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

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Abstract

Variation in the concentration of antioxidants and hormones of maternal origin in the eggs of birds can have profound influences on offspring phenotype both pre- and post-natally. Egg maternal substances, however, 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 considered. We manipulated the yolk concentrations of vitamin E and corticosterone, which are the main antioxidants and, respectively, 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, yet physiological doses of corticosterone depressed embryo growth (total body mass, tarsus length and liver mass) while administration of vitamin E in association with corticosterone restored normal growth. Vitamin E did not affect embryo growth when administered alone. We further analyzed independent and combined effects of vitamin E and corticosterone on liver and brain total antioxidant capacity, 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 post-natal rather than pre-natal life stages, our findings indicate that the effects of a physiological increase in the egg concentrations of these substances can be life stage- and dose-specific, implying that generalizing prenatal effects of egg compounds may not be feasible.

Keywords: corticosterone; embryo growth, yellow-legged gull; oxidative status; vitamin E

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 carry-over in post-natal life, as well as into adulthood (Royle, Surai & Hartley, 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 trans-generational effects whereby conditions experienced by the mothers are translated into phenotypic variation in the offspring (Mousseau & Fox, 1998). Such early maternal effects, which may adaptive or, conversely, reflect constraints on the ability of mothers to produce eggs of optimal composition, have therefore not only attracted the interest of animal production and physiology research but also that of ecologists and evolutionary biologists. Importantly, egg compounds are expected to act in concert on offspring development and post-natal growth, and the effect of individual components is therefore expected to depend on the concomitant effect of other components, as determined by their relative concentrations (Royle, Surai & Hartley, 2001; Surai, 2002; Possenti et al., 2017, 2018). However, the combined, besides the independent effects of egg constituents has been very seldom subjected to experimental analysis (e.g. Giraudeau et al., 2016; Possenti et al., 2018).

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 pre-natal life stages (Surai, 2002, Costantini, 2014). In birds, vitamin E, a class of compounds which 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 post-natal 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 to circumvent such potentially confounding effects (Groothuis et al., 2005).

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 & Groothuis, 2003; Groothuis et al., 2005). Studies of corticosterone in particular have shown that increased egg concentrations can impair post-natal somatic growth and immune function, ultimately depressing survival (Eriksen et al. 2003; Janczak, Braastad & Bakken, 2006; Rogers & Deng 2005; Rubolini et al., 2005; Saino et al., 2005; Henriksen, Groothuis, & Rettenbacher, 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, Costantini et al., 2008, Costantini, Marasco & Møller, 2011; Haussmann et al., 2012; Stier et al. 2009; Monaghan, 2014). For this reason, the effect of an increase in corticosterone levels on oxidative status of the offspring is expected to depend on the concomitant concentration of antioxidants, including vitamin E (Possenti et al., 2018). On the other hand, 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 been very seldom investigated (Williams and Groothuis, 2015; Giraudeau et al., 2016; Possenti et al., 2018) 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 investigate the combined effects of an experimental manipulation of corticosterone and vitamin E concentration on embryo morphology and oxidative status in the yellow-legged gull (*Larus michahellis*), a species that has been previously subjected to experimental investigation of 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, 2018).

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 of that used in the present study) and found that body mass but not tarsus length at the eggshell cracking stage (i.e. the same 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, 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 third (last) laid eggs in a clutch, consistent with the expectation because last-laid eggs contain smaller concentrations of vitamin E than first and second laid eggs (Parolini et al., 2015). This experiment also showed a significant positive effect of egg vitamin E on plasma total antioxidant capacity 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, Martinelli & Saino, 2006).

In another experiment we aimed at testing if egg vitamin E and corticosterone have antagonistic effects on post-natal development and oxidative status, by increasing egg concentration of either or both compounds simultaneously by two standard deviations (Possenti et al. 2018). 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. 2018). Importantly, in the Possenti et al. (2018) experiment a larger, still physiological dose than that used in the other experiments was injected (Parolini et al., 2015, 2017a,b, 2018).

Because corticosterone and vitamin E can interact in affecting offspring phenotype, but these effects apparently differ even between closely spaced life stages (see Parolini et al., 2017a,b), in the present study we analyzed the independent and combined effects of vitamin E and corticosterone on morphology (embryo mass, tarsus length, brain mass, liver mass) and oxidative status (total antioxidant capacity, amount of pro-oxidant molecules and lipid peroxidation in the brain and the liver) at the pre-hatching, embryonic stage. To this goal, we established three groups of eggs where, immediately after laying, we increased the concentration of vitamin E alone, of corticosterone alone, or of both 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 oxidative status at two organs. This is important because the effect of vitamin E can vary with its concentration, also 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 soon before hatching because the effects experienced during the prenatal developmental stage can result in consequences at early postnatal stage and adulthood. Moreover, as our previous studies of the yellow-legged gull have shown contrasting effects due to the supplementation of a focal antioxidant or putatively pro-oxidant molecule on different phenotypic traits (Table 1), in the present study we aim at investigating if the consequences on embryos of the combined Vitamin E and corticosterone *in-ovo* injection return dissimilar results compared to hatchlings (Possenti et al., 2018). According to previous findings on the yellow-legged gull, we expected that vitamin E treatment enhanced embryonic growth (Parolini et al., 2017), corticosterone treatment depressed it (Rubolini et al., 2015), and treatment with both substances restored normal growth. As for the effect of experimental manipulations on oxidative status variables, we expected that corticosterone reduced total antioxidant capacity and increased the concentration of reactive oxygen species and lipid peroxidation, whereas vitamin E treatment had opposite effects and that treatment with both compounds restored the oxidative status observed among controls.

Materials and Methods

Study species

The yellow-legged gull is a monogamous, semi-colonial charadriiform (Cramp, 1998). Clutch size varies between 1-3, which are laid at 1-3 days intervals. Modal clutch size is 3 eggs. Eggs hatch asynchronously, over 1-4 days) after 27-31 days of incubation by both parents. 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 position in the laying sequence. Specifically, vitamin E yolk concentration declines with the laying sequence, while corticosterone concentration does not vary between first-, second- and third-laid eggs (Rubolini et al., 2011). Hatching of sibling eggs occurs over 1-4 days and hatch order reflects laying order.

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 under the permission of Parco Regionale Delta del Po. We visited the colony every other day to record and individually mark newly laid eggs. The day when a newly laid egg was found, it was temporarily removed from its nest and replaced with a dummy egg in order to minimize perturbation of behaviour of 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-clutches variation in egg quality and parental incubation behaviour. Each egg was assigned to one of the following treatments: control injection (Cont), injection with vitamin E (VitE), injection with corticosterone (Cort), 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., 2018). Treatment schemes with corticosterone and vitamin E plus corticosterone were: nest 1, a-egg: VitE+Cort; b-egg: Cort; c-egg: Cont; nest 2, Cort – VitE+Cort - Cont; nest 3, Cont - Cort – VitE+Cort; nest 4, Cont – VitE+Cort - Cort; nest 5, Cort- Cont – VitE+Cort; nest 6, VitE+Cort – Cont - Cort and so forth with the following nests. Treatment schemes with vitamin E and vitamin E plus corticosterone were: nest 1, a-egg: VitE+Cort; b-egg: VitE; c-egg: Cont; nest 2, VitE - VitE+Cort - Cont; nest 3, VitE+Cort – VitE - Cort; nest 4, Cont – VitE - VitE+Cort; nest 5, VitE – Cont - VitE+Cort; nest 6, VitE+Cort – Cont - VitE and so forth with the following nests. We aimed at increasing the concentration of both vitamin E and corticosterone by 2 standard deviations

of their concentrations recorded in the same 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 bearing 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 9 bird species ranged between 0.1 and 0.53 ng/g (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 was up to 75 pg/g (Larsen et al., 2015). Thus, the concentration we injected could be considered as supraphysiological. Because the variance in the concentrations of these substances varied both among positions in the laying sequence (for Vitamin E only, not for corticosterone) and with yolk mass, we decided to tune the amount of the substances due to be injected based on both these variables. We grouped a-, b- and c-eggs each 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 x egg mass – 1.815. (see Parolini et al. 2015; Possenti et al. 2018). The absolute amount of vitamin E and corticosterone due to be injected in each individual egg was then computed as the product between the relevant standard deviation value and the estimated yolk mass (Possenti et al., 2018). The absolute amounts of vitamin E and corticosterone that were injected are reported in Possenti et al. (2018; specifically in Table 1). The doses of vitamin E and corticosterone were dissolved in 30 µl corn oil. The solutions for all the different laying order by egg mass classes of eggs were prepared in advance and stored in sterile vials. A single vial was used in each treatment day and then disposed. Control eggs were injected with 30 µl corn oil. The injection procedures were performed as reported in Romano et al. (2008). The same procedures have been applied in several other experiments performed by our group on the yellow-legged gull (e.g. Parolini et al., 2015; 2017a,b; Possenti et al., 2018). When the egg manipulation procedures were completed, the eggs were returned to their original nest within 2 hours after 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 hours after 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 calipers prior to the 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

Total antioxidant capacity (TAC), the amount of pro-oxidant molecules (TOS) and lipid peroxidation (LPO) 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 and liver (~ 0.1 g) was homogenized in 100 mM phosphate buffer added with 1 mM EDTA and 100 mM 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 remainder 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 ($ABTS^{*+}$) in presence of antioxidant molecules within the sample. The amount of pro-oxidant molecules (TOS) 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 reacting with xylenol orange returns a blue complex. Lipid peroxidation was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979), adapted to embryos' 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, whit 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 been 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, false discovery rate correction for the effect of egg treatment on embryo phenotype was applied to the ten tests performed on the ten 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 by 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 (Cont), 23 (8; 15) from Vitamin E injected eggs (VitE), 27 (17; 10) from corticosterone injected eggs (Cort), and 52 (21; 31) from eggs injected with vitamin E plus corticosterone (VitE-Cort). 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 the Grubbs' test (extreme studentized deviate method). Due to the presence of significant outliers (see Methods), 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 by 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 sensible, 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 supporting information (Table S1).

Egg treatment significantly affected total embryo mass, tarsus length and liver mass but not brain mass, while controlling for sex, laying order and original egg mass (Table 2). *Post hoc* tests showed that total body mass, tarsus length and liver mass of the embryos from Cort eggs was smaller than mass of the embryos from the other experimental groups (Figure 1). In addition, tarsus length of the embryos from VitE eggs was larger than tarsus length of the embryos from VitE+Cort eggs (Figure 1). Moreover, liver mass of Cont embryos was larger than liver mass of embryos from VitE+Cort eggs (Figure 1).

Total antioxidant capacity in the liver was significantly affected by egg treatment (as a main effect), with embryos from VitE eggs showing smaller antioxidant capacity than those from the other treatment groups (Table 3). However, there was a hint that the effects of treatment depended on sex, as indicated by the effect of the treatment by sex interaction (Table 3). The effect of the sex by treatment interaction was in fact statistically significant before but not after FDR correction. However, false discovery rate correction entails a marked increase in the risk of incurring type II statistical errors and an inspection of the group means (see Figure 2, panel ‘b’) 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 by 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 TAC than males of the other three groups (Figure 2). Conversely, in females the only significant pairwise difference was observed between the Cont and the VitE+Cort groups (Figure 2). 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 (Figure 2).

Lipid peroxidation in the brain was also significantly affected by egg treatment. Embryos from Cort eggs had significantly smaller lipid peroxidation levels than embryos from Cont and VitE+Cort

eggs. The difference between lipid peroxidation in the brain of Cort and VitE embryos was similar to that between Cort and Cont embryos but did not attain statistical significance ($P = 0.127$).

No effect of egg treatment was observed on TAC in the brain, amount of pro-oxidants in both brain and liver, and in LPO in the liver (Table 3).

Sex had a significant main effect on brain mass, with males having larger brains but not overall mass or tarsus length, than females (Table 2). 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 smaller TAC in the liver compared to females (Table 3). No sex-related differences existed for the other traits (Table 2 and 3).

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 2). No significant variation according to laying order was observed for the other traits (Table 2 and 3).

Discussion

We manipulated the yolk concentrations of vitamin E, of corticosterone and of 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 the 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 embryonic growth under natural conditions, although it can be specifically for c-eggs (Parolini et al. 2015) and that beneficial effects of vitamin E supplementation become apparent only under the physiological conditions set by an experimental increase in the concentration of corticosterone. 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 dose as that 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-days-old chicks (whereas it had no effect at hatching; Possenti et al., 2018). 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 age 4 days, again suggesting that an increase in either egg compound concentration must be accompanied by an increase also in the other in order to attain a normal phenotype for the offspring (Possenti et al., 2018). It should also be noted that no effect of experimental treatments on tarsus length was observed among chicks (Possenti et al., 2018), 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 total antioxidant capacity in the brain, on reactive oxygen species concentrations in both focal organs, and in 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 total antioxidant capacity in the liver. This result is consistent with the negative post-natal effect of vitamin E supplementation at the same dose used here on plasma total antioxidant capacity of chicks (Possenti et al., 2018). However, such effect was not observed in Parolini et al. (2017a), where half the dose used in the present study did not affect total antioxidant capacity in the liver of the embryos. The causes why high, physiological concentrations of vitamin E cause negative effects on total antioxidant capacity both in the liver of embryos (present study) and in the plasma of young chicks (Parolini et al., 2017a) remain open to speculation. As noted in Possenti et al. (2018), excess vitamin E within physiological limits is expected not to have detrimental effects, based on studies of domestic animals, but very little is known on 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, Martinelli & Saino, 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 of vitamin E changes the allocation of antioxidants among body districts, resulting in lowered antioxidant capacity in the liver. It should also be noted that the negative effects of high concentrations of vitamin E on total antioxidant capacity in the liver were mainly experienced by males, as suggested by the treatment by 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 Figure 2 (panel ‘b’), we emphasize that the statistical effect of the treatment by 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 it appears from Figure 1 (panel ‘c’), 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 also after controlling for embryo size or mass. Sex difference in brain mass is consistent with evidence from previous studies (Parolini et al., 2017c), although it was not affected by the yolk supplementation of 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 the generality of the eggs (but see Parolini et al., 2015 for 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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Conflict of interest

The authors declare no conflict of interest.

Data accessibility

The data will be included as supporting information upon the acceptance of the manuscript.

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Table 1: effect of *in-ovo* injection of antioxidant and putatively pro-oxidant molecules in the yolk of the yellow-legged gull on different phenotypic traits at prenatal (embryo) and postnatal (hatchling) stage. '+' indicates a positive significant effect of the yolk supplementation compared to control group, '-' indicates a negative significant effect of the yolk supplementation compared to control group, while '=' indicates a null non-significant effect of the yolk supplementation compared to control group on a specific phenotypic trait.

Injected molecule	**Concentration range**	**Developmental stage**	**Phenotypic trait**	**Effect**	**Reference**
Vitamin E	305-748 µg (+ 1 SD)	Hatchlings	Body mass	+	Parolini et al., 2015
			Tarsus length	+	
Vitamin E	305-748 µg (+ 1 SD)	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 SD)	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 SD)	Hatchlings	Begging frequency	-	Possenti et al., 2018a
			Reversal-to-Prone response	=	
Vitamin E	567-1392 µg (+2 SD)	Hatchlings (0-d old)	Body mass	=	Possenti et al., 2018b
			Tarsus length	=	
		Hatchlings (4-d old)	Body mass	-	
			Tarsus length	=	

Corticosterone	66-150 ng (+2 SD)	Hatchlings (0-d old)	Body mass	=
			Tarsus length	=
		Hatchlings (4-d 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 SD)	Hatchlings (0-d old)	Body mass	=
			Tarsus length	=
		Hatchlings (4-d old)	Body mass	=
			Tarsus length	=
			Total antioxidant capacity	+
			Amount of pro-oxidant molecules	=

Table 2: Linear mixed models of embryo morphological traits in relation to egg treatment (Control, Vitamin E, Corticosterone, Vitamin E + Corticosterone), sex and laying order. 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 for either sex or positions in the laying sequence and the coefficient for egg mass are reported. For least squares means of the treatment groups see Figure 1. Superscript asterisks indicate that the effect was statistically significant after FDR correction.

				Least squares means					
	F	df	P	Males	Females	a-eggs	b-eggs	c-eggs	Coefficient
Embryo body mass									
Treatment	3.33	3,145	0.0214*						
Sex	0.15	1,145	0.695	46.88 (0.41)	46.67 (0.41)				
Laying order	2.62	2,145	0.0761			46.99 (0.48)	47.42 (0.47)	45.92 (0.51)	
Egg mass	111.34	1,145	<0.0001						0.50 (0.05)
Tarsus length									
Treatment	6.23	3,145	0.0005*						
Sex	1.54	1,145	0.216	23.41 (0.21)	23.10 (0.21)				
Laying order	7.02	2,145	0.0012*			23.47 (0.24)	23.71 (0.23)	22.57 (0.26)	
Egg mass	1.12	1,145	0.291						0.03 (0.02)
Brain mass									
Treatment	1.68	3,145	0.175						
Sex	9.37	1,145	0.0026*	1.68 (0.02)	1.62 (0.02)				
Laying order	9.86	2,145	<0.0001*			1.69 (0.02)	1.67 (0.02)	1.59 (0.02)	

Egg mass	0.97	1,145	0.327						0.002 (0.002)
Liver mass									
Treatment	5.32	3,145	0.0017*						
Sex	2.19	1,145	0.141	0.98 (0.03)	0.93 (0.03)				
Laying order	1.12	2,145	0.329			0.99 (0.03)	0.95 (0.03)	0.93 (0.03)	
Egg mass	0.97	1,145	0.327						0.001 (0.003)

Table 3: Linear mixed models of embryo oxidative status traits in relation to egg treatment (Control, Vitamin E, Corticosterone, Vitamin E + Corticosterone), sex and laying order. 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 for either sex or positions in the laying sequence and the coefficient for egg mass are reported. For least squares means of the treatment groups see Figure 2. Superscript asterisks indicate that the effect was statistically significant after FDR correction.

				Least squares means					
	F	df	P	Males	Females	a-eggs	b-eggs	c-eggs	Coefficient
TAC in the brain									
Treatment	1.72	3,141	0.166						
Sex	2.24	1,141	0.137	1.61 (0.16)	1.86 (0.16)				
Laying order	2.11	2,141	0.125			1.92 (0.17)	1.55 (0.17)	1.75 (0.17)	
TAC in the liver									
Treatment[a]	3.70	3,137	0.013*						
Sex	6.91	1,137	0.0052*	1.52 (0.17)	2.00 (0.16)				
Laying order	2.97	2,137	0.055			1.94 (0.17)	1.51 (0.17)	1.82 (0.18)	
Treatment x Sex[b]	3.70	3,137	0.0135						
ROS in the brain									
Treatment	1.92	3,142	0.130						
Sex	0.10	1,142	0.756	55.6 (3.6)	56.9 (3.6)				
Laying order	0.15	2,142	0.863			56.2 (4.0)	55.1 (3.9)	57.5 (4.0)	

ROS in the liver								
Treatment	0.51	3,141	0.678					
Sex	0.73	1,141	0.394	255.8 (9.8)	244.2 (9.6)			
Laying order	0.61	2,141	0.545			254.3 (11.4)	239.8 (11.5)	255.8 (11.8)
LPO in the brain								
Treatment	3.19	3,145	0.026					
Sex	2.93	1,145	0.089	10.57 (0.58)	9.65 (0.58)			
Laying order	0.82	2,145	0.443			10.54 (0.61)	9.93 (0.61)	9.86 (0.61)
LPO in the liver								
Treatment	0.12	3,144	0.946					
Sex	0.00	1,144	0.966	13.34 (0.70)	13.31 (0.70)			
Laying order	2.31	2,144	0.103			14.37 (0.76)	12.92 (0.75)	12.69 (0.76)

[a] TAC of VitE embryos was significantly smaller than TAC from the other groups at LSD tests.

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

Figures

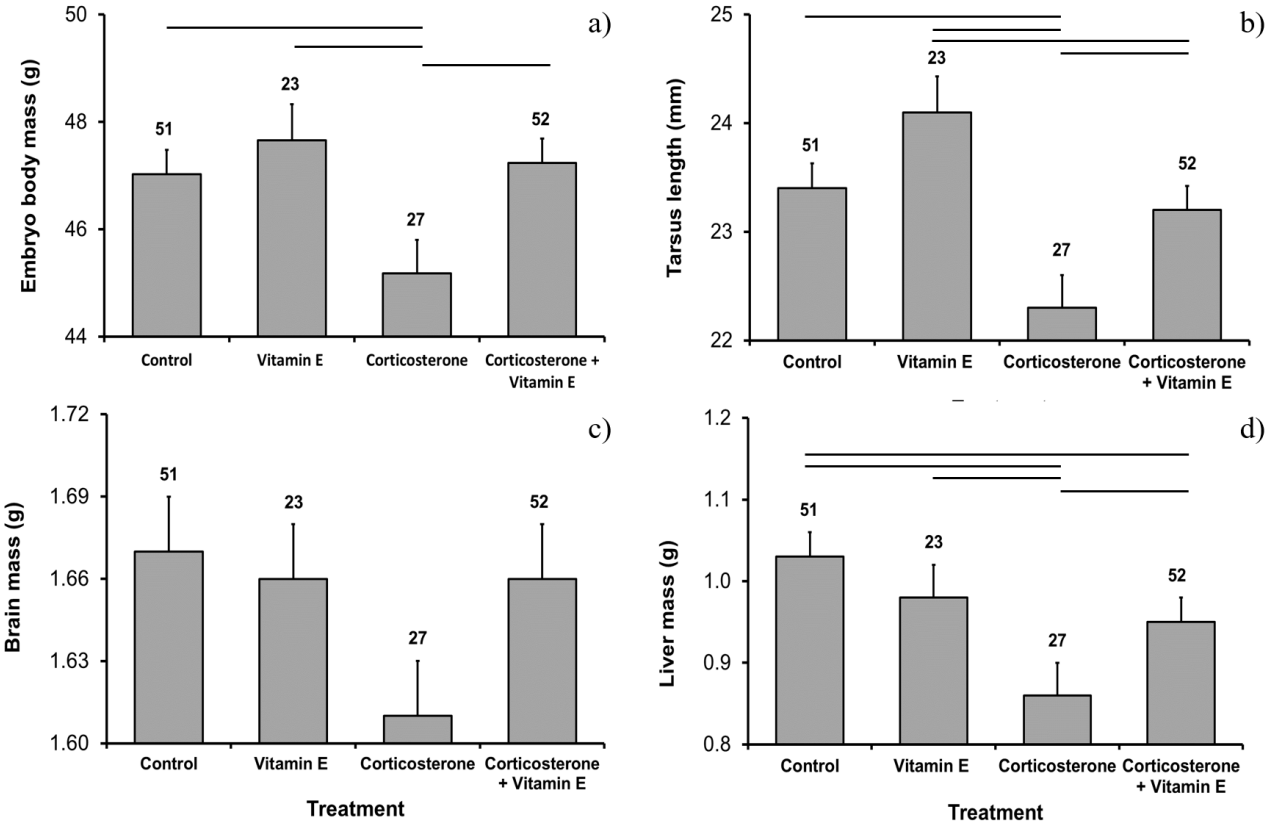

Figure 1: Mean (+ standard error bar) for morphological variables (body mass, tarsus length, brain mass, liver mass; panels a-d) of embryos from control eggs or eggs injected with vitamin E, corticosterone or vitamin E plus corticosterone. Horizontal lines in the body of the panels connect pairs of groups that significantly differed at LSD post hoc tests. Numbers above histograms indicate sample sizes.

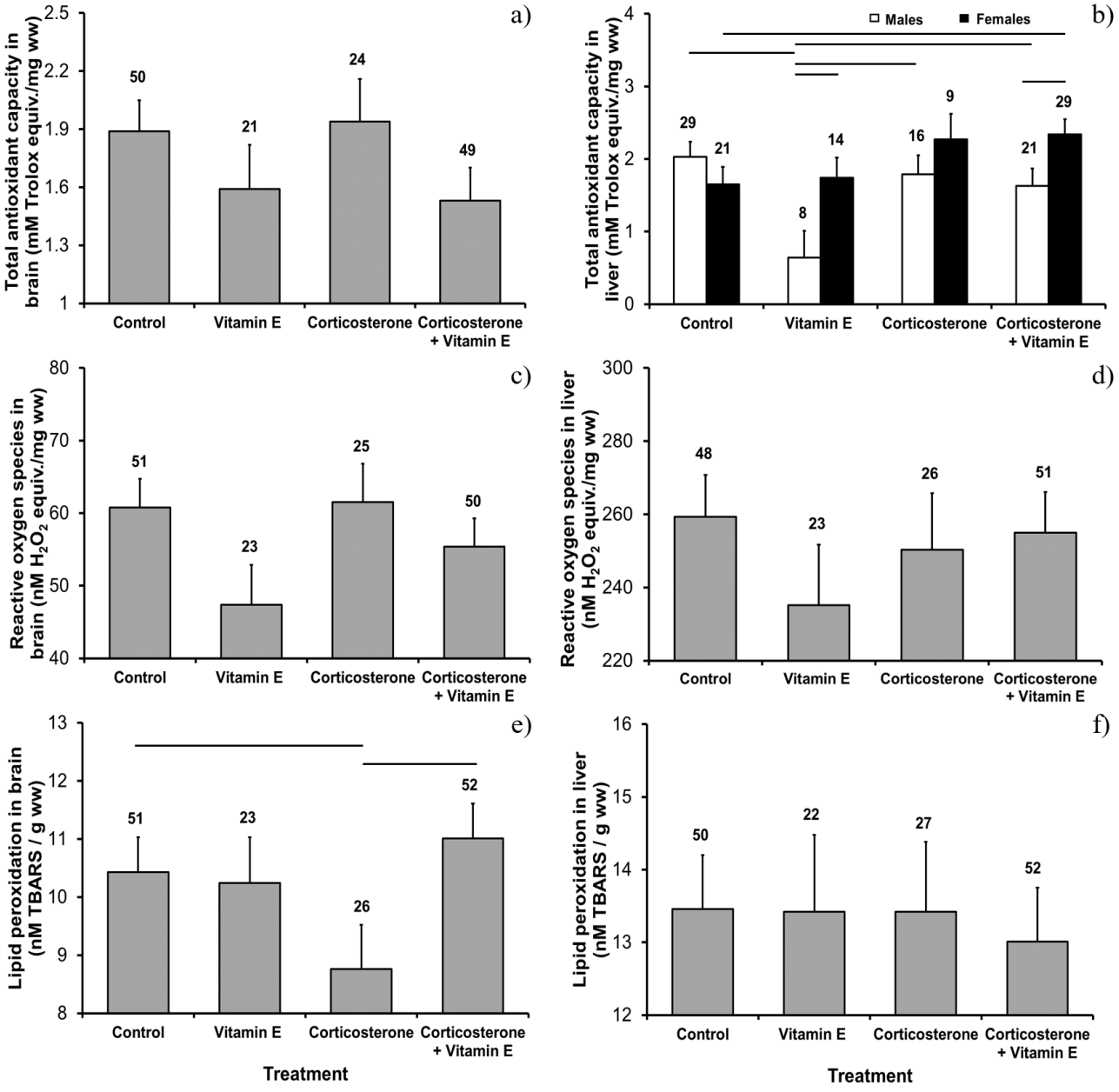

Figure 2: Mean (+ standard error bar) for oxidative status variables; total antioxidant capacity in the brain a) and the liver b), amount of pro-oxidant molecules in the brain c) and in the liver d), and lipid peroxidation in the brain e) and the liver f) of embryos from control eggs or eggs injected with vitamin E, corticosterone or vitamin E plus corticosterone. Horizontal lines in the body of the panels connect pairs of groups that significantly differed at LSD post hoc tests. Numbers above histograms indicate sample sizes.

Table S1: Full statistics for the post hoc tests of differences in least squares means reported in Figures 1 and 2. CONT = control group; VitE = Vitamin E injected; CORT = corticosterone injected; VitE+CORT = injection of vitamin E and Corticosterone mixture.

	Phenotypic trait	**Treatment**	**P**
Figure 1A	Embryo body mass		
	CONT	CORT	0.011
	VitE	CORT	0.007
	CORT	VitE+CORT	0.006
Figure 1B	Tarsus length		
	CONT	CORT	0.001
	VitE	CORT	<0.001
	VitE	VitE+CORT	0.018
	CORT	VitE+CORT	0.006
Figure 1D	Liver mas		
	CONT	CORT	<0.001
	CONT	VitE+CORT	0.023
	VitE	CORT	0.04
	CORT	VitE+CORT	0.052
Figure 2B	TAC liver		
	CONT males	VitE males	<0.001
	CONT females	VitE+CORT females	0.013
	VitE males	VitE+CORT males	0.019
	VitE males	CORT males	0.009
	VitE males	VitE females	0.016
	VitE+CORT males	VitE+CORT females	0.018
Figure 2E	LPO brain		
	CONT	CORT	0.021
	CORT	VitE+CORT	0.003

Table S1: Full statistics for the post hoc tests of differences in least squares means reported in Figures 1 and 2. CONT = control group; VitE = Vitamin E injected; CORT = corticosterone injected; VitE+CORT = injection of vitamin E and Corticosterone mixture.

	Phenotypic trait	**Treatment**	**P**
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	VitE	CORT	<0.001
	VitE	VitE+CORT	0.018
	CORT	VitE+CORT	0.006
Figure 1D	Liver mas		
	CONT	CORT	<0.001
	CONT	VitE+CORT	0.023
	VitE	CORT	0.04
	CORT	VitE+CORT	0.052
Figure 2B	TAC liver		
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	CONT females	VitE+CORT females	0.013
	VitE males	VitE+CORT males	0.019
	VitE males	CORT males	0.009
	VitE males	VitE females	0.016
	VitE+CORT males	VitE+CORT females	0.018
Figure 2E	LPO brain		
	CONT	CORT	0.021
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