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RESEARCH ARTICLE

Prenatal yolk corticosterone exposure promotes skeletal growth and induces oxidative imbalance in yellow-legged gull embryos

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ABSTRACT

Maternally derived hormones induce variation in offspring phenotype, with consequences that can carry over into post-natal life and even into adulthood. In birds, maternal egg corticosterone (CORT) is known to exert contrasting effects on offspring morphology, physiology and behaviour after hatching. However, information on the effects of CORT exposure on pre-hatching embryonic development is limited. We experimentally increased yolk CORT levels in yellow-legged gull (*Larus michahellis*) eggs, and assessed the effects on embryo pre-hatching development and oxidative status of brain and liver. CORT-supplemented embryos reached a larger skeletal size and liver mass compared with controls. Embryos from CORT-injected last-laid eggs showed decreased activity of the hepatic antioxidant enzymes superoxide dismutase and catalase, while intermediate-laid eggs showed increased levels of lipid peroxidation. However, elevated yolk CORT did not affect oxidative stress endpoints in the brain. Our results indicate that elevated yolk CORT levels affect prenatal embryo development by promoting skeletal growth, and induce laying sequence- and organ-specific oxidative imbalance, with potential adverse consequences during postnatal life, especially for late-hatched offspring.

KEY WORDS: Maternal effects, Embryo growth, Oxidative stress, Maternally derived hormones

INTRODUCTION

Cleidoic eggs of vertebrates are relatively sealed environments with limited exchange of materials with the external environment. Embryo development and the sustainment of its physiological processes are therefore based uniquely on substances transmitted by the mothers to the eggs. Although some classes of egg components, such as maternally derived antioxidants and hormones, occur in the eggs at a relatively low concentration, variation in their level has been shown to play a key role in embryo developmental trajectories, with effects that can also carry over into post-natal life, and even into adulthood (Royle et al., 2001; Saino et al., 2003; Rubolini et al., 2005; Groothuis et al., 2005, 2006). The transfer of several types of maternal substances to the eggs is known to depend on environmental conditions experienced by mothers. For example, some substances, such as vitamins and carotenoids, are usually acquired through the diet, and therefore their circulating concentration should vary according to their availability in the environment (Parolini et al., 2015). Egg components of maternal origin can therefore act as transgenerational mediators between conditions experienced by the mothers and the phenotypic variation of the offspring (Mousseau and Fox, 1998). Such early maternal effects can be adaptive if they predispose the offspring to develop specific phenotypic traits promoting fitness under predicted environmental conditions to which they will be exposed to during postnatal life. However, they can also reflect ecological constraints possibly limiting the ability of mothers to produce eggs of optimal composition, thus resulting in the production of offspring of sub-optimal quality (Mousseau and Fox, 1998; Groothuis et al., 2005; Love and Williams, 2008; Love et al., 2013).

Among the substances transferred by mothers to their eggs, steroid hormones, such as androgens and glucocorticoids, are functionally important in regulating several processes underpinning offspring growth and physiology (e.g. Eising and Groothuis, 2003; Groothuis et al., 2005). The concentration of such substances can vary among mothers according to the environment experienced, their age and physiological condition (Bonier et al., 2009a,b), as well as paternal quality and attractiveness (e.g. Petrie et al., 2001; Müller et al., 2002; Gil et al., 2003). In addition, a mother can transfer different amounts of steroid hormones to different eggs within the same clutch according to, for example, embryo sex and egg position in the laying sequence (Schwabl, 1999; Royle et al., 2001; Groothuis et al., 2006; Rubolini et al., 2011).

Glucocorticoids in particular are known to play a crucial role in mediating the physiological and behavioural responses of organisms to changes in their environment (Scheuerlein et al., 2001; Schoech et al., 2009; Bonier et al., 2009a,b; Boonstra, 2013; Paitz et al., 2014). In birds, the main glucocorticoid hormone is corticosterone (CORT), which is secreted by the adrenal glands under stimulation of the hypothalamo-pituitary–adrenocortical axis (Romero and Wingfield, 2001; Henriksen et al., 2011; Costantini et al., 2014). CORT levels increase during metabolically demanding activities, such as reproduction and migration, but also as a consequence of experiencing stressful environmental conditions, such as decreases in food abundance or increases in parasite load and predation risk (Wingfield et al., 1994; Scheuerlein et al., 2001; Pereyra and Wingfield, 2003; Schoech et al., 2011). Indeed, females exposed to stressful conditions during egg formation increase circulating CORT levels, which may result in higher amounts of this hormone in both the yolk and albumen of their eggs (Hayward and Wingfield, 2004; Rubolini et al., 2005, 2011; Saino et al., 2005; Love et al., 2008; Navara and Pinson, 2010; Almasi et al., 2012).

Development of avian embryos in eggs with elevated levels of this stress hormone is known to shape post-natal phenotype, whose effects may potentially last until adult stages (e.g. Bonier et al., 2009a,b; Schoech et al., 2011; Bowers et al., 2016). Prenatal exposure to high levels of CORT has been shown to affect growth

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trajectories and physiological traits of offspring during the early phases of life (Rubolini et al., 2005, 2011; Saino et al., 2005; Bowers et al., 2016), as well as several behavioural traits (Rubolini et al., 2005; Davis et al., 2008; Love and Williams, 2008; Possenti et al., 2018a,b). Although the literature on prenatal CORT effects on the growth and behaviour of young individuals has flourished in recent decades, the results are often conflicting (see Henriksen et al., 2011; Groothuis et al., 2020). Indeed, some studies increasing egg CORT via *in ovo* inoculation or via a maternal increase through diet or using implanted capsules have documented impaired immune system functioning and somatic growth after hatching, with negative consequences for survival (Eriksen et al., 2003; Hayward and Wingfield, 2004; Rogers and Deng, 2005; Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006; Love et al., 2008; Henriksen et al., 2011). Other studies, however, have highlighted positive, rather than negative, effects of pre-natal CORT exposure on post-natal growth and physiology (Chin et al., 2009; Bowers et al., 2016; Weber et al., 2018; Noguera, 2021). Hence, prenatal CORT exposure may be adaptive under certain environmental circumstances. To further complicate this scenario, it is also known that the effects of pre-natal CORT exposure vary among different stages of development (Groothuis et al., 2020). For example, house wren (*Troglodytes aedon*) nestlings from CORT-injected eggs had a lighter hatching mass than control nestlings, but they become heavier near fledging (Strange et al., 2016). In addition, in the yellow-legged gull (*Larus michahellis*), a likely supra-physiological increase of CORT depressed embryo development (Parolini et al., 2019), but not chick growth after hatching (Possenti et al., 2018a,b).

Considering its role in affecting growth rate and/or influencing other physiological pathways, egg CORT has been suggested to influence offspring oxidative status (Costantini and Møller, 2008; Costantini et al., 2014; Stier et al., 2009; Costantini et al., 2011; Haussmann et al., 2012; Monaghan and Spencer, 2014). In particular, similar to other steroid hormones, such as androgens, CORT has pro-oxidant effects, thus increasing oxidative stress and damage (Costantini and Møller, 2008; Costantini et al., 2011; Lin et al., 2009; Stier et al., 2009; Haussmann et al., 2012; Costantini et al., 2014; Monaghan and Spencer, 2014; Monaghan and Haussmann, 2015). The effects of administration of exogenous CORT into eggs on the post-hatching phenotype have been examined only in very few studies, showing a general increase in oxidative stress or a reduced resistance to oxidative stress in the offspring (Stier et al., 2009; Haussmann et al., 2012; but see Possenti et al., 2018a,b).

Compared with the increasing knowledge of the effects of maternal egg CORT on offspring growth, physiology and oxidative status after hatching, there is still a dearth of studies on its consequences in terms of the development of avian embryos before hatching. This is unfortunate because embryonic developmental stages preceding the moment when the hatchlings start to exchange substances with the external environment are the only ones to be affected uniquely by the substances contained in the eggs. The knowledge of these early developmental stages is therefore fundamental to complement the increasing information about the effects of CORT when nestlings start to interact with the external environment. To the best of our knowledge, only two studies of birds have focused on the effects of egg CORT increase on embryo development. The first, performed on the yellow-legged gull, showed that a relatively large, probably supra-physiological, increase of CORT depressed embryo growth but did not affect oxidative status (Parolini et al., 2019). The second, conducted on the swan goose (*Anser cygnoides*), showed that *in ovo* injection of CORT resulted in an increase of growth hormone in embryonal somatotropic cells (Yu et al., 2018).

In order to contribute to filling this gap in the knowledge, in this study, we investigated the effects of physiological elevated CORT (ca. 1 s.d. of the mean concentration of the study population) in the egg yolk on morphology (e.g. skeletal and organ size) and oxidative stress-related endpoints in the brain and liver isolated from embryos just before hatching. In particular, we focused on the analysis of oxidative status, in terms of the amount of reactive oxygen species (ROS) and the activity of two antioxidant enzymes involved in the antioxidant machinery counteracting ROS toxicity: superoxide dismutase (SOD) and catalase (CAT). In addition, we also measured lipid peroxidation, which reflects oxidative damage to lipid membranes and is considered an indicator of oxidative stress.

MATERIALS AND METHODS

Field and experimental procedures

The yellow-legged gull (*Larus michahellis* Naumann 1840) is a monogamous colonial seabird (Cramp, 1998). Clutch size ranges between 1 and 3 eggs (modal size 3), which are laid at 1–4 day (most frequently 2 days) intervals and hatch 27–31 days after laying. Hatching is asynchronous and spans 1–4 days. The chicks are semi-altricial: they can move outside the nest just a few hours after hatching but they are fed by both parents until fledging, occurring at 35–40 days of age (Cramp, 1998). We studied a large colony (>400 pairs) breeding on an island in the Comacchio lagoon (NE Italy, 44°20′N, 12°11′E) during March–May 2014. We visited the colony every second day starting from 23 March 2014. When a new nest was found, it was marked and the newly laid egg was temporarily removed, and replaced with a 'dummy' egg to avoid interference with parental incubation behaviour. The removed egg was marked and taken to a nearby tent for experimental manipulation.

We used a well-established protocol of *in ovo* injection of yellow-legged gull eggs (e.g. Rubolini et al., 2005; Parolini et al., 2015, 2017a,b; Possenti et al., 2018a,b) to increase the yolk concentration of CORT by 1 s.d. compared with that measured in the egg yolk of individuals from the same colony (Rubolini et al., 2011). The injection was done directly into the yolk and this procedure returned a post-manipulation yolk concentration within the natural range of variation for the species. Although the concentration of CORT in the yolk of yellow-legged gull eggs did not vary according to egg size and the position in the laying sequence (Rubolini et al., 2011), we tuned the dose due to be injected according to these factors. Using information from Rubolini et al. (2011), we estimated yolk mass based on total egg mass for each class of position in laying sequence as follows: yolk mass=0.227±0.039 egg mass+1.815±3.46 (means±s.e.m.; $F_{1,88}$=34.38, P<0.001; see Parolini et al., 2015). Then, we grouped first (a), second (b) or third (c) laid eggs into three classes (tertiles) of size according to egg mass and calculated the standard deviation of CORT concentration in the yolk for each tertile within each class of position in the laying sequence (Rubolini et al., 2011). The amount of CORT due to be injected was computed as the product of the s.d. (in ng g^{-1}) of CORT concentration for each tertile and position in the laying sequence, and the estimated yolk mass. Corn oil was used as the carrier solvent for CORT, and it was used as a control treatment in the control group of eggs. We adopted a within-clutch design, whereby both sham- (control) and CORT-injected groups were established within each clutch to minimize the confounding effects of environmental and parental effects. The following treatment schemes (nest, a-egg, b-egg, c-egg) were assigned sequentially to the clutches according to the order in which the first egg was found

(nest, a-egg, b-egg, c-egg): nest 1, corticosterone injection (CORT)–control injection (oil)–CORT; nest 2, oil–CORT–oil; nest 3, CORT–oil–oil; nest 4, oil–CORT–CORT and so forth with the subsequent nests.

After the *in ovo* CORT injection, all the nests were visited every day starting 5 days before the earliest expected hatching date to check for any sign of imminent hatching such as eggshell fractures (i.e. 'cracking stage'). When the eggshell was fractured, eggs were weighed (to the nearest g), collected and frozen at −20°C within 3 h of sampling. At the end of the field procedure, the eggs were transferred to the lab where they were dissected. We first removed and weighted the residual yolk sac from each egg, then the embryos were weighted (to the nearest g), and tarsus and occiput–beak (i.e. a proxy of head size) length were measured by callipers prior to the dissection of liver and brain, which were immediately weighed (to the nearest mg) and frozen at −80°C until biochemical analyses. All the measurements were taken by the same person to ensure consistency. Molecular sexing of embryos and chicks was performed according to Rubolini et al. (2015).

Eggs from 86 nests were manipulated. Overall, 65 manipulated eggs failed to hatch. The proportion of failed eggs did not significantly differ between experimental groups (proportion of failed eggs for control group was 31/132=0.23 and for the CORT-injected group was 34/126=0.27; χ^2_1<0.130, P>0.717). The analyses on biometric traits and oxidative stress-related endpoints were performed on embryos from 33 nests having 3 eggs that reached the so-called 'cracking stage', for a total of 99 embryos, in order to obtain measures for each embryo per position in the laying sequence (except for a few outliers). Overall, the sex ratio (proportion of males) of embryos did not significantly differ either between the two experimental groups (control group: 20/40=0.50; CORT-treated group: 30/59=0.51; χ^2_1=0.01, P=0.90) or according to the position in the laying sequence (a-egg 14/33=0.42; b-egg 14/33=0.42; c-egg 15/33=0.45; χ^2_1<0.06, P>0.81 for all pairwise comparisons).

The study was carried out under permission of the Parco Regionale del Delta del Po (#252015, 20 February 2015), which allowed both the manipulation and the collection of eggs when the eggshell showed signs of imminent hatch (eggshell fractures). According to the Guidelines for the Euthanasia of Animals by the American Veterinary Medical Association, physical methods of euthanasia may be necessary in some field situations if other methods are impractical or impossible to implement. In this case, embryos were euthanized by placing eggs into a −20°C freezer within 2 h of collection.

Assessing oxidative status

Oxidative stress assays were performed on homogenates of embryo liver and brain. About 0.1 g tissue was homogenized in 100 mmol l^{-1} phosphate buffer pH 7.4, containing 1 mmol l^{-1} EDTA and 100 mmol l^{-1} KCl, with an automatic homogenizer. An aliquot of raw homogenate was used for determination of the total amount of ROS and lipid peroxidation. The amount of ROS in brain samples was measured through the dichlorofluorescein-diacetate (DCFH-DA) method developed by Deng and co-authors (2009). Change in fluorescence of DCFH-DA due to the presence of pro-oxidant molecules was measured at λ=485 nm (excitation) and λ=536 nm (emission) and the amount of ROS was expressed as arbitrary units (a.u.) DCF-H mg^{-1} protein. The levels of lipid peroxidation were measured according to the thiobarbituric acid reactive substances (TBARS) method developed by Ohkawa et al. (1979) and expressed as nmol TBARS g^{-1} wet mass.

A second aliquot of homogenate was used for measuring the activity of the antioxidant enzymes SOD and CAT, according to the method reported by Possenti et al. (2019). CAT activity was measured only in the liver because during the prenatal period it is expressed at low levels in birds (see Surai, 2002). The homogenates were centrifuged at 15,000 ***g*** for 1 h at 4°C. The obtained supernatant was processed to measure the total protein content according to the Bradford (1976) method and enzymatic activity. CAT activity was assessed by monitoring the consumption of 50 mmol l^{-1} H_2O_2 at λ=240 nm. SOD activity was measured by the inhibition of cytochrome *c* reduction (10 mmol l^{-1}) at λ=550 nm that was induced by the superoxide anion generated by the reaction between xanthine oxidase (1.87 mU ml^{-1}) and hypoxanthine (50 mmol l^{-1}). All the investigated endpoints were measured in duplicate in both brain and liver homogenates.

Statistical analysis

The effect of CORT injection on embryo time to reach the cracking stage (days elapsed between laying and egg cracking), morphological traits and oxidative stress-related endpoints was analysed through linear mixed models (LMM), including clutch identity as a random intercept effect. Preliminary checks of data normality and homogeneity of variances were performed through Shapiro–Wilk and Bartlett tests, respectively. Egg mass, embryo and residual yolk mass, and mass of the liver and the brain were $\log_{10}$-transformed to account for allometry in models controlling for size. Similarly, data of the amount of ROS, lipid peroxidation and SOD activity were $\log_{10}$-transformed to reduce skewness. Egg treatment (CORT versus sham injected), embryo sex and egg laying order were included as fixed-effect factors, as well as their two-way interactions. Egg mass at the time of laying was included as a covariate in models of body mass and skeletal growth only, while embryo body mass was included as a covariate in models of brain and liver mass. Non-significant (P>0.05) interaction terms were removed from the models in a single step. Final models are reported. After running the Tietjen–Moore test to detect multiple outliers in a univariate dataset (Tietjen and Moore, 1972), we excluded some outliers (n=1–3 depending on the considered endpoint) from each single analyses. In detail, we excluded one outlier for ROS levels measured in the liver and the brain, as well as in hepatic CAT and SOD activity and lipid peroxidation levels in the brain. Three outliers emerged and were excluded for lipid peroxidation levels in the liver only. However, qualitatively similar results were found after running the models including the outlier values (details not shown for brevity). Models were fitted using the 'lme4' package for R 3.6.1 (http://www.R-project.org/).

RESULTS

Developmental timing and embryo morphology

Time to reaching the cracking stage was not affected by CORT treatment ($F_{1.66}$=0.32, P=0.57) or embryo sex ($F_{1.78}$=3.23, P=0.08). As expected, a significant effect of laying order was found ($F_{1.77}$=56.36, P<0.001): embryos from a-eggs took longer to reach the cracking stage than their siblings from b- and c-eggs. The absorption of yolk during development did not significantly differ between control and CORT-injected groups, although a lower amount of residual yolk was measured in CORT-treated embryos compared with controls, after controlling for sex and laying order (Table 1). As expected, a significant laying order effect was noted for all morphometric traits, whereby embryos from c-eggs were smaller than those from a- and b-eggs (Table 1). Whilst no significant effects on body mass were noted, embryos from CORT-injected eggs showed longer tarsi and a marginally significant larger

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Table 1. Linear mixed models of morphological embryo traits in relation to the main and two-way interaction effects of egg corticosterone treatment, sex, laying order and egg/embryo mass in the yellow-legged gull

	F	d.f.	*P*	Parameter estimate/estimated marginal means (s.e.)		
Chick body mass						
Laying order	4.899	2,75.6	**0.009**	a-egg: 1.65 (0.007)	b-egg: 1.66 (0.007)	c-egg: 1.63 (0.008)
Treatment	1.544	1,68.6	0.218	Control: 1.64 (0.006)	CORT: 1.65 (0.006)	
Sex	1.582	1,91.1	0.211	Males: 1.64 (0.007)	Females: 1.65 (0.006)	
Egg mass	10.160	1,58.3	**0.002**	1.64 (0.005)		
Tarsus length						
Laying order	4.667	2,75.1	**0.014**	a-egg: 2.305 (0.005)	b-egg: 2.304 (0.005)	c-egg: 2.285 (0.006)
Treatment	6.222	1,66.5	**0.015**	Control: 2.292 (0.004)	CORT: 2.304 (0.004)	
Sex	0.064	1,89.1	0.800	Males: 2.297 (0.005)	Females: 2.297 (0.004)	
Egg mass	0.001	1,61.7	0.976	2.298 (0.004)		
Head size						
Laying order	4.361	2,74.8	**0.016**	a-egg: 2.636 (0.003)	b-egg: 2.634 (0.003)	c-egg: 2.622 (0.004)
Treatment	3.839	1,71.8	0.053	Control: 2.627 (0.003)	CORT: 2.634 (0.003)	
Sex	0.607	1,92.9	0.437	Males: 2.632 (0.003)	Females: 2.629 (0.003)	
Egg mass	0.082	1,51.9	0.928	2.632 (0.002)		
Residual yolk						
Laying order	4.040	2,75.1	**0.021**	a-egg: 1.16 (0.015)	b-egg: 1.15 (0.014)	c-egg: 1.21 (0.016)
Treatment	3.493	1,71.0	0.065	Control: 1.19 (0.012)	CORT: 1.16 (0.012)	
Sex	1.727	1,92.9	0.191	Males: 1.18 (0.013)	Females: 1.16 (0.011)	
Egg mass	57.460	1,52.7	**<0.001**	1.17 (0.009)		
Liver mass						
Laying order	1.968	2,67.0	0.147	a-egg: 0.020 (0.011)	b-egg: 0.038 (0.011)	c-egg: 0.050 (0.012)
Treatment	5.536	1,71.1	**0.021**	Control: 0.022 (0.009)	CORT: 0.050 (0.009)	
Sex	1.250	1,91.8	0.266	Males: 0.043 (0.010)	Females: 0.029 (0.009)	
Embryo mass	76.346	1,90	**<0.001**	0.039 (0.010)		
Brain mass						
Laying order	2.269	2,64.6	0.111	a-egg: 0.227 (0.004)	b-egg: 0.227 (0.004)	c-egg: 0.217 (0.005)
Treatment	1.313	1,64.4	0.256	Control: 0.222 (0.002)	CORT: 0.226 (0.004)	
Sex	12.799	1,77.4	**<0.001**	Males: 0.232 (0.004)	Females: 0.216 (0.004)	
Embryo mass	73.029	1,88.5	**<0.001**	0.224 (0.004)		

Clutch identity was included in the model as a random intercept effect. Significant effects are reported in bold. CORT, corticosterone.

head size compared with the control group. Elevated CORT affected the mass of the liver, but not of the brain (Table 1). As shown in our previous study (Parolini et al., 2017a,b), males had a larger brain than females (Table 1).

Oxidative status

Hepatic ROS levels were unaffected by elevated CORT (Table 2). Hepatic SOD and CAT activity were significantly affected by elevated CORT but the effect differed according to laying order (Table 2, Fig. 1B,C). *Post hoc* tests showed that the activity of both enzymes measured in the liver of embryos developing in the CORT-injected third-laid eggs was significantly lower than that of control embryos (P=0.016 for SOD and P=0.002 for CAT). Moreover, hepatic SOD activity of embryos developing in a-eggs was significantly higher compared with that in embryos from the b- (P=0.003) and c-eggs (P=0.027). Furthermore, females showed higher CAT activity than males (P<0.001).

Hepatic lipid peroxidation levels were significantly higher in the liver of embryos developing in CORT-treated eggs compared with controls, and the treatment by laying order interaction was significant (Table 2). *Post hoc* tests showed that lipid peroxidation of embryos developing in the CORT-injected second-laid eggs was significantly higher than that of corresponding control embryos (P≤0.001; Fig. 1A), while this was not the case for embryos developing in a- or c-eggs. Brain oxidative stress-related biomarkers were not significantly affected by elevated CORT (Table 2).

DISCUSSION

We investigated whether a physiological increase in yolk CORT affected prenatal development and oxidative stress-related endpoints in yellow-legged gull embryos. Our manipulation resulted in significant changes in phenotypic traits and oxidative status of CORT-treated embryos compared with controls, with some patterns depending on the laying sequence.

Effects on morphology and development

The first main result is that eggs supplemented with a physiological dose of CORT produced embryos with greater skeletal growth and a heavier liver. These results were not expected because high CORT levels are generally associated with a decrease in growth and condition in hatchlings of several bird species, but most of these experimental studies increased the level of this hormone above the physiological range, thus making their results somewhat questionable (see Henriksen et al., 2011). However, a number of studies showed no measurable negative effects on body growth, suggesting that prenatal exposure to high CORT levels is not necessarily detrimental (e.g. Love and Williams, 2008; Chin et al., 2009; Carter et al., 2016) – it can also be positive (Chin et al., 2009; Bowers et al., 2016; Weber et al., 2018; Noguera, 2021; for positive effects of cortisol in other taxa see also Meylan and Clobert, 2005; Dantzer et al., 2013; Capelle et al., 2016). Under the hypothesis of an adaptive transfer of maternal CORT to the eggs, the level of CORT experienced by the embryos during development would prepare offspring for the stressful environment that they will encounter, and a positive relationship between increased maternal egg CORT and progeny fitness is therefore expected (Bonier et al., 2009a,b; Sherif et al., 2017). A larger embryo skeletal growth might be helpful for the chicks at hatching because it can favour a more efficient escape from predators (Saino et al., 2005; Pitk et al., 2012). In addition, larger individuals can prevail over siblings in

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Table 2. Linear mixed models of the effects of treatment, sex and laying order, and their two-way interaction, on oxidative stress-related biomarkers in the liver and brain isolated from yellow-legged gull embryos

	F	d.f.	*P*	Estimated marginal means (s.e.)		
LPO – liver						
Laying order	2.725	2,56.5	0.074	a-egg: 1.09 (0.03)	b-egg: 1.15 (0.03)	c-egg: 1.19 (0.03)
Treatment	5.578	1,68.4	**0.021**	Control: 1.10 (0.03)	CORT treated: 1.18 (0.03)	
Sex	0.804	1,83.4	0.372	Males: 1.12 (0.03)	Females: 1.16 (0.03)	
Treatment×laying order	4.476	2,75.1	**0.014**	Fig. 1C		
LPO – brain						
Laying order	0.229	2,62.9	0.796	a-egg: 0.943 (0.039)	b-egg: 0.924 (0.039)	c-egg: 0.922 (0.039)
Treatment	1.052	1,62.9	0.309	Control: 0.914 (0.037)	CORT treated: 0.945 (0.037)	
Sex	1.188	1,74.1	0.279	Males: 0.949 (0.040)	Females: 0.910 (0.037)	
ROS – liver						
Laying order	2.087	2,60.6	0.133	a-egg: 5.10 (0.03)	b-egg: 5.04 (0.03)	c-egg: 5.02 (0.03)
Treatment	1.747	1,66.7	0.191	Control: 5.07 (0.03)	CORT treated: 5.03 (0.03)	
Sex	0.457	1,85.1	0.500	Males: 5.06 (0.03)	Females: 5.04 (0.03)	
ROS – brain						
Laying order	0.510	2,60.4	0.603	a-egg: 4.65 (0.04)	b-egg: 4.63 (0.04)	c-egg: 4.67 (0.04)
Treatment	2.399	1,65.8	0.126	Control: 4.68 (0.03)	CORT treated: 4.62 (0.03)	
Sex	0.017	1,80.3	0.896	Males: 4.65 (0.04)	Females: 4.65 (0.03)	
SOD activity – liver						
Laying order	5.148	2,57.8	**0.009**	a-egg: 1.25 (0.02)	b-egg: 1.19 (0.02)	c-egg: 1.21 (0.02)
Treatment	0.329	1,62.5	0.568	Control: 1.22 (0.02)	CORT treated: 1.21 (0.02)	
Sex	0.409	1,74.4	0.524	Males: 1.21 (0.02)	Females: 1.22 (0.02)	
Treatment×laying order	4.174	2,79.3	**0.019**	Fig. 1A		
SOD activity – brain						
Laying order	0.457	2,58.0	0.636	a-egg: 1.36 (0.05)	b-egg: 1.38 (0.05)	c-egg: 1.33 (0.05)
Treatment	0.001	1,62.7	0.981	Control: 1.35 (0.04)	CORT treated: 1.35 (0.04)	
Sex	1.122	1,82.3	0.293	Males: 1.33 (0.05)	Females: 1.38 (0.04)	
CAT activity – liver						
Laying order	0.787	2,57.8	0.460	a-egg: 7.99 (0.47)	b-egg: 7.45 (0.48)	c-egg: 8.01 (0.47)
Treatment	2.172	1,63.3	0.145	Control: 8.13 (0.43)	CORT treated: 7.50 (0.43)	
Sex	11.866	1,77.9	**<0.001**	Males: 6.96 (0.48)	Females: 8.68 (0.43)	
Treatment×laying order	4.603	2,81.3	**0.013**	Fig. 1B		

Clutch identity was included in the models as a random intercept effect. Only significant interaction terms were included in the final model and reported in the table. Significant effects are reported in bold.

CORT, corticosterone; LPO, lipid peroxidation; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase.

competition for parental food and/or because it allows them to resist starvation for longer. However, considering that CORT can have opposite effects on different life stages (see Strange et al., 2016; Possenti et al., 2018a,b; Parolini et al., 2019), and therefore such putative positive effects might disappear after hatching, we refrain from interpreting our results under an adaptive scenario, as we do not have information on the consequences of our experimental manipulation for chick phenotype at later life stages.

Such an increase in skeletal growth might be partially due to an more rapid yolk consumption in CORT-supplemented embryos relative to controls. However, more rapid yolk consumption was not a proxy for advanced development, as indicated by the lack of an effect of treatment on the time to reaching the cracking stage, a result which contrasts with previous studies administering exogenous corticosteroids in eggs (Mashaly, 1991; Kaltner et al., 1993; Heiblum et al., 2001; Eriksen et al., 2003; Rubolini et al., 2005). A possible physiological mechanism explaining this enhanced skeletal growth (and liver mass) of embryos from CORT-supplemented eggs involves the activity of the growth hormone during embryogenesis, which was recently shown to be elicited by *in ovo* physiological injection of CORT (Yu et al., 2018). The same study also showed that a larger dose of CORT resulted in a smaller concentration of plasma CORT. Such a dose-dependent effect can also explain the difference in the effect of CORT on embryo growth between the present study and a previous one on the same population in which a supra-physiological egg supplementation of CORT (ca. 2 s.d.) negatively affected embryo growth, and decreased liver size (Parolini et al., 2019). Such a potential beneficial effect of prenatal CORT on growth may therefore become apparent only under physiological conditions, while higher doses may have negative effects, reflecting a potential hormetic effect (Calabrese and Baldwin, 1998; Mattson, 2008). We can indeed envisage that, if high CORT levels have strong negative effects on offspring development, selection should act against the transfer of large amounts of this hormone to the eggs and/or favour the embryo's capacity to limit its detrimental consequences (e.g. through metabolism; Vassallo, et al., 2014, 2019).

In addition, our finding of a larger liver mass in CORT-treated embryos is in line with previous experimental studies performed in captivity where dietary exposure to different CORT concentrations resulted in hepatomegaly in chickens (*Gallus gallus domesticus*; Davison et al., 1983; Maurice et al., 2007). Hepatomegaly could be due to an increase in protein and energy intake, leading to a decrease in protein accretion and total lipid content in organs, as suggested by a study of dietary CORT administration to broiler chickens (Covasa and Forbes, 1995). This hypothesis is again partially supported by the more rapid, marginally significant, yolk adsorption experienced by embryos from CORT-supplemented eggs relative to controls.

Effects on oxidative status

A second main finding of the present study concerns that embryos developed in CORT-supplemented eggs showed higher levels of hepatic lipid peroxidation compared to control group, with a

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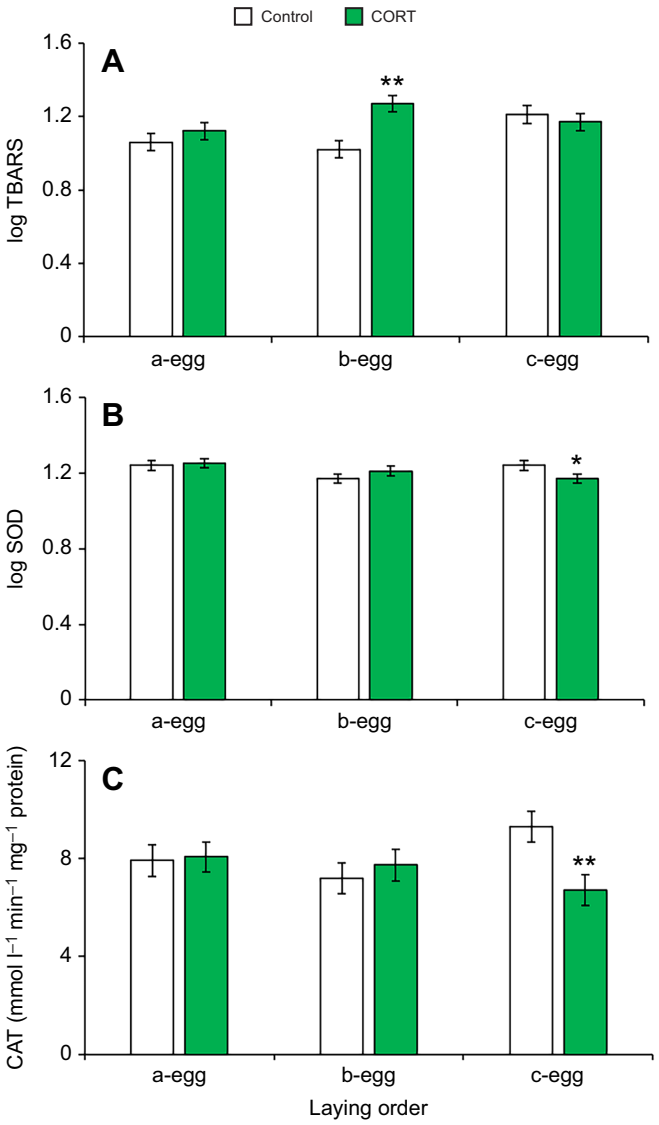

Fig. 1. Effect of corticosterone and laying order on lipid peroxidation and activity of superoxide dismutase and catalase in the yellow-legged gull. Levels of lipid peroxidation (log LPO; A) and activity of superoxide dismutase (log SOD; B) and catalase (CAT; C) measured in the liver from the yellow-legged gull in relation to treatment [control and corticosterone (CORT) injected] and laying order (a-, b- and c-eggs). Significant pairwise differences (LSD test) between embryos of the same laying order are indicated by asterisks (*P<0.05, **P<0.01).

particularly strong effect in b-eggs. The onset of oxidative damage might be due to an imbalance of the oxidative status, but our data only partly supported this hypothesis. In fact, whilst CORT supplementation did not induce ROS overproduction, a significant modulation of the hepatic activity of the antioxidant enzymes SOD and CAT occurred only in embryos from CORT-supplemented c-, but not b-eggs. However, the increase in hepatic lipid peroxidation found in embryos from the CORT b-eggs was not unexpected considering that antioxidant defences, both enzymatic and non-enzymatic ones, decrease according to the position in the laying sequence (Rubolini et al., 2011; Parolini et al., 2017a,b; the present study). Indeed, the activity of both SOD and CAT is smaller in b- than in a-eggs. A previous study showed that the total, non-enzymatic, antioxidant capacity of gull embryos developed in b-eggs is smaller compared to that recorded in a-eggs, but also in c-eggs (Parolini et al., 2017a,b), suggesting that these embryos may be highly susceptible to oxidative damage. This latter observation might help to explain the result that an increase of lipid peroxidation was not observed in CORT-supplemented c-eggs relative to controls, although they showed a smaller activity of both SOD and CAT. In addition, control c-eggs also paid a larger cost in term of lipid peroxidation in hepatic cells than other eggs, thus indicating that last-laid eggs are naturally under a condition of oxidative damage, irrespectively of the level of CORT. Therefore, mothers seem to deposit suboptimal amounts of antioxidant substances (or a relative composition of different interacting compounds; Possenti et al., 2018a,b; Parolini et al., 2019) in c-eggs. Indeed, the concentration of non-enzymatic antioxidants not measured here, such as lutein and vitamin E, decreases with laying order in yellow-legged gull eggs (Rubolini et al., 2011), and embryos developing in c-eggs, irrespectively of their level of CORT, may not efficiently balance their oxidative status, leading to oxidative damage to their hepatic cells. This is not the case, however, for embryos of a-eggs which seem well-equipped to sustain the putative larger costs of developing in a CORT-enriched environment.

In contrast to results from the liver, neither oxidative status modulation nor oxidative damage was induced by CORT supplementation to embryo brain, confirming that organs differ in their sensitivity to CORT (Hull et al., 2007). These results were somewhat unexpected as the brain is considered to be the most susceptible tissue to glucocorticoid-induced oxidative stress because it is the main target of glucocorticoids, and has low antioxidant capacity, high metabolic activity and cell membranes susceptible to peroxidation (e.g. Surai, 2002; Pamplona et al., 2004). Despite this susceptibility, the increase of CORT levels in egg yolk was probably not sufficiently high to induce oxidative stress in embryo brain during prenatal development.

An alternative explanation relies on the observation that embryos possess substantial metabolic capacities, which may allow them to modulate their exposure to maternal steroid hormones (Paitz et al., 2011), including CORT (Vassallo, et al., 2014, 2019). Indeed, *in ovo* metabolism of maternal CORT can considerably reduce its concentrations in developing embryos, such that they are exposed to high CORT levels only when elevated amounts of this hormone are transferred by the mother (Vassallo, et al., 2014, 2019). It is therefore possible that the combination of our manipulation within the physiological range and the fact that the liver is the primary site of metabolism resulted in some effects on this organ but not on the brain.

Finally, we cannot totally exclude that the lack of a marked effect of CORT supplementation on the oxidative status in either the liver and the brain might at least partly be the result of an unintended effect of the use corn oil as a vehicle for injecting CORT into the eggs. In fact, corn oil represents an important source of minor bioactive lipids, such as phytosterols, tocopherols, tocotrienols and carotenoids (Barrera-Arellano et al., 2019), which are all antioxidant molecules involved in contrasting ROS toxicity, and which could have therefore mitigated any effect of CORT on ROS levels.

Comparison with previous studies

The present findings contrast somewhat with those of our previous investigations in which levels of CORT in yolk of yellow-legged gull eggs were supra-physiologically increased (ca. 2 s.d.; Possenti et al., 2018a,b; Parolini et al., 2019). Indeed, after a supra-physiological CORT supplementation, gull embryos benefited from a smaller lipid peroxidation in the brain, but not the liver (Parolini et al., 2019), suggesting that different doses can impact on different target organs during the same life stage. In addition, although ROS

concentration did not differ between treated and control embryos, the total antioxidant capacity, which does not result from the activity of antioxidant enzymes, was unaltered by the experimental manipulation (Parolini et al., 2019).

It is worth mentioning that our results are also in contrast with two previous studies in the same population examining the consequences of elevated egg CORT on chicks, rather than embryos, which respectively reported negative (Rubolini et al., 2005) and no effects (Possenti et al., 2018a,b) on body growth, as well as no significant effects on oxidative status (Possenti et al., 2018a,b). However, these studies cannot be properly compared with the present one because of discrepancies in the experimental approach. In fact, Rubolini et al. (2005) manipulated albumen (rather than yolk) CORT levels, which could exert different effects during the prenatal stage. In Possenti et al. (2018a,b), the injected dose of CORT was much larger (ca. 2 s.d.) than that used in the present study. A similar inconsistency across studies has previously been observed on the behavioural consequences of exposure to variable amounts of pre-natal CORT either in the yolk or in the albumen (e.g. Rubolini et al., 2005; Janczak et al., 2006; Davis et al., 2008; Henriksen et al., 2011; Possenti et al., 2018a,b).

Concluding remarks

Our findings, together with previous work, indicate that the effects of CORT are highly dependent not only on its concentration and on the moment when the effects are recorded but also on the egg component where it is administered and on the endpoint considered (see Henriksen et al., 2011; Groothuis et al., 2020). A major role in such a complex scenario might be played by oxidative status, as some effects emerge at and/or carry-over into different life stages. For example, a 'positive' effect on development during the embryonic stage might translate into a 'negative' effect on post-hatching growth, as a result of a latency of the consequences of accumulation of irreversible oxidative damage. Future studies on the same sample of individuals, using the same procedures and testing the same endpoints, but carefully varying the above variables, could return a comprehensive picture of how this hormone acts during embryogenesis and how its consequences may carry over across later life stages.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.R., D.R., M.P.; Methodology: M.P.; Formal analysis: M.P., A.R.; Investigation: A.R., C.D.P., M.C., B.D.F., M.P.; Writing - original draft: A.R., M.P.; Writing - review & editing: D.R.

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