.d. of the concentrations of the two compounds that were previously recorded in the same population.

Based on previous evidence from other species, corticosterone administration was expected to have a negative effect on chick body growth (as gauged by body mass and tarsus length; Eriksen et al., 2003; Janczak et al., 2006; Saino et al., 2005; see review by Henriksen et al., 2011). If the negative effect of a physiological dose of corticosterone is mediated by a pro-oxidant effect, we also expected a reduction in plasma antioxidant capacity and an increase in the plasma concentration of oxidative compounds in chicks from the corticosterone group compared with the control group.

The prediction of the effect of vitamin E was conditional on whether the dose that we applied here had any detrimental effect on the traits under scrutiny, i.e. was toxic or not. In the former case, a depression of body mass and size was expected, whereas we had no unequivocal expectation of the effect on oxidative status variables (TAC and TOS) as such a prediction would depend on the specific mechanisms of toxicity. In the latter case, a positive effect on body mass and size was expected, and also a positive effect on TAC and/or a negative effect on TOS.

The expected combined effect of vitamin E plus corticosterone treatment was again conditional on the effect of the specific vitamin E dose that we applied and on the mechanism behind any effect of corticosterone on body growth. If a negative effect of corticosterone on the morphological and oxidative status variables was mediated by oxidative status, we would expect simultaneous administration of both vitamin E and corticosterone to cause an amelioration of phenotypic trait values relative to the administration of corticosterone and of vitamin E alone. This is because an excess of vitamin E may be advantageous in the case of oxidative stress caused by a physiological dose of corticosterone. In this scenario, we specifically expected chicks from the vitamin E plus corticosterone treatment to be of the same size or larger than chicks from the corticosterone treatment group and also from the vitamin E group, if the vitamin E dose in the latter group was so large as to cause detrimental effects on body growth. In contrast, if the effect of corticosterone on body growth was not mediated by oxidative status and the vitamin E dose was detrimental, we expected an additive/synergistic negative effect of vitamin E and corticosterone on body growth, causing chicks from the vitamin E plus corticosterone groups to be smaller and in poorer oxidative status than those from the other groups. Finally, if the effect of vitamin E at the dose that we applied was favourable on the traits that we scrutinized and corticosterone had no effect, we expected chicks from the vitamin E plus corticosterone treatment group to have better phenotypic trait values (i.e. large body size and low concentration of pro-oxidants) than controls and similar to those of chicks from the vitamin E treatment group.

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MATERIALS AND METHODS

Model species

The study was performed on the yellow-legged gull (*Larus michahellis* Naumann 1840), a monogamous, mainly colonial bird, inhabiting coastal habitats across the Mediterranean Sea. The breeding period usually lasts from March until the end of June. Females generally lay from one to three eggs (modal clutch size is three eggs), at 1–3 day intervals, and the egg size declines along the laying sequence (mass range: 80–100 g). The incubation period lasts 27–32 days and hatching is asynchronous over 1–4 days. The chicks are semi-precocial, receive biparental care and fledge at 35–40 days of age (see Cramp, 1998).

Field procedures

The study was carried out on a large colony in the Comacchio lagoon (NE Italy; 44°20′N, 12°11′E) during the breeding period. The study area was visited every second day to monitor the progress of laying and mark the newly laid eggs, which were temporarily removed (for ca. 3 h) from the nest for the experimental manipulation, during which time they were replaced by 'dummy' eggs.

We aimed at increasing the vitamin E (α-tocopherol) and corticosterone concentrations in the yolk, both independently and in combination (vitamin E plus corticosterone), by 2 s.d. of the concentrations of the two compounds that were previously recorded in the same population (Rubolini et al., 2011). Thus, the final concentration of vitamin E, corticosterone and vitamin E plus corticosterone was within the natural range of variation for the vast majority of the eggs. We varied the dose to be injected according to the egg size and the position in the laying sequence. Therefore, based on Rubolini et al. (2011), we grouped first- (a), second- (b) or third-laid (c) eggs each into three classes (tertiles) of size according to egg mass and we calculated 2 s.d. of both vitamin E and corticosterone concentrations in the yolk for each tertile, within each position in the laying sequence. Then, we estimated yolk mass based on total egg mass according to the following equation: yolk mass=0.227(0.039 s.e.)×egg mass+1.815(3.461 s.e.) ($F_{1,88}$=34.38, P<0.001) (Parolini et al., 2015). The amount of vitamin E and corticosterone to be injected was calculated as the product of the relevant standard deviation values and yolk mass as estimated using the above equation. The amount of vitamin E and corticosterone injected for the three classes of egg mass for each of the three positions in the laying sequence is reported in Table 1. The same amounts of vitamin E and corticosterone were mixed in a single solution (vitamin E plus corticosterone) in order to simultaneously inject and increase the yolk concentrations of both compounds by 2 s.d.

The vitamin E, corticosterone and vitamin E plus corticosterone solutions, which were solubilized in corn oil, were prepared in advance and stored in sterile vials. In the field, the freshly laid eggs were weighed to the nearest gram. This allowed us to assign the appropriate dose according to the class of egg mass and the position in the laying sequence. Treated eggs were injected with 30 µl of the appropriate solution of vitamin E, corticosterone or vitamin E plus corticosterone, while control eggs were injected with 30 µl of corn oil only. The injection procedure was performed in the yolk as reported by Romano et al. (2008). We adopted a within-clutch experimental design, whereby both control and treated eggs were set in the same nest to minimize the consequences of both environmental and parental effects. The following treatment schemes were sequentially assigned to the clutches according to the order in which the first egg was found (nest, laying order). Treatment schemes with corticosterone and vitamin E plus corticosterone were: nest 1, a-egg, vitamin E plus corticosterone injection (mix); b-egg, corticosterone injection; c-egg, control injection; nest 2, corticosterone-mix-control; nest 3, control-corticosterone-mix; nest 4, control-mix-corticosterone; nest 5, corticosterone-control-mix; nest 6, mix-control-corticosterone, and so on with subsequent nests. Treatment schemes with vitamin E and vitamin E plus corticosterone were: nest 1, a-egg (mix); b-egg, vitamin E injection; c-egg (control); nest 2, vitamin E-mix-control; nest 3, control-vitamin E-mix; nest 4, control-mix-vitamin E; nest 5, vitamin E-control-mix; nest 6, mix-control-vitamin E, and so on with subsequent nests.

Table 1. Amount of vitamin E and corticosterone injected into the yolk of yellow-legged gull eggs according to egg mass at the time of deposition and laying order

Laying order	Egg mass (g)	Vitamin E (µg)	Corticosterone (ng)
a-egg	84–91	1227	108
	92–95	1392	126
	96–108	1296	114
b-egg	80–88	947	77
	89–92	1300	66
	93–99	1280	150
c-egg	75–82	567	82
	83–87	1146	91
	88–98	1196	75

a-, b- and c-egg refer to the first, second and third egg laid, respectively.

When the eggshell was found fractured by the chick at the start of the hatching event (i.e. the 'pipping' stage), the egg was injected with a drop of atoxic coloured food dye through the small egg fracture to associate the hatchling with its original egg. As soon as the chick had hatched, it was made individually recognizable by banding either tarsus with a coloured elastic rubber ring, which was removed after the trial period. Then, we measured body mass and tarsus length as proxies of body growth and we collected a blood sample at hatching age and at ca. 4 days after hatching (0–1 days and 3–5 days, respectively, depending on the day when the hatchling was found). The blood sample was centrifuged at 15,000 rpm for 10 min to separate the plasma from the blood cells. Analyses of markers of oxidative status were performed on the plasma, and molecular sexing on red blood cells (Saino et al., 2008).

The study was carried out under permission of the Parco Regionale del Delta del Po (no. 450/2016, 4 January 2017), which allowed both the manipulation and the collection of blood samples.

Analysis of TAC and TOS

As markers of oxidative status, we measured the TAC and the TOS on plasma of 4 day old chicks. Briefly, TAC was measured according to a colorimetric method developed by Erel (2004). The colour of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) bleaches depending on the concentration of antioxidants in the plasma sample. The reaction was monitored spectrophotometrically (λ=750 nm) and the final absorbance was inversely related to the TAC of the sample. The assay was calibrated using a standard curve of Trolox and the results were expressed as µmol l^{-1} Trolox equivalent. Mean (±s.d.) TAC intra-assay coefficient of variation (CV) was 2.5±1.7% (n=5 replicates), while the mean inter-assay CV was 5.2±3.7% (n=3 assay plates). TOS was also measured according to a colorimetric method (Erel, 2005). This assay relies on the oxidation of ferrous ion-*o*-dianisidine complex to the ferric ion due to the presence of oxidant molecules, which react with Xylenol Orange to form a blue complex. The change in absorbance was measured by a spectrophotometer at λ=535 nm and it was directly proportional to the pro-oxidant amount in the sample. The assay was calibrated using a standard curve of hydrogen

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peroxide (H_2O_2) and the results were expressed as nmol l^{-1} H_2O_2 equivalents. The mean (±s.d.) intra-assay CV was 3.8±2.9% (n=5 replicates) and the inter-assay CV was 5.3±3.9% (n=3 assay plates).

Statistical analyses

The effect of treatment (four levels, fixed effect factor) on chick body mass and tarsus length at hatching age (range 0–1 days) and at ∼4 days after hatching (range 3–5 days), and on TAC and TOS (4 days) was analysed in linear mixed models where we included sex and position in the laying sequence as fixed effect factors, and egg mass and exact age at measurement as covariates. The two-way interaction terms between factors were initially included in the models and then excluded in a single step as they did not significantly contribute to the models. In all models, brood identity was included as a random effect factor to account for non-independence of the data from sibling chicks. The effect of brood identity on the phenotypic variables was tested by likelihood ratio tests comparing the log-likelihood values associated with models including or, respectively, excluding the random effect of brood identity. Pairwise comparisons between experimental treatment groups were performed by least-square difference (LSD) tests. The analyses of morphological traits recorded at either age were run separately because mortality or failure to locate the chicks hiding in the dense herbaceous vegetation caused the number of chicks included in the sample to markedly drop between hatching (control, vitamin E, corticosterone, vitamin E plus corticosterone groups: 42, 19, 19, 48, respectively) and 4 days (control, vitamin E, corticosterone, vitamin E plus corticosterone groups: 30, 13, 12, 38, respectively) (see Fig. 1). In order to maximize the information obtained from the analyses, we first ran all the analyses on the maximum sample available at either age. However, when we restricted the analyses at hatching to include only the chicks that were also in the analyses at 4 days, the results remained qualitatively unaltered (details not shown). The analyses on oxidative status are based on the largest sample available (TAC: control, vitamin E, corticosterone, vitamin E plus corticosterone groups: 30, 13, 12, 37, respectively; TOS: control, vitamin E, corticosterone, vitamin E plus corticosterone groups: 30, 12, 12, 37, respectively).

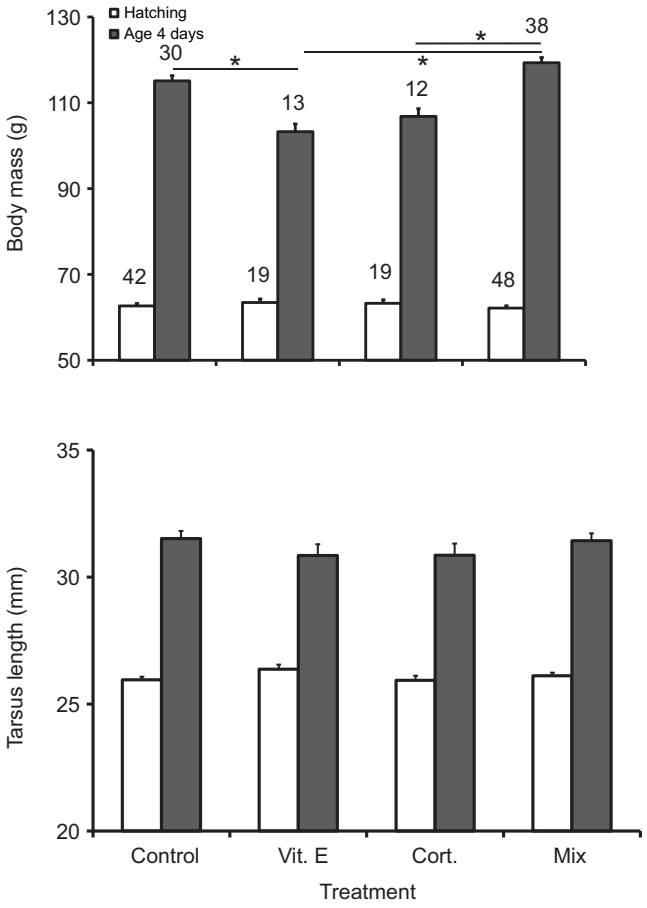

Fig. 1. Body mass and tarsus length of yellow-legged gull chicks in relation to treatment. Mean (+s.e.) body mass (A) and tarsus length (B) at hatching or 4 days of control chicks or chicks from the vitamin E (Vit. E), corticosterone (Cort.), or vitamin E plus corticosterone (Mix) treatment groups. Numbers above bars are sample sizes for the traits. Asterisks indicate the pairwise differences between groups that were significant in least-square difference (LSD) tests.

RESULTS

Body mass and tarsus length at hatching (age ≤1 day) were not affected by experimental treatment (Table 2). Both body mass and tarsus length significantly increased with mass of the original egg, as expected (Table 2). In addition, while controlling for egg mass, tarsus length showed a marginally significant variation with laying order, with c-chicks being smaller than a- and b-chicks, again as expected (Table 2).

At 4 days, experimental treatment had a significant effect on body mass (Table 2). Pairwise comparisons showed that control chicks were heavier than those in the vitamin E and corticosterone groups, although the difference was statistically significant between control and vitamin E groups (P=0.022; Table 2, Fig. 1), while it was marginally non-significant between control and corticosterone groups (Table 2, Fig. 1). Chicks from the vitamin E plus corticosterone treatment group were significantly heavier than those from either the vitamin E (P=0.002) or the corticosterone (P=0.015) group (Table 2, Fig. 1). Finally, there was no statistically significant difference (P=0.271) in body mass of control and vitamin E plus corticosterone treatment groups (Table 2, Fig. 1). Treatment did not significantly affect tarsus length (Table 2, Fig. 1). In addition, at age 4 days, both body mass and tarsus length significantly positively co-varied with age and mass of the original egg (Table 2).

Experimental treatment significantly affected plasma antioxidant capacity (TAC) at ca. 4 days after hatching (Table 3). Pairwise comparisons showed that chicks from the vitamin E plus corticosterone group had greater antioxidant capacity than those from both the control (P=0.019) and vitamin E (P=0.001) groups (Fig. 2), whereas the other pairwise comparisons were statistically non-significant (Table 3, Fig. 2). TAC significantly declined with size of the original egg (Table 3). Finally, TOS was not affected by experimental treatment (Table 3, Fig. 2).

DISCUSSION

The present study showed that an increased concentration of vitamin E caused a statistically significant reduction in body mass of the chicks at 4 days of age but not around hatching, relative to control chicks. Corticosterone administration also caused a reduction in body mass relative to the controls but this effect was statistically marginally non-significant. However, the simultaneous administration of vitamin E and corticosterone reversed the independent negative effects of both compounds and led to chick phenotypic trait values similar to those of controls. In addition, the combined administration of vitamin E and corticosterone boosted plasma antioxidant capacity relative to that of chicks in the control

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Table 2. Linear mixed models of body mass and tarsus length in relation to experimental treatment, sex and laying order (factors) around hatching (0–1 days) and ∼4 days of age (3–5 days)

	Body mass					Tarsus length				
	χ^2	*F*	d.f.	*P*	Coefficient (s.e.)	χ^2	*F*	d.f.	*P*	Coefficient (s.e.)
Hatching										
Nest	0.00		1	0.999		6.08		1	0.014	
Treatment		0.77	3,119	0.515			1.54	3,82.8	0.210	
Sex		0.00	1,119	0.967			0.00	1,113.7	0.978	
Laying order		1.08	2,119	0.341			3.30[a]	2,76.2	0.042	
Egg mass		287.60	1,119	<0.001	0.752 (0.044)		59.29	1,96.9	<0.001	0.843 (0.109)
Age		1.30	1,119	0.257	0.751 (0.659)		4.11	1,90.0	0.046	2.819 (1.390)
Age 4										
Nest	0.00		1	0.999		2.27		1	0.132	
Treatment		4.44[b]	3,84	0.006			0.98	1,55.0	0.409	
Sex		2.62	1,84	0.109			1.41	1,83.1	0.239	
Laying order		1.95	2,84	0.148			1.78	2,49.1	0.179	
Egg mass		15.75	1,84	<0.001	0.886 (0.224)		29.16	1,69.0	<0.001	1.355 (0.251)
Age		12.14	1,84	0.001	39.319 (11.287)		8.33	1,70.9	0.005	33.210 (11.504)

The effect of age at the time of measurement and original egg mass are included as covariates. The effect of nest is included as a random factor (see also Materials and Methods, 'Statistical analyses').

[a]Tarsus length (means±s.e.): a-chicks: 26.001±0.126 mm; b-chicks: 26.311±0.114 mm; c-chicks: 25.970±0.158 mm.

[b]Body mass, least-square difference (LSD) tests: control versus vitamin E: *P*=0.022; control versus corticosterone: *P*=0.105; vitamin E versus mix (vitamin E plus corticosterone): *P*=0.002; corticosterone versus mix: *P*=0.015; control versus nix: *P*=0.271 (see Fig. 1).

and vitamin E groups, whereas it did not affect the plasma concentration of oxidative compounds.

Vitamin E is a major exogenous antioxidant that is acquired by animals through the diet. It is involved in a number of antioxidant mechanisms mediated by scavenging of free radicals. It thus prevents oxidative damage mostly to polyunsaturated fatty acids and nucleic acids (Surai, 2002). Excess vitamin E is thought not to be detrimental, even in large doses, mainly based on studies of laboratory animal models or domestic animals including poultry (Surai, 2002). However, extremely little is known about the effects of physiological doses on animals not artificially selected and under a natural (i.e. non-artificial) selection regime in the wild. In a food supplementation experiment of barn swallow nestlings, it was shown that supplementation with a physiological dose (1 s.d. of the normal food intake of vitamin E) boosted body growth but a larger physiological dose (2 s.d. of normal food intake) reversed the positive effect of low doses (1 s.d.; de Ayala et al., 2006). Similar to these previous findings, the present results suggest that a physiological increase (2 s.d.) in vitamin E concentration can depress body mass gain, though not skeletal body growth of gull chicks. This effect may be due to an overproduction of pro-oxidant molecules caused by an excess of vitamin E, as observed from previous *in vitro* studies performed on human cells (Bowry and Stocker, 1993; Bowry et al., 1995). Alternatively, the negative effect on somatic growth may be caused by the displacement of other antioxidants (Huang and Appel, 2003), leading to an impairment of the antioxidant defence system and an increase in the susceptibility to oxidative stress (Miller et al., 2005). However, these hypotheses are not corroborated by our findings on oxidative status markers, possibly because of large variation in TAC and TOS measurements, the relatively small sample size and the imbalance of the samples compared with the control and vitamin E plus corticosterone groups, potentially reducing the statistical power of the tests. We speculate that vitamin E concentration in yellow-legged gull eggs is normally close to the maximum for growth and that vitamin E is therefore not limiting for laying females. The effect of vitamin E was detected at 4 days of age but not around hatching, implying that the consequences of egg quality in terms of antioxidants carry over into post-hatching life stages and may not be detectable in the early post-natal period. The mechanisms for such delayed effects, however, are unclear at present.

The adaptive function, if any, of transmission of maternal corticosterone to the eggs in birds is largely unknown. One possibility is that transmission of corticosterone is the inevitable consequence of maternal egg-laying physiology (Haussmann et al., 2012; Monaghan and Haussmann, 2015; Mousseau and Fox, 1998). In fact, increasing egg corticosterone concentrations have been shown to translate mainly into negative consequences for the

Table 3. Linear mixed models of TAC and TOS in relation to experimental treatment, sex and laying order (factors) at ∼4 days (age 3–5 days)

	TAC					TOS				
	χ^2	*F*	d.f.	*P*	Coefficient (s.e.)	χ^2	*F*	d.f.	*P*	Coefficient (s.e.)
Nest	0.00		1	0.999		0.33		1	0.566	
Treatment		4.33[a]	3,83	0.007			0.74	3,78.9	0.531	
Sex		0.94	1,83	0.334			0.14	1,81.4	0.707	
Laying order		0.42	2,83	0.657			0.48	2,75.7	0.622	
Egg mass		6.70	1,83	0.011	–12.938 (5.000)		0.28	1,74.7	0.599	6.976 (13.225)
Age		0.03	1,83	0.857	45.52 (252.27)		0.03	1,81.6	0.861	–114.22 (649.576)

TAC, plasma antioxidant capacity; TOS, total oxidation status (the concentration of oxidant compounds).

The effect of age at measurement and original egg mass are included as covariates. The effect of nest is included as a random factor (see also Materials and Methods, 'Statistical analyses').

[a]TAC LSD tests: control versus vitamin E: *P*=0.129; control versus corticosterone: *P*=0.727; vitamin E versus mix: *P*=0.001; corticosterone versus mix: *P*=0.156; control versus mix: *P*=0.019 (see Fig. 2).

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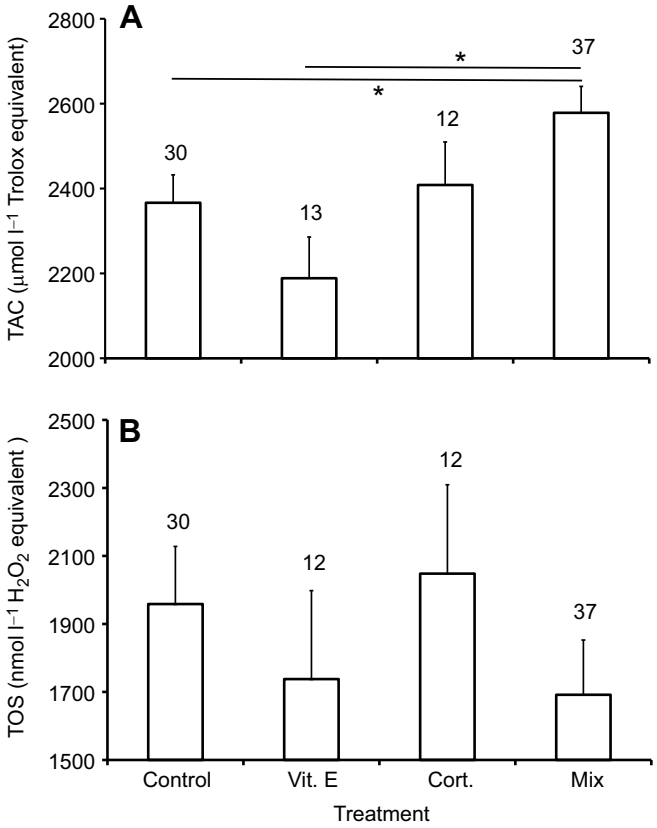

Fig. 2. Total antioxidant capacity (TAC) and total oxidation status (TOS) of yellow-legged gull chicks in relation to treatment. Mean (+s.e.) TAC (A) and TOS (B; concentration of oxidative compounds) of control chicks or chicks from the vitamin E (Vit. E), corticosterone (Cort.) or vitamin E plus corticosterone (Mix) treatment groups at 4 days. Numbers above bars are sample sizes for the traits. Asterisks indicate the pairwise differences between groups that were significant in LSD tests.

developing embryo and offspring during the post-natal stage, including somatic growth and oxidative status (e.g. Costantini, 2014; Costantini et al., 2011; Eriksen et al., 2003; Haussmann et al., 2012; Janczak et al., 2006; Saino et al., 2005). Of course, such negative effects may be compensated by yet unexplored positive effects on other traits and functions during the post-natal stage or later in life. Indeed, egg corticosterone may mediate maternal effects to adaptively prime offspring physiology and behaviour according to the stress conditions experienced by the mother (Groothuis et al., 2005; Hayward and Wingfield, 2004; Love and Williams, 2008; Mousseau and Fox, 1998). Our present results thus add to previous evidence that increasing egg corticosterone concentrations have negative effects on offspring growth during the post-natal stage by showing that chicks in the corticosterone group were smaller, though marginally non-significantly, compared with controls. This finding suggests that corticosterone may reduce the ability of chicks from corticosterone-treated eggs to use metabolic resources for development (Janczak et al., 2006) and/or limit food supply by decreasing begging rate (Rubolini et al., 2005; Possenti et al., 2018). An alternative interpretation is that corticosterone may have an inhibitory effect on the growth hormone (Sapolsky, 1993).

However, the combination of physiological doses of both corticosterone and vitamin E in the egg yolk led to a reversal of the negative effect of corticosterone and of vitamin E, separately, on body mass, with chicks from the corticosterone plus vitamin E groups being larger than those for the two groups that were treated with either compound, and also larger, though not statistically significantly so, than those for control chicks. We interpret these findings as evidence of the interaction among maternally transferred egg components, such as antioxidants and steroid hormones (Surai, 2002; Saino et al., 2011; Possenti et al., 2017), and as an indication that the imbalance in the concentrations of these compounds can lead to adverse effects on offspring phenotypic traits (Giraudeau et al., 2016). Hence, mothers need to balance the transfer of substances to their eggs in order to achieve optimal offspring phenotype (Postma et al., 2014; Possenti et al., 2017).

These observed patterns of variation of the independent and combined effects of vitamin E and corticosterone suggest that, in order to retain normal rates of body mass gain after hatching, relatively large egg concentrations of corticosterone should be accompanied by a proportional increase in egg vitamin E concentrations. A previous correlational study showed that this expectation is indeed largely fulfilled because the concentrations of vitamin E significantly positively co-varied with those of corticosterone in first-laid eggs (r=0.43) and the correlations were also positive for second- (r=0.23) and third-laid (r=0.28) eggs, although they did not attain statistical significance, in bivariate linear mixed models where the effect of inter-clutch variation on egg composition was controlled for statistically (Rubolini et al., 2011).

Consistent with the idea that excess vitamin E is detrimental, including having negative effects on the antioxidant system, we showed that chicks in the vitamin E group had poorer plasma TAC than chicks from the vitamin E plus corticosterone group. This suggests that the mechanism that produced a negative effect of vitamin E on body mass growth was mediated by an effect on the antioxidant system and that an excess of vitamin E caused an increase in the use of antioxidant compounds, and thus a reduction in TAC, possibly to counter the mechanism of toxicity of vitamin E itself.

In the yellow-legged gull, the body size at hatching has important consequences on fitness and survival in later life stages because it strongly positively predicts body size at subsequent ages (correlation coefficients among body size or tarsus length measures at hatching and 4 or 8 days after hatching >0.55 in all cases; C.D.P., A.R., N.S. and M.P., unpublished data), consistent with results from previous studies performed on two other gull species (*Larus glaucescens* and *Larus fuscus*) (Hunt and Hunt, 1976; Nager et al., 2000).

In conclusion, in the present study we have shown for the first time that independent physiological increases of both vitamin E and corticosterone in the egg yolk of the yellow-legged gull have detrimental effects on body growth and on plasma antioxidant capacity (vitamin E only). However, a physiological increase of the two compounds combined reversed these negative effects, possibly because the amount of these maternal compounds allocated to the eggs must be balanced, as suggested by the positive covariation between corticosterone and vitamin E concentration in the eggs of our study species.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.D.P., D.R., A.R., N.S., M.P.; Methodology: C.D.P., M.C., M.P.; Investigation: C.D.P., S.S., A.S., M.C., A.R., N.S., M.P.; Resources: N.S.; Writing - original draft: C.D.P., N.S., M.P.; Supervision: N.S.

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