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RESEARCH ARTICLE

Prenatal independent and combined effects of yolk vitamin E and corticosterone on embryo growth and oxidative status in the yellow-legged gull

Marco Parolini[1,*], Cristina Daniela Possenti[1], Simona Secomandi[1], Silvia Carboni[1], Manuela Caprioli[1], Diego Rubolini[1], Andrea Romano[1,2] and Nicola Saino[1]

ABSTRACT

Variation in the concentration of antioxidants and hormones of maternal origin in the eggs of birds can have a profound influence on offspring phenotype both prenatally and postnatally. Egg maternal substances can have interacting effects, but experimental studies of the consequences of the combined variation in the egg concentration of such molecules are extremely rare, particularly as far as prenatal stages are concerned. We manipulated the yolk concentration of vitamin E and corticosterone, which are, respectively, the main antioxidant and the main glucocorticoid hormone in bird eggs, both independently and simultaneously, and we tested their separate and combined effects on growth and oxidative status in the liver and in the brain of yellow-legged gull (*Larus michahellis*) embryos. Egg supplementation of relatively large physiological doses of corticosterone depressed embryo growth (total body mass, tarsus length and liver mass), whereas administration of vitamin E in association with corticosterone restored normal growth. Vitamin E did not affect embryo growth when administered alone. We further analysed the independent and combined effects of vitamin E and corticosterone on liver and brain total antioxidant capacity, the concentration of reactive oxygen molecules and lipid peroxidation. Vitamin E significantly reduced liver total antioxidant capacity, while corticosterone depressed brain lipid peroxidation. Prenatal exposure to vitamin E and corticosterone appears to have antagonistic effects on body growth, although vitamin E is not limiting in yellow-legged gull eggs. In combination with the results of previous experiments on the same species applying smaller experimental doses or focusing on the postnatal rather than prenatal life stages, our findings indicate that the effects of a physiological increase in the egg concentration of these substances can be life stage and dose specific, implying that generalizing prenatal effects of egg compounds may not be feasible.

KEY WORDS: Antioxidant, Glucocorticoid hormone, *Larus michahellis*, Egg maternal substance, Embryo development

INTRODUCTION

Cleidoic eggs of vertebrates are a sealed environment with very limited exchange of materials with the outer environment. Egg maternal substances are therefore the major source of materials to sustain embryo development and physiological processes. While some classes of egg components, like hormones and antioxidants of maternal origin, are quantitatively minor, variation in their concentration in the egg, even within physiological limits, can have a major impact on embryo development and can carry over into postnatal life, as well as into adulthood (Royle et al., 2001; Saino et al., 2003; Groothuis et al., 2005, 2006; Rubolini et al., 2005, 2006a,b). Maternal transfer of several classes of compounds to the eggs depends on maternal experience of environmental conditions. For example, vitamins are acquired via the diet and may be available to mothers in different amounts (Parolini et al., 2015), while maternal hormones may be transmitted to the eggs in concentrations that vary with ecological conditions (e.g. Saino et al., 2005; Williams and Groothuis, 2015). Thus, the egg components of maternal origin have the potential to mediate transgenerational effects whereby conditions experienced by the mothers are translated into phenotypic variation in the offspring (Mousseau and Fox, 1998). Such early maternal effects, which may be adaptive or, conversely, reflect constraints on the ability of mothers to produce eggs of optimal composition, have therefore attracted the interest not only of animal production and physiology researchers but also of ecologists and evolutionary biologists. Importantly, egg compounds are expected to act in concert on offspring development and postnatal growth, and the effect of individual components is therefore presumed to depend on the concomitant effect of other components, as determined by their relative concentrations (Royle et al., 2001; Surai, 2002; Possenti et al., 2017, 2018a,b). However, the combined as opposed to the independent effects of egg constituents have seldom been subjected to experimental analysis (e.g. Giraudeau et al., 2016; Possenti et al., 2018a,b).

A major class of egg compounds of maternal origin, which can profoundly impact on embryo development, is that of antioxidants, which mainly act to prevent oxidative damage to embryo biological molecules caused by intense metabolic activity during prenatal life stages (Surai, 2002; Costantini, 2014). In birds, vitamin E, comprising a class of compounds that includes tocopherols and tocotrienols, is the quantitatively most relevant exogenous (i.e. acquired through the diet) antioxidant of maternal origin in the egg yolk (Surai, 2002). Experiments on the effects of vitamin E have mainly focused on the postnatal stages and have been carried out on domestic animals (e.g. poultry), mainly by administration to the laying mothers, potentially generating confounding effects due to the consequences of vitamin E administration on maternal physiology, with potential cascading effects on egg components other than vitamin E (Surai, 2002). Experiments where the concentration of focal compounds is manipulated directly *in ovo* within physiological limits allow such potentially confounding effects to be circumvented (Groothuis et al., 2005).

[1]Department of Environmental Science and Policy, University of Milan, via Celoria 26, I-20133 Milan, Italy. [2]Department of Ecology and Evolution, University of Lausanne, Building Biophore, CH-1015 Lausanne, Switzerland.

*Author for correspondence (marco.parolini@unimi.it)

M.P., 0000-0003-0226-1709

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Maternal egg steroid hormones are also functionally important in regulating offspring growth and physiology, as shown by studies where the concentration of androgens or corticosterone has been manipulated directly in the egg (e.g. Eising and Groothuis, 2003; Groothuis et al., 2005). Studies of corticosterone in particular have shown that increased egg concentrations can impair postnatal somatic growth and immune function, ultimately depressing survival (Eriksen et al., 2003; Janczak et al., 2006; Rogers and Deng, 2005; Rubolini et al., 2005; Saino et al., 2005; Henriksen et al., 2011). By influencing growth rates or via other physiological pathways, egg corticosterone, like other egg steroid hormones, is considered to have the potential to affect offspring oxidative status (Costantini, 2014, 2008; Costantini et al., 2011; Haussmann et al., 2012; Stier et al., 2009; Monaghan, 2014). For this reason, the effect of an increase in corticosterone levels on the oxidative status of the offspring is expected to depend on the concomitant concentration of antioxidants, including vitamin E (Possenti et al., 2018a,b). Conversely, because antioxidants are often sequestered in order to accomplish their physiological functions, the effect of an increase in antioxidant concentration may depend of the concomitant action of pro-oxidants as influenced by their concentration. However, the concomitant effects of experimental manipulation of egg components with potentially antagonistic effects has seldom been investigated (Williams and Groothuis, 2015; Giraudeau et al., 2016; Possenti et al., 2018a,b) and, to the best of our knowledge, no single study of birds has focused on the effects at the embryonic stage. In the present study, we therefore investigated the combined effects of experimental manipulation of corticosterone and vitamin E concentration on embryo morphology and oxidative status in the yellow-legged gull (*Larus michahellis* Naumann 1840), a species that has previously been subjected to experimental investigation into the effects of diverse egg components in the wild (e.g. Rubolini et al., 2005; Parolini et al., 2015, 2017a,b; Possenti et al., 2017, 2018a,b).

In the single previous experiment focusing on the effect of administration of vitamin E into the eggs on embryo phenotype, we increased the concentration of vitamin E by one standard deviation of the natural concentration (i.e. a dose half that used in the present study) and found that body mass but not tarsus length at the eggshell cracking stage (as considered in the present study) was significantly increased relative to controls (Parolini et al., 2017a). In that experiment, we found no significant effect of vitamin E administration on total antioxidant capacity (TAC), concentration of reactive oxygen molecular species, protein carbonylation or lipid peroxidation both in the liver and in the brain (Parolini et al., 2017a).

In other experiments, we focused on the effects of vitamin E administration in the egg on post-hatching, rather than pre-hatching yellow-legged gull chick phenotype. An experimental increase in vitamin E concentration in the egg by one standard deviation of the natural concentration (see above) boosted post-hatching growth, particularly of chicks from the third (last) laid eggs in a clutch, consistent with expectations because last-laid eggs contain smaller concentrations of vitamin E than first- and second-laid eggs (Parolini et al., 2015). These experimental conditions also showed a significant positive effect of egg vitamin E on plasma TAC and a negative effect on the concentration of pro-oxidant molecules after hatching, but no effect on protein carbonylation, lipid peroxidation and telomere length (Parolini et al., 2017b). Importantly, the effect of vitamin E can be dose dependent and large, yet physiological doses may not have the same positive effects on offspring growth and physiology as low doses, as previously documented (Surai, 2002; de Ayala et al., 2006).

In another experiment, we aimed at testing whether egg vitamin E and corticosterone have antagonistic effects on postnatal development and oxidative status, by increasing the egg concentration of either or both compounds simultaneously by two standard deviations (Possenti et al., 2018a,b). We found that separate administration of corticosterone and vitamin E caused a reduction of body mass 4 days after hatching but not at hatching, whereas the combined administration of the two compounds reversed these negative effects, suggesting that these two egg components interact and their egg amounts must be balanced to enhance offspring phenotypic quality (Possenti et al., 2018a,b). Importantly, in that experiment (Possenti et al., 2018a,b), a larger (but still physiological) dose than that used in the other experiments was injected (Parolini et al., 2015, 2017a,b,c).

Because corticosterone and vitamin E can interact to alter offspring phenotype, but these effects apparently differ even between closely spaced life stages (see Parolini et al., 2017a,b), in the present study we analysed the independent and combined effects of vitamin E and corticosterone on morphology (embryo mass, tarsus length, brain mass, liver mass) and oxidative status (TAC, amount of pro-oxidant molecules and lipid peroxidation in the brain and the liver) at the pre-hatching, embryonic stage. To this end, we established three groups of eggs where, immediately after laying, we increased the concentration of vitamin E alone, of corticosterone alone, or of the two compounds simultaneously, by two standard deviations of the natural concentration (i.e. within the natural limits of variation). However, a recent study has demonstrated that the concentrations of corticosterone in the yolk of the yellow-legged gull reported by Rubolini et al. (2011) could be overestimated. For this reason, the concentration we injected could be considered as supraphysiological. In addition, we established an appropriate control group of sham-inoculated eggs. Thus, the present study provides novel information on the effect of a higher physiological vitamin E dose than that applied by Parolini et al. (2017a) on embryo growth and the oxidative status of two organs. This is important because the effect of vitamin E can vary with its concentration, within the physiological range of variation. In addition, the present study provides entirely novel information on the independent and combined effects of vitamin E and corticosterone on pre-hatching embryo morphology and oxidative status, a topic that has never been tackled experimentally in any species before. We focused on embryos close to hatching because the effects experienced during the prenatal developmental stage can result in consequences in the early postnatal stage and adulthood. Moreover, as our previous studies of the yellow-legged gull have shown contrasting effects due to supplementation with a focal antioxidant or putatively pro-oxidant molecule on different phenotypic traits (Table 1), in the present study we aimed at investigating whether the consequences on embryos of combined vitamin E and corticosterone *in ovo* injection return dissimilar results compared with those for hatchlings (Possenti et al., 2018a,b). According to previous findings on the yellow-legged gull, we expected that vitamin E treatment would enhance embryonic growth (Parolini et al., 2017a), corticosterone treatment would depress it (Rubolini et al., 2005), and treatment with both substances would restore normal growth. As for the effect of the experimental manipulations on oxidative status variables, we expected that corticosterone would reduce TAC and increase the concentration of reactive oxygen species (ROS) and lipid peroxidation, whereas vitamin E treatment would have opposite effects and that treatment with both compounds would restore the oxidative status observed among controls.

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Table 1. Effect of *in ovo* injection of antioxidant and putatively pro-oxidant molecules into the yolk on different phenotypic traits at prenatal (embryo) and postnatal (hatchling) stages of the yellow-legged gull

Injected molecule	Concentration range	Developmental stage	Phenotypic trait	Effect	Reference
Vitamin E	305–748 µg (+1 s.d.)	Hatchlings	Body mass	+	Parolini et al., 2015
			Tarsus length	+	
Vitamin E	305–748 µg (+1 s.d.)	Hatchling blood	Total antioxidant capacity	+	Parolini et al., 2017b
			Amount of pro-oxidant molecules	+	
			Lipid peroxidation	=	
			Protein carbonylation	=	
			Telomere length	=	
Vitamin E	305–748 µg (+1 s.d.)	Embryos	Body mass	+	Parolini et al., 2017a
			Tarsus length	=	
		Embryo liver and brain	Amount of pro-oxidant molecules	=	
			Lipid peroxidation	=	
			Protein carbonylation	=	
Corticosterone	33–75 ng (+1 s.d.)	Hatchlings	Begging frequency	–	Possenti et al., 2018a
			Reversal-to-prone response	=	
Vitamin E	567–1392 µg (+2 s.d.)	Hatchlings (0 days old)	Body mass	=	Possenti et al., 2018b
			Tarsus length	=	
		Hatchlings (4 days old)	Body mass	–	
			Tarsus length	=	
Corticosterone	66–150 ng (+2 s.d.)	Hatchlings (0 days old)	Body mass	=	
			Tarsus length	=	
		Hatchlings (4 days old)	Body mass	=	
			Tarsus length	=	
			Total antioxidant capacity	=	
			Amount of pro-oxidant molecules	=	
Vitamin E+corticosterone	Vitamin E: 567–1392 µg Corticosterone: 66–150 ng (+2 s.d.)	Hatchlings (0 days old)	Body mass	=	
			Tarsus length	=	
		Hatchlings (4 days old)	Body mass	=	
			Tarsus length	=	
			Total antioxidant capacity	+	
			Amount of pro-oxidant molecules	=	

'+' indicates a positive significant effect of yolk supplementation compared with the control group, '–' indicates a negative significant effect of yolk supplementation compared with the control group, and '=' indicates a null non-significant effect of yolk supplementation compared with the control group on a specific phenotypic trait.

MATERIALS AND METHODS

Study species

The yellow-legged gull is a monogamous, semi-colonial charadriiform (Cramp, 1998). Clutch size varies between 1 and 3, with a modal size of 3 eggs, and eggs are laid at 1–3 days intervals. Sibling eggs hatch asynchronously over 1–4 days, after 27–31 days of incubation by both parents, and hatching order reflects laying order. Egg size normally declines with laying order: third (c-) eggs are considerably smaller than first (a-) and second (b-) laid eggs. The concentration of yolk antioxidants and androgen hormones varies according to the position in the laying sequence; specifically, vitamin E yolk concentration declines with laying sequence. In contrast, corticosterone concentration does not vary between first-, second- and third-laid eggs (Rubolini et al., 2011).

General field procedures and egg manipulation

The study was performed in the Comacchio lagoon (44°20′N, 12°11′E, NE Italy) during spring 2017 with the permission of Parco Regionale Delta del Po. We visited the colony every other day to record and individually mark newly laid eggs. When a newly laid egg was found, it was temporarily removed from its nest for manipulation and replaced with a dummy egg in order to minimize perturbation of the behaviour of the parents. The eggs were moved to a nearby tent for manipulation procedures. We adopted a within-clutch design whereby different treatment groups were established within each clutch. This approach served to reduce the confounding effects of among-clutch variation in egg quality and parental incubation behaviour. Each egg was assigned to one of the following treatments: control injection, injection with vitamin E (VitE), injection with corticosterone (Cort), and injection with vitamin E and corticosterone (VitE+Cort). Because, with very rare exceptions, maximal clutch size in yellow-legged gulls is 3 eggs, only three of the four treatment groups could be established in each clutch. Thus, we assigned each clutch to different treatment schemes according to the order in which the first egg of the clutch was found (see Possenti et al., 2018a,b). Treatment schemes with corticosterone and vitamin E plus corticosterone, and with vitamin E and vitamin E plus corticosterone are shown in Table 2. We aimed at increasing the concentration of both vitamin E and corticosterone by 2 standard deviations of their concentrations recorded in the same

Table 2. Clutch treatment scheme

		Egg order		
Treatment	Nest	a	b	c
Corticosterone and vitamin E plus corticosterone	1	VitE+Cort	Cort	Control
	2	Cort	VitE+Cort	Control
	3	Control	Cort	VitE+Cort
	4	Control	VitE+Cort	Cort
	5	Cort	Control	VitE+Cort
	6	VitE+Cort	Control	Cort
Vitamin E and vitamin E plus corticosterone	1	VitE+Cort	VitE	Control
	2	VitE	VitE+Cort	Control
	3	VitE+Cort	VitE	Cort
	4	Control	VitE	VitE+Cort
	5	VitE	Control	VitE+Cort
	6	VitE+Cort	Control	VitE

The format was continued for subsequent nests. VitE, vitamin E; Cort, corticosterone.

colony in a previous study (Rubolini et al., 2011), so that the post-manipulation concentration of both substances fell within the natural range of variation in the vast majority of the eggs. However, it is important to bear in mind that concentrations of corticosterone reported by Rubolini et al. (2011) could be overestimated because of a methodological limitation (see Larsen et al., 2015). A recent study has shown that the yolk concentration of corticosterone in nine bird species ranged between 0.1 and 0.53 ng g^{-1} (Merrill et al., 2018). Moreover, analyses performed in liquid chromatography coupled with tandem mass spectrometric detection (LC-MS/MS) have revealed that in the eggs of the black-backed gull *Larus fuscus*, a closely related species to the yellow-legged gull, the concentration of corticosterone in the yolk reached 75 pg g^{-1} (Larsen et al., 2015). Thus, the concentration we injected could be considered as supraphysiological. Because the variance in the concentrations of these substances differed both among position in the laying sequence (for vitamin E only, not for corticosterone) and with yolk mass, we decided to tune the amount of the substance for injection based on both these variables. We grouped a-, b- and c-eggs into three classes of egg mass (tertiles) and computed 2 s.d. of the concentration of the substances within each class of laying order and egg mass, based on data from Rubolini et al. (2011). We then estimated yolk mass based on the equation yolk mass=0.227×egg mass−1.815 (see Parolini et al., 2015; Possenti et al., 2018a,b). The absolute amount of vitamin E and corticosterone for injection into each individual egg was then computed as the product of the relevant standard deviation value and the estimated yolk mass (Possenti et al., 2018a,b). The absolute amounts of vitamin E and corticosterone that were injected are reported in Possenti et al. (2018a,b). The doses of vitamin E and corticosterone were dissolved in 30 µl corn oil. The solutions for all the different laying order×egg mass classes of eggs were prepared in advance and stored in sterile vials. A single vial was used on each treatment day and then discarded. Control eggs were injected with 30 µl corn oil. Details of the injection procedures have been reported in previous studies performed by our group on the yellow-legged gull (e.g. Parolini et al., 2015, 2017a,b; Possenti et al., 2018a,b). Eggs were returned to their original nest when the egg manipulation procedures were completed, within 2 h of collection, and the dummy eggs were removed.

Nests were visited every second day throughout the incubation period and daily starting around the time when the eggshell was expected to start showing the typical minute fracturing that precedes hatching. When the first signs of fracturing were observed, the eggs were collected and stored frozen within 5 h of collection. Egg collection occurred on average on day 24.2 after laying (1.5 s.d.).

Embryo morphological measurements

In the lab, we gently thawed eggs and, after removing the eggshell, we detached and weighed the residual yolk sac from each embryo. Then, the embryos were weighed and tarsus length was measured by callipers prior to dissection of the liver and the brain, which were immediately weighed and frozen at −80°C until the analysis of oxidative status markers. All the measurements were taken by the same person to ensure consistency. Embryos were sexed molecularly according to Saino et al. (2008).

Analysis of oxidative status markers in focal organs

TAC, the amount of pro-oxidant molecules and lipid peroxidation were measured in both liver and brain homogenates according to the methods reported in detail by Parolini et al. (2017a). A small piece of brain or liver (~0.1 g) was homogenized in 100 mmol l^{-1} phosphate buffer with 1 mmol l^{-1} EDTA and 100 mmol l^{-1} KCl (pH 7.4), by an automatic homogenizer. The homogenate was centrifuged at 16,000 ***g*** for 10 min and an aliquot of the obtained supernatant was processed for protein content determination according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The remaining supernatant was used for analysis of oxidative status markers. Briefly, TAC was measured according to the method developed by Erel (2004) based on the bleaching of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) in presence of antioxidant molecules within the sample. The amount of pro-oxidant molecules was measured according to a colorimetric method adapted by Erel (2005). The pro-oxidants in the sample oxidize the ferrous ion–*o*-dianisidine complex to ferric ion, which reacts with xylenol orange to produce a blue complex. Lipid peroxidation was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979), adapted to embryo tissue homogenates.

Statistical analysis

We mainly relied on linear mixed models (LMM) where experimental treatment, sex and laying order were included as fixed effect factors, with nest identity as a random effect factor. In the analysis of morphological traits we also included in the models the effect of original egg mass as a covariate. The two-way interactions between factors were also initially included in the models. All the non-significant interaction effects were removed from the models in a single step, to reduce the number of tests on the same variables, which would have increased by a stepwise procedure. To reduce the risk of incurring type I statistical errors due to multiple tests, we corrected significance levels associated to the effect of factors by the false discovery rate (FDR) procedure. Thus, for example, FDR correction for the effect of egg treatment on embryo phenotype was applied to the 10 tests performed on the 10 embryo phenotypic variables. However, in investigating the effects of interactions between main effects, we also relied on visual inspection of variation in mean values among groups (see also Results). This led to the retention of a single (treatment×sex) interaction effect in the models of TAC in the liver (see Results). Statistical parameters are reported with their associated standard error unless otherwise specified. The experiment included 153 embryos from 59 nests. Of these embryos, 51 (29 males, 22 females) originated from control eggs, 23 (8, 15) from vitamin E-injected eggs, 27 (17, 10) from corticosterone-injected eggs and 52 (21, 31) from eggs injected with vitamin E plus corticosterone. For all these embryos, information on all morphological variables was available. Statistically significant outliers (1–6 data points, depending on considered assay and focal organ) were removed after running Grubbs' test (extreme studentized deviate method). Because of the presence of significant outliers, however, the number of embryos included in the analyses of oxidative status variables was reduced to 147–152, depending on the specific variable considered. Statistical analyses were performed with SPSS 21 software.

RESULTS

In a linear mixed model with nest as a random factor, laying date did not significantly vary among egg treatment groups ($F_{3,149}$=0.03, P=0.992). Similarly, eggs mass at laying did not significantly vary among groups ($F_{3,149}$=0.04, P=0.987). In addition, time elapsed between laying and egg collection, which occurred when eggshell fracturing started to become apparent, did not differ among experimental groups ($F_{3,145}$=0.09; P=0.967). Full statistics for the *post hoc* tests of differences in least-squares means are provided in Table S1.

Egg treatment significantly affected total embryo mass, tarsus length and liver mass but not brain mass, when controlling for sex,

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Table 3. Linear mixed models of embryo morphological traits in relation to egg treatment, sex and laying order

Morphological trait	*F*	d.f.	*P*	Males	Females	a-eggs	b-eggs	c-eggs	Coefficient
Embryo body mass									
Treatment	3.33	3145	0.0214*						
Sex	0.15	1145	0.695	46.88 (0.41)	46.67 (0.41)				
Laying order	2.62	2145	0.0761			46.99 (0.48)	47.42 (0.47)	45.92 (0.51)	
Egg mass	111.34	1145	<0.0001						0.50 (0.05)
Tarsus length									
Treatment	6.23	3145	0.0005*						
Sex	1.54	1145	0.216	23.41 (0.21)	23.10 (0.21)				
Laying order	7.02	2145	0.0012*			23.47 (0.24)	23.71 (0.23)	22.57 (0.26)	
Egg mass	1.12	1145	0.291						0.03 (0.02)
Brain mass									
Treatment	1.68	3145	0.175						
Sex	9.37	1145	0.0026*	1.68 (0.02)	1.62 (0.02)				
Laying order	9.86	2145	<0.0001*			1.69 (0.02)	1.67 (0.02)	1.59 (0.02)	
Egg mass	0.97	1145	0.327						0.002 (0.002)
Liver mass									
Treatment	5.32	3145	0.0017*						
Sex	2.19	1145	0.141	0.98 (0.03)	0.93 (0.03)				
Laying order	1.12	2145	0.329			0.99 (0.03)	0.95 (0.03)	0.93 (0.03)	
Egg mass	0.97	1145	0.327						0.001 (0.003)

Egg treatments were: control, vitamin E, corticosterone, vitamin E+corticosterone. The non-significant two-way interaction terms between treatment, sex and laying order were excluded from the models in all cases. The least-squares means (s.e.) for either sex or position in the laying sequence and the coefficient for egg mass are reported. For least-squares means of the treatment groups, see Fig. 1. Asterisks indicate that the effect was statistically significant after false discovery rate (FDR) correction.

laying order and original egg mass (Table 3). *Post hoc* tests showed that total body mass, tarsus length and liver mass of the embryos from Cort eggs were smaller than those of the embryos from the other experimental groups (Fig. 1). In addition, tarsus length of the embryos from VitE eggs was larger than tarsus length of the embryos from VitE+Cort eggs (Fig. 1). Moreover, liver mass of control embryos was larger than liver mass of embryos from VitE+Cort eggs (Fig. 1).

TAC in the liver was significantly affected by egg treatment (as a main effect), with embryos from VitE eggs showing smaller

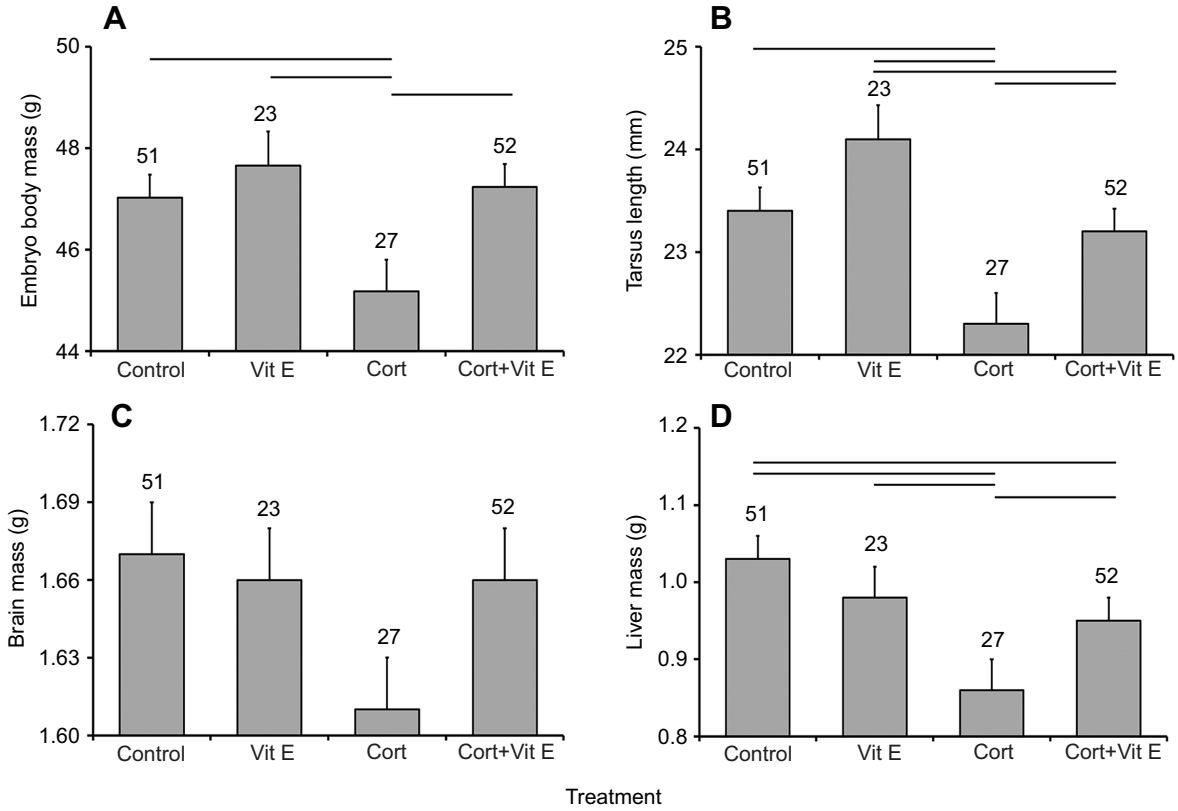

Fig. 1. Morphological variables of embryos from control eggs and eggs injected with vitamin E, corticosterone or vitamin E plus corticosterone. (A) Body mass, (B) tarsus length, (C) brain mass and (D) liver mass. Data are means±s.e.m. Horizontal lines connect pairs of groups that significantly differed in LSD *post hoc* tests. Numbers above histograms indicate sample size. VitE, vitamin E; Cort, corticosterone.

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antioxidant capacity than those from the other treatment groups (Table 4). However, there was an indication that the effects of treatment depended on sex, as shown by the treatment×sex interaction (Table 4). The effect of the sex×treatment interaction was in fact statistically significant before but not after FDR correction. However, FDR correction entails a marked increase in the risk of incurring type II statistical errors, and an inspection of the group means (see Fig. 2B) does indeed suggest that variation according to treatment depended on sex and that the relatively weak effect was due to large variance and small sample size, particularly for VitE males. We therefore retained the treatment×sex effect in the model and present the data while emphasizing that they should be considered with this caveat in mind.

In fact, VitE males had significantly smaller liver TAC than males of the other three groups (Fig. 2B). Conversely, in females the only significant pairwise difference was observed between the control and the VitE+Cort groups (Fig. 2B). In addition, VitE and VitE+Cort females had larger TAC than males from their group, while no significant sex-related differences were observed within the other treatment groups (Fig. 2B).

Lipid peroxidation in the brain was also significantly affected by egg treatment (Fig. 2E). Embryos from Cort eggs had significantly lower lipid peroxidation levels than embryos from control and VitE+Cort eggs. The difference between lipid peroxidation in the brain of Cort and VitE embryos was similar to that between Cort and control embryos but did not attain statistical significance (P=0.127).

No effect of egg treatment was observed on TAC in the brain, the amount of pro-oxidants in both brain and liver, and in lipid peroxidation in the liver (Table 4).

Sex had a significant main effect on brain mass, with males having larger brains but not greater overall mass or tarsus length, than females (Table 3). This effect persisted when we also controlled in the analysis for tarsus length as a proxy for embryo size or for embryo mass (details not shown). In addition, independent of treatment effects, males had a lower TAC in the liver compared with females (Table 4). No sex-related differences existed for the other traits (Tables 3 and 4).

Egg laying order had significant effects on tarsus length and brain mass, with embryos from c-eggs having smaller phenotypic values than embryos from both a- and b-eggs, while controlling for the concomitant effects of treatment, sex and original egg mass (Table 3). No significant variation according to laying order was observed for the other traits (Tables 3 and 4).

DISCUSSION

We manipulated the yolk concentrations of vitamin E, corticosterone and both substances simultaneously in the eggs of yellow-legged gulls and tested the effects on embryo morphology and oxidative status shortly before hatching.

The effects of experimental manipulations on morphological traits were largely consistent with our expectations. The injection of a high corticosterone concentration significantly reduced embryo mass, tarsus length and liver (though not brain) mass relative to controls, while administration of vitamin E in combination with corticosterone restored normal morphological traits as observed in controls. Vitamin E supplementation did not increase size traits relative to controls, suggesting that vitamin E is not generally limiting for embryonic growth under natural conditions, although this may be the case specifically for c-eggs (Parolini et al., 2015), and beneficial effects of vitamin E supplementation may become apparent only under the physiological conditions set by an experimental increase in the concentration of corticosterone.

Table 4. Linear mixed models of embryo oxidative status traits in relation to egg treatment, sex and laying order

	F	d.f.	P	Males	Females	a-eggs	b-eggs	c-eggs
TAC in the brain								
Treatment	1.72	3141	0.166					
Sex	2.24	1141	0.137	1.61 (0.16)	1.86 (0.16)			
Laying order	2.11	2141	0.125			1.92 (0.17)	1.55 (0.17)	1.75 (0.17)
TAC in the liver								
Treatment[a]	3.70	3137	0.013*					
Sex	6.91	1137	0.0052*	1.52 (0.17)	2.00 (0.16)			
Laying order	2.97	2137	0.055			1.94 (0.17)	1.51 (0.17)	1.82 (0.18)
Treatment×sex[b]	3.70	3137	0.0135					
ROS in the brain								
Treatment	1.92	3142	0.130					
Sex	0.10	1142	0.756	55.6 (3.6)	56.9 (3.6)			
Laying order	0.15	2142	0.863			56.2 (4.0)	55.1 (3.9)	57.5 (4.0)
ROS in the liver								
Treatment	0.51	3141	0.678					
Sex	0.73	1141	0.394	255.8 (9.8)	244.2 (9.6)			
Laying order	0.61	2141	0.545			254.3 (11.4)	239.8 (11.5)	255.8 (11.8)
LP in the brain								
Treatment	3.19	3145	0.026					
Sex	2.93	1145	0.089	10.57 (0.58)	9.65 (0.58)			
Laying order	0.82	2145	0.443			10.54 (0.61)	9.93 (0.61)	9.86 (0.61)
LP in the liver								
Treatment	0.12	3144	0.946					
Sex	0.00	1144	0.966	13.34 (0.70)	13.31 (0.70)			
Laying order	2.31	2144	0.103			14.37 (0.76)	12.92 (0.75)	12.69 (0.76)

Egg treatments were: control, vitamin E, corticosterone, vitamin E+corticosterone. TAC, total antioxidant capacity; ROS, reactive oxygen species; LP, lipid peroxidation. The non-significant two-way interaction terms between treatment, sex and laying order were excluded from the models in all cases except for the model of TAC in the liver. The least-squares means (s.e.) for either sex or position in the laying sequence and the coefficient for egg mass are reported. For least-squares means of the treatment groups, see Fig. 2. Asterisks indicate that the effect was statistically significant after FDR correction.

[a]TAC of vitamin E-treated embryos was significantly smaller than TAC from the other groups in LSD tests.

[b]Retained in the model although not statistically significant after FDR correction.

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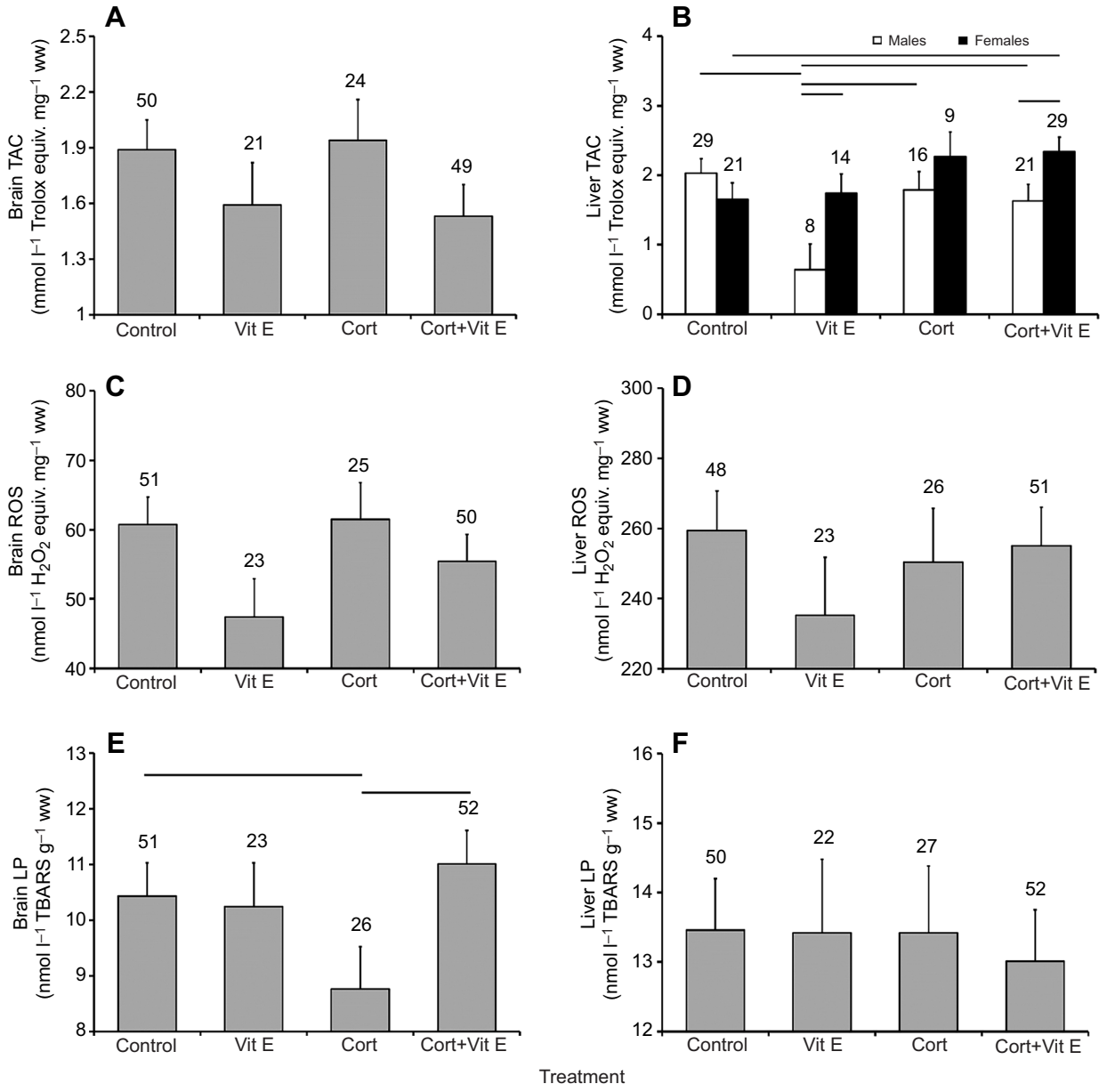

Fig. 2. Oxidative status variables of embryos from control eggs and eggs injected with vitamin E, corticosterone or vitamin E plus corticosterone. (A,B) Total antioxidant capacity (TAC) in the brain (A) and the liver (B). (C,D) Amount of pro-oxidant molecules (reactive oxidant species, ROS) in the brain (C) and in the liver (D). (E,F) Lipid peroxidation (LP) in the brain (E) and the liver (F). Data are means±s.e.m. Horizontal lines connect pairs of groups that significantly differed in LSD *post hoc* tests. Numbers above histograms indicate sample size.

However, it should be noted that vitamin E supplementation did not fully restore normal liver mass when administered together with corticosterone, implying that the effect of vitamin E differs among morphological traits. Interestingly, the correlation between corticosterone and vitamin E concentrations in the eggs of the gull population that we studied here is relatively high (r=0.30; Rubolini et al., 2011), though marginally non-significant, suggesting that mothers tend to allocate more vitamin E to the eggs that also contain larger concentrations of corticosterone, possibly to also compensate for the negative effect of the latter on growth.

In a previous study, we showed that vitamin E at half the dose used in the present study boosted body mass but not tarsus length of the embryos at the same developmental stage (Parolini et al., 2017a). Thus, different vitamin E doses seem to differentially affect different traits. In addition, another experiment showed that the same doses used in the present experiment depressed body mass but not tarsus length in 4 day old chicks (whereas it had no effect at hatching; Possenti et al., 2018a,b). Hence, the same physiological excess of vitamin E has markedly different effects at different, though close life stages. However, also in the previous experiment where we focused on chicks rather than embryos, supplementation of both compounds restored a normal phenotype in terms of body mass at 4 days of age, again suggesting that an increase in either egg compound concentration must be accompanied by an increase in the other in order to attain a normal phenotype for the offspring (Possenti et al., 2018a,b). It should also be noted that no effect of experimental treatment on tarsus length was observed among chicks (Possenti et al., 2018a,b), whereas it was very clearly observed here among embryos, again implying that the effects of the two egg compounds varies with life stage.

The analyses of oxidative status variables showed no effect of experimental treatment on TAC in the brain, on ROS concentrations in both focal organs, and on lipid peroxidation in the liver. These results of no effect of vitamin E treatment on oxidative status endpoints are consistent with a previous study where we applied half the dose of vitamin E used here (Parolini et al., 2017a).

In the present study, vitamin E supplementation depressed TAC in the liver. This result is consistent with the negative postnatal effect of vitamin E supplementation at the same dose as used here on plasma TAC of chicks (Possenti et al., 2018a,b). However, such an effect was not observed in Parolini et al. (2017a), where half the dose used in the present study did not affect TAC in the liver of the embryos. The reason why high, physiological concentrations of vitamin E cause negative effects on TAC both in the liver of embryos (present study) and in the plasma of young chicks (Parolini et al., 2017a) remains open to speculation. As noted in Possenti et al. (2018a,b), excess vitamin E within physiological limits is expected not to have detrimental effects, based on studies of domestic animals, but very little is known about the effects of vitamin E supplementation in wild animals. In a previous study of the barn swallow, while supplementation of nestlings with moderate doses of vitamin E boosted body growth, supplementation with larger amounts within physiological limits reversed the positive effects of moderate doses (de Ayala et al., 2006). Hence, the effect of vitamin E seems not to change linearly according to dose even within physiological limits, not only for body growth but also for antioxidant capacity. An alternative interpretation is that supplementation with vitamin E changes the allocation of antioxidants among body tissues, resulting in lowered antioxidant capacity in the liver. It should also be noted that the negative effects of high concentrations of vitamin E on TAC in the liver were mainly experienced by males, as suggested by the treatment×sex interaction effect and by the *post hoc* comparisons. While this pattern seems quite obvious from the observation of the within-group and -sex mean data presented in Fig. 2B, we emphasize that the statistical effect of the treatment×sex interaction was significant before but not after FDR correction, and this piece of evidence should therefore be considered with this caveat in mind.

Corticosterone treatment appeared to lower lipid peroxidation in the brain while normal lipid peroxidation levels were restored when corticosterone was administered in conjunction with vitamin E. The interpretation of this effect is open to speculation. Corticosterone treatment caused a marked reduction in brain size, relative to the other treatments, as shown in Fig. 1C, although the effect of treatment was not statistically significant. We may speculate that reduced lipid peroxidation in the brain caused by corticosterone treatment resulted from reduced brain growth rates. Again, however, treatment with vitamin E in association with corticosterone administration restored normal brain lipid peroxidation while vitamin E administration per se did not affect lipid peroxidation. These result suggest that corticosterone and vitamin E have contrasting effects on embryo and young chick physiology, and that an excess of vitamin E does not affect physiological perinatal offspring physiological traits.

Finally, we observed that males had significantly larger brains than females after controlling for embryo size or mass. A sex difference in brain mass is consistent with evidence from previous studies (Parolini et al., 2017c), although it was not affected by yolk supplementation with vitamin E, corticosterone or their mixture.

In conclusion, the present experiment suggests for the first time in any wild bird species that corticosterone depresses prenatal growth but such negative effects are countered by vitamin E supplementation, implying antagonistic effects of these egg constituents on embryonic growth. However, vitamin E seems not to be limiting to yellow-legged gull eggs (but see Parolini et al., 2015, for data on c-eggs). Although we injected a high, potentially supraphysiological corticosterone concentration, it does not undermine the conclusion that vitamin E can neutralize the detrimental effect of this molecule. In addition, the present results, in combination with those from previous studies, highlight differences in the effects of different doses of vitamin E and corticosterone on morphological and physiological traits at different, albeit close, life stages, implying that the effects of variation in the concentration of egg components on offspring phenotype should not be generalized across life stages and egg concentrations of these important compounds.

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Conceptualization: M.P., A.R., N.S.; Methodology: M.P., C.D.P.; Formal analysis: M.P., N.S.; Investigation: M.P., C.D.P., S.S., S.C., M.C., D.R., A.R., N.S.; Resources: N.S.; Data curation: N.S.; Writing - original draft: M.P.; Supervision: N.S.

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