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RESEARCH ARTICLE

Contrasting effects of increased yolk testosterone content on development and oxidative status in gull embryos

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ABSTRACT

Hormone-mediated maternal effects generate variation in offspring phenotype. In birds, maternal egg testosterone (T) exerts differential effects on offspring traits after hatching, suggesting that mothers experience a trade-off between contrasting T effects. However, there is very little information on T pre-natal effects. In the yellow-legged gull (*Larus michahellis*), we increased yolk T concentration within physiological limits and measured the effects on development and oxidative status of late-stage embryos. T-treated embryos had a larger body size but a smaller brain than controls. Males had a larger brain than females, controlling for overall size. T treatment differentially affected brain mass and total amount of pro-oxidants in the brain depending on laying order. T-treatment effects were not sex dependent. For the first time in the wild, we show contrasting T pre-natal effects on body mass and brain size. Hence, T may enforce trade-offs between different embryonic traits, but also within the same trait during different developmental periods.

KEY WORDS: Brain mass, Embryo, Growth, Oxidative status, Sexual dimorphism, *Larus michahellis*, Testosterone

INTRODUCTION

Epigenetic maternal effects mediated by egg size and biochemical composition have consequences for the phenotypic composition of the next generations, and can therefore have complex effects on the evolutionary dynamics of populations (Mousseau and Fox, 1998; Muriel et al., 2015). Ecological and evolutionary studies have framed the interpretation of maternal effects via the egg mostly in terms of their functional value to maximize parental fitness via adaptive transgenerational phenotypic plasticity (Mousseau and Fox, 1998; Müller et al., 2007). However, maternal effects evolve and are expressed under a number of potentially constraining conditions, suggesting that egg quality may be sub-optimal to Darwinian fitness of parents and/or individual offspring. In fact, maternal allocation of substances to the eggs may entail costs to the mother, and thus enforce trade-offs between offspring quality and maternal condition (Mousseau and Fox, 1998). In addition, mediators of maternal effects may have 'pleiotropic' and potentially contrasting effects on offspring fitness traits, thereby imposing trade-offs on maternal allocation strategies (Navara and Mendonça, 2008).

Maternal effects can occur via variation in egg mass and macro-constituents (e.g. albumen content) that have major effects on post-natal performance, as shown by correlational evidence and also egg-manipulation experiments (Bonisoli-Alquiati et al., 2007, 2008; Christians, 2002). Studies of the consequences of variation in quantitatively minor egg constituents have focused on antioxidants and steroid hormones (Groothuis and Schwabl, 2008; Groothuis et al., 2005; Navara and Mendonça, 2008; Romano et al., 2008; Saino et al., 2003). Androgens, in particular, have a special appeal in the evolutionary ecological study of maternal effects for at least three reasons. First, they are transferred to the eggs in amounts that partly depend on extrinsic conditions such as predation risk (Coslovsky et al., 2012), population density (van Dijk et al., 2013) and mate sexual attractiveness (Krištofik et al., 2014; but see Saino et al., 2006). Therefore, they have the potential to mechanistically and functionally link maternal experience of environmental conditions to offspring phenotype (Marshall and Uller, 2007). The observation that androgen deposition in the eggs varies with position in the laying sequence, and that these patterns of variation change across species, corroborates the idea that egg androgen concentrations are strategically modulated by females (Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). Second, androgens are of pivotal importance to regulation of embryo differentiation and development of physiological and behavioural traits (Arnold, 2002; Groothuis and Schwabl, 2008; Pfannkuche et al., 2011). Third, androgens are drivers of sexual phenotypic differentiation, and can therefore participate in strategies of maternal sex allocation (e.g. Adkins-Regan et al., 2013; Navara and Mendonça, 2008; Riedstra et al., 2013; Ruuskanen and Laaksonen, 2010; Schweitzer et al., 2013).

The cleidoic egg of birds affords an ideal study system for maternal effects because it is isolated from the maternal physiological milieu. *In ovo* manipulation experiments on birds have provided extensive evidence for the pervasiveness and also for the differential and sex-dependent effects of androgens, and testosterone (T) in particular, on diverse offspring traits. Most studies have suggested that androgens are anabolic for muscle and skeletal growth (Eising et al., 2001; Lipar and Ketterson, 2000; Navara et al., 2006), and boost post-natal body mass gain (Navara et al., 2005; Pilz et al., 2004; Schwabl, 1996). However, other studies have failed to show such a positive effect or have even shown a negative effect on post-natal growth (Henry and Burke, 1999; Podlas et al., 2013; Possenti et al., 2016; Rubolini et al., 2006). In addition, *in ovo* T effects on growth have been shown to be sex dependent (Müller et al., 2009; Ruuskanen, 2015; Saino et al., 2006; Sockman et al., 2008). Elevated egg T levels are mostly suppressive to acquired immune processes (Groothuis et al., 2005; Navara and Mendonça, 2008; Navara et al., 2005; but see Rubolini et al., 2006; Tobler et al., 2010), with evidence for sex- and age-dependent effects (Tobler et al., 2010). Few studies have investigated the effect of T on oxidative status, with partly different outcomes. Some studies postulated that high amounts of maternally transferred androgens, including testosterone, may represent a cost for offspring in terms of increased susceptibility

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List of abbreviations

LMM	linear mixed model
ROS	reactive oxygen species
T	testosterone
TAC	total antioxidant capacity
TOS	total pro-oxidant molecules

to oxidative stress as a consequence of accelerated growth (e.g. Groothuis et al., 2006; Martin and Schwabl, 2008). Enhanced offspring development rate mediated by egg androgens is associated with increased cell metabolism and a concomitant overproduction of reactive oxygen species (ROS; see Martin and Schwabl, 2008), which are produced during normal metabolic processes by the mitochondria and can cause severe toxic effects because they can oxidize cellular macromolecules (e.g. Finkel and Holbrook, 2000). Additionally, testosterone may directly induce oxidative stress in some tissues (Alonso-Alvarez et al., 2007 and references therein). For instance, yolk T decreased post-natal lipid peroxidation and increase total plasma antioxidant capacity (TAC) in one study (Noguera et al., 2011) but had no or negative sex-specific effects on post-natal TAC in other studies (Tobler and Sandell, 2009; Tobler et al., 2013). In addition, high egg T levels during pre-natal stages depress DNA repair after damage from acute stress (Treidel et al., 2013). Egg T enhances pre-natal (Boncoraglio et al., 2006) and post-natal begging behaviour (Eising et al., 2001; Rice et al., 2013; but see Boncoraglio et al., 2006; Saino et al., 2006; Smiseth et al., 2011) also in a sex-dependent way (Ruuskanen and Laaksonen, 2013; Ruuskanen et al., 2009), and affects chick territorial, neophobic, dispersal and other behaviours (e.g. Bertin et al., 2015; Müller et al., 2009; Tobler and Sandell, 2007). Finally, studies of egg T effects in adulthood have also provided evidence of contrasting effects both within and among fitness traits, such as fecundity (Müller et al., 2009; Rubolini et al., 2007), expression of male secondary sexual traits (Bonisoli-Alquati et al., 2011; Rubolini et al., 2006) and survival (Hegyi et al., 2011; Ruuskanen et al., 2012).

Thus, prominent features of experiments manipulating egg T on different fitness traits are that the effects are multi-tiered, can vary in sign, as well as according to the ontogenetic stage when the effects are measured, and they can be sex specific. In addition, there is evidence for mothers not being able to tune egg androgen concentration according to embryo sex (Aslam et al., 2013; Rubolini et al., 2011; but see Badyaev et al., 2008). The fact that egg androgens can have differential, and even opposite, effects on fitness traits, and that these effects can be sex dependent, may therefore enforce trade-offs in the amount of androgens that mothers allocate to their eggs.

Studies on prenatal effects of T are very rare (Hegyi and Schwabl, 2010; Muriel et al., 2013). This is unfortunate for several reasons. First, embryonic development and growth is a dynamic process where developmental conditions can preemptively imprint development and growth trajectories. In addition, the peri-natal period is often critical to individual offspring performance and survival because social relationships with competing siblings are established after birth. Furthermore, the effect of androgens may change over time even on the same trait (e.g. Hegyi and Schwabl, 2010). 'Longitudinal' trade-offs at high androgen levels may therefore occur, and, in order to interpret maternal allocation in androgen transfer to the eggs, we need to expand our view of the effects of androgens not only across traits but also over the entire ontogenetic process, starting from pre-natal development.

In the present study of the yellow-legged gull (*Larus michahellis* Naumann 1840), we aimed at contributing to fill this gap of knowledge of the effect of T on pre-natal development and physiology. Using a within-clutch experimental design, we administered a physiological dose of T and recorded the effects on embryo size and oxidative status while establishing a group of sham-injected control eggs. Shortly before hatching, the eggs were dissected to measure total embryo size and mass, residual yolk mass as an inverse measure of yolk absorption, as well as size of the liver and the brain. In addition, we measured TAC and the concentration of pro-oxidant molecules in the brain and liver.

Brain size was measured because the brain is a major target organ for the organizing effects of pre-natal androgens (Gahr et al., 1996; Garamszegi et al., 2007; Godsave et al., 2002) and also because inter-specific comparative evidence exists for coevolution between maternal effects mediated by androgens and brain size (Garamszegi et al., 2007). Liver size was measured because it is the main repository of antioxidants (Surai, 2002), and evidence exists that egg T can affect oxidative status after hatching (see above). Imbalance between antioxidant defenses and oxidative challenge to biological molecules is a major factor affecting bodily performance and fitness (Costantini, 2014; Halliwell and Gutteridge, 1999; Surai, 2002). We focused on TAC and the amount of pro-oxidant molecules (i.e. TOS according to terminology in Erel, 2005) in the liver, because of its role in antioxidant defense, and in the brain, because it is believed to be particularly sensitive to peroxidation of phospholipids (Surai, 2002; Surai et al., 1999).

Because of the heterogeneity in the observed effects of T, of the scanty number of studies on pre-hatching stages, and the absence of a general theoretical framework of the effects of T on the specific endpoints that we focused on, we refrained from making explicit predictions on the outcome of T manipulation on pre-natal morphology and physiology, and on its sex-dependent variation.

MATERIALS AND METHODS

Study organism

The yellow-legged gull is a large, mainly colonial, monogamous gull with altricial offspring and biparental care of progeny. Females lay one to three eggs (modal clutch size=3) that hatch asynchronously (hatching span 1–4 days) after 27–32 days of incubation (Cramp, 1998). Previous studies that are relevant to the present experiment have shown that eggs are a source of important maternal effects mediated by carotenoids, vitamins, corticosterone as well as T (e.g. Bonisoli-Alquati et al., 2007; Parolini et al., 2015; Romano et al., 2008; Rubolini et al., 2011; Saino et al., 2010). Specifically, a previous experiment, in which we only measured offspring phenotype after hatching and no oxidative stress endpoints were analysed, showed that *in ovo* T depressed body mass 4 days post-hatching (Rubolini et al., 2006). A subsequent experiment showed that *in ovo* T manipulation affected behavioural lateralization but had only weak effects on body mass at hatching (Possenti et al., 2016). In addition, *in ovo* manipulation of T boosts peri-natal signals of solicitation of care that offspring address to their parents at pipping-egg stage, but not post-natal begging behaviour (Boncoraglio et al., 2006). T levels decline with laying order. However, steroid hormone concentrations do not differ between eggs carrying a male or a female (Rubolini et al., 2011).

Field procedures

The present study was performed on a colony (>400 breeding pairs) in the Comacchio lagoon (NE Italy) during March–May 2015. The colony was visited every second day to monitor the progress of

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laying and mark the newly laid eggs. When a new egg was found, it was temporarily removed from the nest for experimental manipulation while temporarily replacing it with a 'dummy' egg.

The experimental *in ovo* T manipulation was performed as described in Possenti et al. (2016).

We aimed at increasing the concentration of T by 1 standard deviation (s.d.) of the concentration recorded in the yolk of yellow-legged gull eggs from the same colony (Rubolini et al., 2011), by injecting an appropriate volume of a T solution directly into the yolk. Because the concentration of T in the yolk varies according to egg size and position in the laying sequence, we scaled the dose to be injected accordingly. Thus, we grouped first (a-), second (b-) or third (c-) laid eggs into three classes (tertiles) of size according to egg mass and calculated the s.d. of T concentration in the yolk for each tertile within each position in the laying sequence. We estimated the yolk mass for each class size and position in laying sequence according to the following equation: yolk mass=0.227 (0.039 s.e.) egg mass+ 1.815 (3.461 s.e.) ($F_{1,88}$=34.38, P<0.001). The amount of T due to be injected was computed as the product of the s.d. (in ng g^{-1}) of T concentration for each tertile and position in the laying sequence and the estimated yolk mass. The doses injected were as follows [laying order: class of size according to egg mass (g): amount of T injected (ng per egg)]: a-eggs: 84–91 g: 57 ng, 92–95 g: 59 ng, 96–108 g: 42 ng; b-eggs: 80–88 g: 74 ng, 89–92 g: 73 ng, 93–99 g: 81 ng; and c-eggs: 75–82 g: 95 ng, 82–87 g: 84 ng, 88–98 g: 76 ng.

T was injected in the yolk according to the validated procedure reported by Romano et al. (2008). Before being injected, the egg was weighed (to the nearest g) and placed with the longitudinal axis vertical. After disinfecting the eggshell, a hole was drilled using a sterile pin close to the acute pole. *In ovo* injection was performed by means of a 1-ml sterile syringe mounting a 0.6×30 mm needle while the egg was held firmly with its longitudinal axis vertical. Immediately after extracting the needle from the egg, the hole was sealed with a drop of epoxidic glue and a small piece of eggshell was superimposed on the hole. T solutions were prepared in sterile vials dissolving the hormone in corn oil to the final dilution required. Each vial contained the concentration of T to be injected in egg yolk depending on egg mass and position in the laying sequence. We adopted a within-clutch design, whereby both control and T-treated eggs were established within each clutch. We sequentially assigned the following treatment schemes to the clutches, according to the order in which the first egg was found (nest, a-, b-, c-egg): nest 1, T injection (T), control injection (C), T; nest 2, C-T-C; nest 3, T-C-C; nest 4, C-T-T and so forth with the following nests. T-treated eggs were injected with 30 µl of the appropriate T solution, while control eggs were injected with the same volume of corn oil only.

After T level manipulation, each egg was brought back to its nest of origin and regularly monitored until any sign of imminent hatching appeared. When eggshell fractures were observed, eggs were collected and frozen at −20°C until dissection.

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 any sign of imminent hatching appeared. Eggs were experimentally manipulated by injecting a physiological dose of T at the time of laying. Even though the Guideline on The Use and Euthanasia Procedures of Chicken/Avian Embryos draft by Animal Care and Use Committee discourages hypothermia for euthanasia of avian embryos, we had to euthanize embryos through this procedure, placing the eggs into a −20°C freezer within 2 h of collection due to facility constraints. The Guidelines for the Euthanasia of Animals by American Veterinary Medical Association, physical methods of euthanasia, agree that this procedure may be necessary in some field situations if other methods are impractical or impossible to implement. This is the case because we performed a field experiment and we did not have a field laboratory with equipment to euthanize embryos by other methods [i.e. carbon dioxide (CO_2), anesthetic agents or decapitation], to dissect organs and to store them appropriately until biochemical analyses.

Laboratory procedures

In the laboratory, the eggs were left at room temperature for ca. 15 min and weighed (to the nearest g). Then, we removed the eggshell and the residual yolk sac was detached from the embryo and weighed (to the nearest g). Before dissection, the embryo was weighed (to the nearest g) and tarsus and head size (occipital-beak length) were measured by a caliper (to the nearest mm). The liver and brain were isolated from the embryo, weighed (to the nearest mg) and frozen at −80°C until biochemical analyses. All the measurements were performed by a single operator to ensure consistency. Molecular sexing of the embryo was performed according to Saino et al. (2008).

TAC and the concentration of total pro-oxidant molecules (TOS according to Erel, 2005) were measured in liver and brain homogenates from each embryo. Organs were homogenized in an appropriate volume of phosphate buffer (100 mmol l^{-1}, pH 7.4, 1 mmol l^{-1} EDTA and 100 mmol l^{-1} KCl) by an automatic homogenizer. Homogenates were centrifuged at 16,200 ***g*** for 10 min and an aliquot of the obtained supernatant was processed for measuring TAC and TOS. Briefly, TAC and TOS were measured according to colorimetric methods developed on plasma by Erel (2004 and 2005 respectively), and adapted to tissue homogenate samples. The TAC assay was calibrated by using Trolox and the results were expressed as µmol l^{-1} Trolox equiv. g^{-1} wet weight, while TOS was calibrated using hydrogen peroxide (H_2O_2) and the results were expressed as nmol l^{-1} H_2O_2 equiv. g^{-1} wet weight. Mean TAC intra-assay percentage coefficient of variation (CV%) was 5.01±4.24% and 5.01±4.24% (n=30 replicates), while the mean inter-assay CV (n=3 assay plates) was 6.97±5.42% and 6.9±2.5% for brain and liver homogenates, respectively. Mean TOS intra-assay CV% was 6.42±4.95% and 5.01±4.24% (n=30 replicates), while the mean inter-assay CV (n=3 assay plates) was 7.9±5.5% and 8.4±6.3% for brain and liver homogenates, respectively.

Statistical analyses

We relied on Gaussian linear mixed models (LMM) to analyze the independent and combined (two-way interaction) effects of treatment (T injection versus control), sex and laying order (fixed effect factors) on embryo traits. Where relevant, we included in the models egg mass at laying or embryo mass as covariates. Nest identity was always included as a random effect to account for non-independence of embryos from the same clutch. Egg mass, embryo and residual yolk mass, and mass of the liver and the brain were $\log_{10}$-transformed to account for allometry. In the analyses of liver and brain mass, embryo mass (covariate) was expressed as embryo mass minus liver or brain mass, respectively. The models in which all interactions were non-significant were simplified by removing all the interaction terms in a single step. We generally refrained from testing three-way interactions to avoid model over-parameterization. However, because of the evidence of an effect of T on brain size and sex differences in brain size, in the analysis of this variable, we tentatively investigated the three-way interaction between sex, treatment and embryo mass (see Results).

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For morphological analyses, the sample consisted of 30 clutches with three eggs each. Information on sex or morphology was not available for one a- and one b-egg. Thus, we considered 29 a-eggs (controls, T-injected: 15, 14), 29 b-eggs (14, 15) and 30 c-eggs (17, 13). Thirty-three eggs carried a female and 55 eggs carried a male embryo.

For analyses of markers of oxidative status, the sample consisted of 30 clutches with three eggs each. Information on sex was not available for one b-egg and information on individual markers of oxidative status was not available for some embryos, yielding the following sample sizes: 30 a-eggs (controls, T-injected: 16, 14), 28–29 b-eggs (14, 14–15) and 29–30 c-eggs (16–17, 13).

RESULTS

Testosterone and embryo morphology

In LMMs with brood as a random effect, T treatment did not affect the length of incubation (from laying to appearance of eggshell fractures) of embryos from T-treated eggs compared with controls ($F_{1,60}$=0.057, P=0.813), after controlling for embryo sex and laying order. However, T treatment was found to significantly enhance embryo mass and tarsus length while controlling for original egg mass (Table 1). These analyses did not disclose any significant effect of sex or laying order, nor any two-way interaction effects among sex, laying order and treatment. Conversely, T treatment caused a reduction in residual yolk mass, again independently of laying order or sex effects (Table 1).

Brain mass showed a complex pattern of variation according to the independent and combined effects of T treatment, sex and laying order. Brain mass increased with total embryo mass, as expected, but the slope of this relationship was more than twice as steep among controls compared with T-treated embryos (Table 1, Fig. 1), resulting in a significant treatment by embryo mass interaction effect (Table 1). The effect of T treatment depended on laying order: brain mass of embryos from T-treated b-eggs was significantly higher compared with that from control b-eggs, while brain mass from control b-eggs was significantly smaller than that from a- and c-control eggs. However, brain mass did not vary according to laying order among T-treated eggs. In addition, brain size was significantly smaller in females (Table 1). Hence, males had significantly larger brains compared with females also after controlling for the effect of general body size. All these significant effects on brain mass were confirmed (i.e. they were still statistically significant) when we controlled for a measured of head size (occipital-beak length) rather than for embryo body mass (details not shown). Differently from brain mass, liver mass was unaffected by T treatment, sex or laying order, whereas it increased with embryo mass, as expected (Table 1).

Inclusion of brain size in the model reported in Table 1 of the three-way interaction between sex, treatment and embryo mass showed that the relationship between brain size and embryo mass did not differentially vary in either sex depending on egg treatment (three-way interaction effect: $F_{1,45}$=1.73, P=0.195). Hence, there was no statistically significant evidence that T had a differential effect on brain mass of male or female embryos.

Testosterone and markers of oxidative status

Testosterone treatment did not affect TAC either in the liver or in the brain. In addition, TAC did not vary between the sexes or between positions in the laying sequence.

Testosterone treatment caused a reduction in TOS in the liver after controlling for the statistically non-significant effects of sex and

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

	F	d.f.	P	Estimated marginal means/Coefficients (s.e.)		
Embryo mass						
Treatment	6.83	1, 53	0.011	Controls: 1.617 (0.007)	T-treated: 1.637 (0.008)	
Sex	0.49	1, 53	0.486	Males: 1.630 (0.006)	Females: 1.624 (0.008)	
Laying order	1.03	2, 53	0.365	a-eggs: 1.621 (0.008)	b-eggs: 1.633 (0.008)	c-eggs: 1.626 (0.009)
Egg mass	21.19	1, 53	<0.001	0.785 (0.171)		
Tarsus length						
Treatment	9.64	1, 53	0.003	Controls: 2.329 (0.005)	T-treated: 2.348 (0.005)	
Sex	0.52	1, 53	0.474	Males: 2.341 (0.005)	Females: 2.336 (0.006))	
Laying order	0.87	2, 53	0.425	a-eggs: 2.333 (0.006)	b-eggs: 2.341 (0.006)	c-eggs: 2.342 (0.007)
Egg mass	9.49	1, 53	0.003	0.402 (0.130)		
Residual yolk mass						
Treatment	6.84	1, 53	0.012	Controls: 1.222 (0.013)	T-treated: 1.178 (0.013)	
Sex	0.23	1, 53	0.635	Males: 1.196 (0.012)	Females: 1.204 (0.015)	
Laying order	0.74	2, 53	0.481	a-eggs: 1.214 (0.016)	b-eggs: 1.190 (0.015)	c-eggs: 1.196 (0.016)
Brain mass						
Treatment	5.60	1, 47	0.022			
Sex	22.88	1, 47	<0.001	Males: 0.171 (0.007)	Females: 0.129 (0.008)	
Laying order	2.77	2, 47	0.073	a-eggs: 0.159 (0.009)	b-eggs: 0.138 (0.009)	c-eggs: 0.153 (0.008)
Embryo mass	21.69	1, 47	<0.001	See Fig. 2		
Treatment×Sex	0.26	1, 47	0.612			
Treatment×Laying order	7.75	2, 47	0.001			
Laying order×Sex	1.29	2, 47	0.284			
Treatment×Embryo mass*	5.37	1, 47	0.025	Controls: 0.676 (0.134)[a]	T-treated: 0.264 (0.135)[b]	
Liver mass						
Treatment	0.85	1, 53	0.400	Controls: –0.149 (0.017)	T-treated: –0.134 (0.017)	
Sex	0.00	1, 53	0.486	Males: –0.142 (0.019)	Females: –0.141 (0.019)	
Laying order	0.53	2, 53	0.365	a-eggs: –0.129 (0.019)	b-eggs: –0.148 (0.019)	c-eggs: –0.148 (0.019)
Embryo mass	3.87	1, 53	0.055	0.471 (0.240)		

*See also Fig. 1.
[a]t=5.06, P<0.001.
[b]t=1.95, P=0.057.

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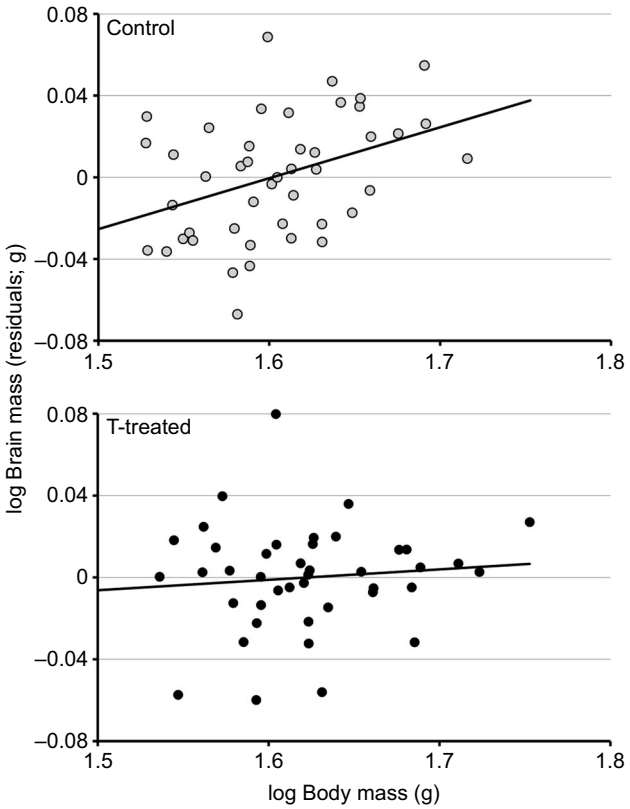

Fig. 1. Relationship between brain mass and embryo body mass in control or testosterone (T)-treated yellow-legged gull eggs. Brain mass is expressed as residuals from a linear mixed model on log brain mass data including treatment, sex, laying order and their interactions as fixed effects. Nest was also included in the model to calculate the residuals. The relationship was significantly positive for controls and marginally non-significant for T-treated embryos. The slope was significantly larger for the controls (see Table 1). Linear regression lines are fitted to better show the trend.

laying order (Table 2). In the brain, T treatment had a highly significant complex differential effect on TOS depending on laying order. In essence, while TOS of embryo from control b-eggs was smaller than that from control a-eggs, the difference between TOS of a- and b-eggs was reversed in the T-treated group, as TOS was significantly larger in b-eggs than in a-eggs.

DISCUSSION

A large body of studies has unveiled the multi-faceted role of maternal effects mediated by transfer of maternal androgens to the egg in causing short- and long-term variation in offspring phenotype after hatching (Groothuis and Schwabl, 2008; Groothuis et al., 2005). Yet, understanding how egg substances affect pre-natal development is of pivotal importance because maternal effects during embryonic life can imprint development of physiological and behavioural traits, and complex longitudinal trade-offs may operate between contrasting androgen effects during pre- and post-natal life (Navara and Mendonça, 2008).

We studied the effect of a physiological increase in yolk T concentration in the yellow-legged gull on embryonic growth and oxidative status. The main result of our experiment was that T has contrasting effects on overall embryo and brain mass. Embryos from T-treated eggs had a larger body size but, for a given body size, had a smaller brain than controls. In addition, T treatment caused a reduction in residual yolk mass, suggesting that an ultimate mechanism by which T enhances body size is acceleration of yolk absorption. T is believed to be anabolic to muscle and bone tissues (Navara and Mendonça, 2008). The present results imply that such anabolic effects are expressed already during the late embryonic stages. Brain tissues, in contrast, are known to be one of the main targets of the organizing effects of pre-natal T. Receptors for T exist in the brain from very early embryo development (Godsave et al., 2002), and major avian body systems, including immune, metabolic, muscular and skeletal systems, are likely to respond to androgens early in embryonic development when the neural and physiological axes are organized (Gahr et al., 1996; see Groothuis and Schwabl, 2008; Navara and Mendonça, 2008). The contrasting effects on body size and brain mass may therefore suggest that T mediates a developmental trade-off between allocation to general somatic growth and energetically demanding brain growth at the embryonic stage (the ‘expensive tissue hypothesis’; Aiello and Wheeler, 1995; see also Isler and van Schaik, 2006). This interpretation is supported by comparative studies in which negative associations between brain size and size of other costly organs have been demonstrated across species (Aiello and Wheeler, 1995; Isler and van Schaik, 2006; Navarrete et al., 2011; Tsuboi et al., 2014). Intriguingly, the present results are also consistent with the conclusion of a comparative study of the coevolution between maternal effects mediated by egg composition and brain size, which suggested that high T levels may be suppressive to brain size (Garamszegi et al., 2007). Developmental trade-offs between brain and somatic growth might therefore be a conserved trait throughout the bird lineage.

In a previous experiment on the same colony, we found a negative effect of T on body mass 4 days after hatching (Rubolini et al., 2006). In addition, in a study of the effect of egg T on peri-natal lateralization, we reported a negative effect on body mass of 1-day-old chicks from a-eggs only, but no effect on body mass of hatchlings from b- or c-eggs (Possenti et al., 2016). Hence, the positive effect of T on pre-natal size seems to vanish around hatching and then turns to negative in early post-natal growth. T effects may therefore enforce not only trade-offs between different traits (body size and brain size) at the embryonic stage, but also longitudinal trade-offs in body size at different ontogenetic stages.

These findings are consistent with those of a previous study of spotless starling (*Sturnus vulgaris*) showing a stronger effect of T during embryo development compared with the nestling period (Muriel et al., 2013; see also Schwabl et al., 2007). The mechanism behind such age-dependent variation may consist of differences in the secretion of metabolizing enzymes between embryos and hatchlings (Bruggeman et al., 2002) and/or an effect of embryonic T on the number and distribution of androgen receptors after hatching (Navara and Mendonça, 2008; Resko and Roselli, 1997). Unfortunately, no study has investigated the differences in the metabolizing pathways of androgens in embryos and hatchlings, although it has been suggested that extensive metabolization occurs during early incubation (Fivizzani et al., 1986; von Engelhardt et al., 2009).

Notably, brain mass was found to differ between the sexes. Present evidence prompted us to analyze brain mass data from the control group from a previous experiment on the same population (M.P., unpublished data). Consistently with the present experiment, we found that brain mass was significantly larger in male than in female embryos (mean±s.e.: males: 1.59±0.03 g n=10; females: 1.49±0.03 g, n=20; $F_{1,25}$=9.06, P=0.006). Sexual dimorphism in size and structure of specific brain nuclei has been reported in several taxa (Gahr, 1994; Jacobs, 1996). Examples of sexual dimorphism in brain size include species where the sexes differ in

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Table 2. Linear mixed models of markers of embryo oxidative status in relation to the main and two-way interaction effects of egg testosterone treatment, sex, laying order and egg/embryo mass in the yellow-legged gull

	F	d.f.	*P*	Estimated marginal means/Coefficients (s.e.)		
TAC in the brain						
Treatment	0.83	1, 55	0.367	Controls: 17.75 (1.34)	T-treated: 18.90 (1.34)	
Sex	0.56	1, 55	0.456	Males: 18.92 (1.32)	Females: 19.73 (1.41)	
Laying order	0.92	2, 55	0.406	a-eggs: 18.54 (1.40)	b-eggs: 19.96 (1.41)	c-eggs: 19.48 (1.39)
TOS in the brain						
Treatment	1.35	1, 50	0.251	Controls: 0.211 (0.030)	T-treated: 0.235 (0.030)	
Sex	3.06	1, 50	0.087	Males: 0.244 (0.029)	Females: 0.203 (0.032)	
Laying order	0.50	2, 50	0.610	a-eggs: 0.226 (0.031)	b-eggs: 0.211 (0.032)	c-eggs: 0.234 (0.031)
Treatment×Sex	0.27	1, 50	0.300	See Fig. 3		
Treatment×Laying order	7.21	2, 50	0.002			
Laying order×Sex	0.95	2, 50	0.394			
TAC in the liver						
Treatment	0.25	1, 54	0.618	Controls: 30.00 (1.86)	T-treated: 29.02 (1.88)	
Sex	0.79	1, 54	0.379	Males: 26.53 (1.78)	Females: 30.49 (2.08)	
Laying order	0.49	2, 54	0.614	a-eggs: 28.63 (2.05)	b-eggs: 30.82 (2.12)	c-eggs: 29.08 (2.04)
TOS in the liver						
Treatment	4.51	1, 54	0.038	Controls: 1.234 (0.073)	T-treated: 1.055 (0.073)	
Sex	0.65	1, 54	0.424	Males: 1.108 (0.069)	Females: 1.183 (0.081)	
Laying order	0.49	2, 54	0.614	a-eggs: 1.109 (0.082)	b-eggs: 1.202 (0.084)	c-eggs: 1.125 (0.082)

TAC, total antioxidant capacity; TOS, total pro-oxidant molecules.

the structural architecture of the brain (e.g. Garamszegi et al., 2005; see Kotrschal et al., 2012), but reports of sex differences on overall brain size are scarce and refer to adult individuals (see Kotrschal et al., 2012). These results are therefore the first, to the best of our knowledge, where a sex difference in brain size at the embryonic stage is documented in any vertebrate species in the wild.

Sex-dependent selection for specific cognitive tasks is believed to cause divergent evolution in brain size in either sex (e.g. Garamszegi et al., 2005; Jones and Healy, 2006; Sherry, 2006) based on the assumption that brain space is positively associated with cognitive ability (Lefebvre et al., 1997; Striedter, 2005). For example, selection experiments have shown that large-brained males have faster learning ability in mate-finding tasks (Kotrschal et al., 2014). In the yellow-legged gull there is no evidence so far that late-stage embryos or newly hatched chicks are exposed to divergent selection for cognitive tasks between the sexes. Post-natal behaviour, including lateralization, anti-predator behaviour and begging, which are major fitness traits during early post-natal stages, show no sex-dependent variation (Possenti et al., 2016). However, sex differences in lateralization have been demonstrated in the 'tonic immobility' response to acute stress (Romano et al., 2015). The function of the sexual dimorphism in brain size that we observed thus remains to be elucidated. At the mechanistic level, differently from oestrogens, which participate in the orchestration of sexual differentiation of several organs, yolk androgens seem not to interfere with sexual differentiation of the brain (Groothuis and Schwabl, 2008). Our results are consistent with the notion of no effect of T on sexual differentiation of the brain, because we found no differential effect of T treatment on brain size in either sex, after controlling for variation in embryo size, and imply that processes not mediated by egg T concentration cause early onset of brain sexual dimorphism. For example, sex-dependent variation in the expression of androgen receptors, which may occur independently of androgen regulation, could cause the observed sexual dimorphism in brain size (Gahr, 2001).

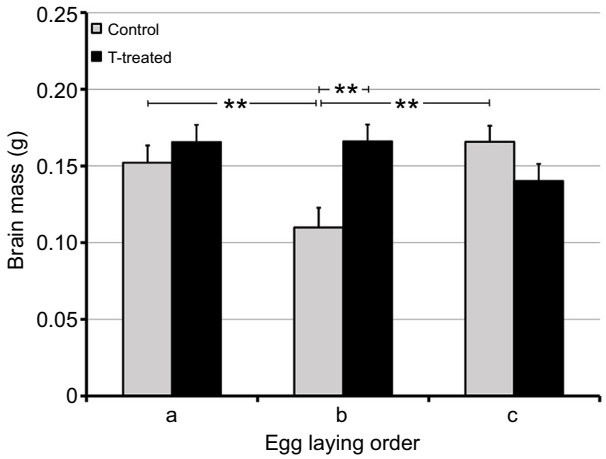

Fig. 2. Yellow-legged gull brain mass (estimated marginal means+s.e.) from a model with the same design as in Table 1 in relation to treatment and laying order. Significant pairwise differences (LSD test) between embryos of the same treatment or laying order are indicated by asterisks (**P<0.01).

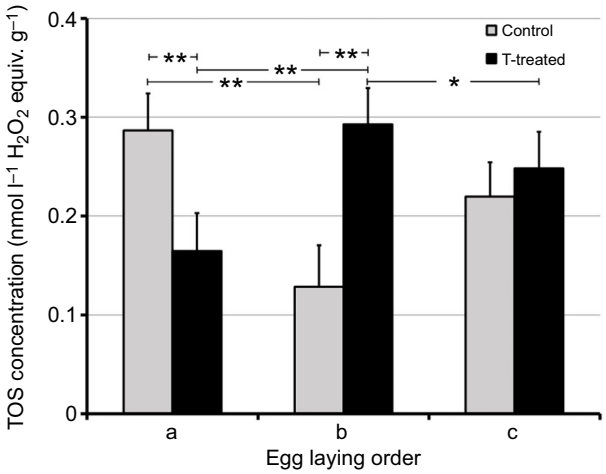

Fig. 3. Total pro-oxidant molecules (TOS; expressed as nmol l^{-1} H_2O_2 equivalents g^{-1} wet weight) in the yellow-legged gull brain in relation to treatment and laying order. Significant pairwise differences (LSD test) between embryos of the same treatment or laying order are indicated by asterisks (*P<0.05; **P<0.01).

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T treatment affected the pattern of variation of brain size according to laying order. Brain size of embryos of control b-eggs was smaller than that of embryos from control a- or c-eggs, whereas no such variation according to laying order was observed among embryos from T-treated eggs. The proximate causes and any possible function, from a parental perspective, of variation in offspring pre-natal brain size according to laying order are not clear. b-Eggs from the same colony are intermediate in size and biochemical composition between a- and c-eggs (Rubolini et al., 2011), suggesting that maternal effects via steroid hormones and antioxidants do not mechanistically cause b-egg embryos to grow smaller brains at the specific developmental stage when we collected them. Yet, the present results show that egg T has the potential to interfere with the developmental processes that cause differential pace of growth of brain tissues, relative to embryo size, according to laying order.

Interestingly, the differential pattern of variation of brain size according to laying order in control as compared with T-treated embryos was paralleled by variation in TOS in the brain. Control embryos in b-eggs had lower TOS compared with TOS in a- or c-eggs. Conversely, TOS of T-embryos from b-eggs did not significantly differ or was significantly larger compared with TOS from c- or a-eggs from the T-treated group, respectively. The studies of the effect of T on markers of oxidative status in young birds are few, have focused on post-natal stages and have provided mixed evidence (Noguera et al., 2011; Tobler and Sandell, 2009; Tobler et al., 2013). One interpretation of the present results is that T, via an unknown mechanism, specifically enhances growth of the brain of b-egg embryos and this causes increased production of oxidative molecules, leading to relatively large TOS estimates. This interpretation is supported by the observation that T is anabolic and that increased growth rate is generally considered to increase the production of molecules of high oxidative potential, although the evidence for an effect of T on metabolic rates is conflicting (Eising et al., 2003; Tobler et al., 2007; Wikelski et al., 1999) and is available for post-natal but not for embryonic life stages. Differently, the significant decrease in TOS levels measured in the brain of embryos from a-eggs could be related to the higher amount of antioxidants (including vitamins and carotenoids) that mothers allocate to the first-laid eggs compared with second- and third-laid eggs (Rubolini et al., 2011), which efficiently counteracted the free radical production imposed by early development.

We found no evidence that T affected the total antioxidant activity in the brain or in the liver. In the same species that we studied here, Noguera et al. (2011) showed that egg T enhanced plasma TAC during chick growth (age 8 days). Because we did not document any effect of egg T on liver or brain TAC in the present study, we conclude that T effects on TAC depend on ontogenetic stage and/or that they vary between tissues. The negative effect of T on liver TOS that we observed here is partly consistent with Noguera et al. (2011), who did not show an effect of T on TOS but showed that T prevented the increase in lipid peroxidation during chick post-natal growth that was observed among control chicks. In fact, these results combined suggest that reduced oxidative damage to lipids could be due to reduced production of oxidative compounds.

However, our results on the effect of T on TAC and TOS indicate that T has no general direct effect on the oxidative status in different organs via an effect on body growth. Hence, an alternative interpretation is that T has independent effects on growth and TOS in selected organs, with no effect of growth on antioxidant capacity or TOS.

In conclusion, this study shows that physiological increase in T levels has major, contrasting effects on pre-natal development, boosting body growth but depressing brain growth, which was differentially affected depending on laying order. In the same population, T has been shown to depress post-natal body size. Such contrasting effects of egg T on different traits and on the same trait during different ontogenetic stages may enforce a trade-off on maternal decisions regarding T transfer to the eggs. Testosterone also had complex effects on the production of pro-oxidant molecules both in the liver and in the brain, which may be independent of any effect on growth. Finally, we documented for the first time sexual dimorphism in pre-natal brain size. Further studies on other species are needed to assess the generality of pre-natal T effects and of sex-related variation in brain size.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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