Yolk testosterone reduces oxidative damages during postnatal development

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Conditions experienced during early life can influence the development of an organism and several physiological traits, even in adulthood. An important factor is the level of oxidative stress experienced during early life. In birds, extra-genomic egg substances, such as the testosterone hormone, may exert a widespread influence over the offspring phenotype. Interestingly, testosterone can also upregulate the bioavailability of certain antioxidants but simultaneously increases the susceptibility to oxidative stress in adulthood. However, little is known about the effects of maternally derived yolk testosterone on oxidative stress in developing birds. Here, we investigated the role of yolk testosterone on oxidative stress of yellow-legged gull chicks during their early development by experimentally increasing yolk testosterone levels. Levels of antioxidants, reactive oxygen species and lipid oxidative damage were determined in plasma during nestlings' growth. Our results revealed that, contrary to control chicks, birds hatched from testosterone-treated eggs did not show an increase in the levels of oxidative damage during postnatal development. Moreover, the same birds showed a transient increase in plasma antioxidant levels. Our results suggest that yolk testosterone may shape the oxidative stress-resistance phenotype of the chicks during early development owing to an increase in antioxidant defences and repair processes.

Keywords: androgens; metabolic programming; maternal effects; oxidative stress

1. INTRODUCTION

Conditions experienced during early life can determine a range of traits in adulthood [1]. In this context, the imbalance between the production of reactive oxygen species (ROS) and the state of the antioxidant machinery (i.e. oxidative stress [2]) could play a critical role. Oxidative stress early in life has been related to several diseases and physiological changes in adulthood in model species including humans [3]. On these lines, a recent experimental study in zebra finches (*Taeniopygia guttata*) has shown that early conditions may

determine resistance to oxidative stress throughout a lifetime as well as life-history trajectories, probably owing to changes in the development of antioxidant defences [4].

In mammals, mothers may influence the foetal environment, which may determine the phenotype of the offspring (i.e. foetal programming [5]). Similarly, female birds exert a widespread effect over offspring characteristics by means of extra-genomic egg substances, which shape offspring phenotype and fitness [6,7]. Experimental studies have shown that increased levels of maternally derived yolk testosterone positively influence offspring traits such as begging behaviour, metabolic rate, growth and survival, although some failed to find the effects (reviewed in [6]). Furthermore, they may improve secondary sexual traits in adulthood [6]. In some bird species, testosterone seems to upregulate the bioavailability of certain antioxidants (i.e. carotenoids) during adulthood (e.g. [8,9]), but simultaneously increase the susceptibility to oxidative stress [8]. Additionally, testosterone may directly induce oxidative stress in some tissues (see [10] and references therein). However, little is known about the effects of testosterone on oxidative stress in developing birds. A recent experimental study showed that eggs whose yolk testosterone levels were increased [11] produced chicks with reduced plasma antioxidant levels during growth, although this effect was only detected in males [11]. Nonetheless, a decline in antioxidant levels does not necessarily mean a higher oxidative stress, because it can also be accompanied by reduced ROS production [12].

To determine the role of maternal yolk testosterone in bird development and oxidative stress, we artificially increased testosterone levels in the last laid eggs of three-egg clutches of yellow-legged gulls (*Larus michahellis*) under free-living conditions. Antioxidant defences, ROS production and oxidative damage were assessed in growing birds. In gulls, amounts of yolk testosterone in third eggs are higher and more variable than in the first two eggs, and third chicks experience the most stressful conditions [13]. Therefore, if testosterone increases susceptibility to oxidative stress (i.e. [10]), we predict that third eggs treated with testosterone should not only develop a weaker antioxidant defence, but also suffer from higher ROS production and oxidative damage.

2. MATERIAL AND METHODS

The experiment was conducted from May to June 2009 in a gull colony at Sálvora Island, Galicia, northwest Spain. The third eggs from 92 three-egg clutches were randomly assigned to either testosterone or control treatment. Eggs were collected on the day of laying and injected with testosterone (261 ng) or vehicle (i.e. sesame oil). The full protocol is described in the electronic supplementary material.

Nests were checked daily beginning 2 days before the estimated hatching date. Egg volume did not differ between treatments ($t_{90} = -0.59$, $p = 0.55$). During the experiment, 23 eggs from the testosterone treatment (T) group and 29 from the control (C) group did not successfully develop. A total of 17 eggs (8 T and 9 C) developed until pipping, but died during hatching. Chicks were molecularly sexed (electronic supplementary material). Treatment did not affect incubation time (generalized linear model (GLMz): $F_{1,21} = 0.483$, $p = 0.495$), sex ratio at hatching (generalized linear model (GLMz): $\chi^2_1 = 0.01$, $p = 0.90$) or hatching success (GLMz: $\chi^2_1 = 3.47$, $p = 0.06$), although T-chicks tended to hatch more successfully than C-chicks. Survival of hatched chicks was recorded until 9 days of age. Chicks were considered dead when carcasses were found. Missing chicks during the experiment were excluded from the analyses.

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Table 1. Repeated-measure analyses of total antioxidant capacity and lipid peroxidation levels in relation to treatment and covariates. (The minimal adequate models are shown. In the minimal model of ROS levels, only the intercept was retained. Full model: age + treatment + sex + body mass (day 1) + hatching date + treatment $\times$ age + treatment $\times$ sex + treatment $\times$ body mass (day 1) + treatment $\times$ hatching date.)

dependent variable	independent variables	estimate	<i>F</i>	d.f.	<i>p</i>
total antioxidant capacity	intercept	0.573			
	age (1)	-0.185	4.90	2,54	0.011
	age (5)	0.233			
lipid peroxidation	intercept	0.536			
	treatment	0.272	7.14	1,55	0.009

Treatment did not affect chick survival until the age of 9 days (GLM: $\chi^2_1 = 2.16$, $p = 0.14$). All chicks were weighed (± 0.01 g), their tarsus length measured (± 0.01 mm) and blood sampled at days 1 (1 day after hatching), 5 and 9 of age (electronic supplementary material). Growth rate was calculated as the increment in body mass and tarsus length between 1–5 and 5–9 days of age.

ROS in plasma was determined per duplicate by following Brambilla *et al.* [14]. ROS level was expressed as millimoles of hydrogen peroxide (H₂O₂) equivalents per litre. Total plasma antioxidant capacity was measured per duplicate by following Erel [15], being expressed as millimoles of Trolox equivalents per litre. Lipid peroxidation in plasma (levels of oxidative damage in lipids) was assessed per triplicate by quantifying malondialdehydes (MDA) by high-performance liquid chromatography following Karatas *et al.* [16], but modifying sample volume and reagents (electronic supplementary material). MDA calibration curves showed a correlation coefficient of $r^2 > 0.999$. Data were expressed as micrograms of MDA per millilitre of plasma.

The post-hatching effects of testosterone on growth rates, ROS, antioxidants and lipid peroxidation levels were analysed using repeated-measure models (PROC MIXED in SAS software), with age as repeated-measure factor and individual as subject term (REPEATED statement). Treatment and sex were included in the models as fixed factors. Hatching date (in Julian days) and body mass (day 1) were included as covariates, as well as first-order interactions. Complementary to this, to analyse individual changes, we also re-ran the models with the change in levels of ROS, antioxidants and lipid peroxidation among age periods (1–5 and 5–9 days; for details, see the electronic supplementary material). Satterthwaite's approximation for degrees of freedom was used and the best covariance structure ('variance component' in SAS) was selected according to the Akaike information criterion [17]. All models were simplified by removing non-significant terms (backward deletion), starting from two-way interactions. Sex was removed from all models as a non-significant term.

3. RESULTS

Body mass, tarsus length, ROS, antioxidants and MDA levels at day 1 of age did not significantly differ between treatments (all $p > 0.14$; for details, see the electronic supplementary material). Testosterone injection did not affect growth during the first 9 days of life (body mass: $F_{1,29} = 0.01$, $p = 0.91$; tarsus length: $F_{1,30} = 0.02$, $p = 0.87$).

During postnatal development, ROS levels were not affected by testosterone treatment ($F_{1,51} = 0.01$, $p = 0.92$) or its interaction with chick age ($F_{2,45} = 0.62$, $p = 0.54$). Antioxidant levels increased with chick age (table 1 and figure 1a), but we did not find significant differences between treatments throughout development (treatment: $F_{1,51} = 0.08$, $p = 0.78$; age $\times$ treatment: $F_{2,48} = 1.55$, $p = 0.20$). Nevertheless, secondary analysis of individual changes in antioxidants levels revealed that antioxidants in T-chicks increased between 1 and 5 days and decreased from 5 to 9 days, whereas C-chicks only showed a slight increase during the growth (age period $\times$ treatment: $F_{1,30} = 6.33$, $p = 0.017$).

Figure 1. Values (mean $\pm$ s.e.) in plasma levels of (a) total antioxidant capacity and (b) lipid peroxidation over age (days after hatching) in T-chicks (dark dots) and C-chicks (white dots).

Lipid peroxidation levels differed significantly between treatments (table 1 and figure 1b). Interestingly, when we analysed the patterns of individual changes in MDA, we found that differences between groups were especially important between 5 and 9 days (age period $\times$ treatment: $F_{1,29} = 4.93$, $p = 0.034$).

4. DISCUSSION

Our results indicate that an increase in testosterone in the egg yolk induced a reduction of oxidative damage, at least in lipids, during postnatal development. Contrary to our predictions, testosterone treatment

reduced lipid peroxidation levels during the first 9 days of age. Results on individual changes revealed that T-chicks experienced an increase in circulating antioxidants levels during the first 5 days of life. Afterwards, T-chicks showed a decline in the levels of lipid peroxidation between 5 and 9 days of age. How these effects are mirrored in other macromolecules susceptible to oxidative stress (proteins or DNA) and how the effects of testosterone may remain until adulthood are however unknown.

Testosterone is assumed to promote oxidative stress [10]. Nevertheless, our testosterone treatment did not increase ROS levels. By contrast, testosterone treatment prevented the increase in MDA levels. In this way, our results suggest that the yolk testosterone induced an early mobilization of antioxidants during development. Our experimental increase of yolk testosterone could have produced an early stress (i.e. in the embryo), promoting a compensatory response later in life [18].

It is interesting to note that third eggs in gulls' clutches not only present higher levels of yolk testosterone than earlier eggs, but higher levels of dietary antioxidants such as vitamin E and carotenoids [13]. It has been argued that high yolk testosterone levels in last laid eggs serve to enhance the competitiveness of the chick within the brood, compensating for the handicap of hatching later and lower antioxidant content in the yolk [6,7]. If testosterone promotes oxidative stress, this might also imply that last chicks compensate their initially low survival prospects at the cost of reduced longevity. However, in contrast to Tobler & Sandell [11], our results suggest that testosterone would not impinge an immediate cost in terms of oxidative damage (see lipid peroxidation). Instead, our results would suggest that mothers could be programming the phenotype of the chicks (e.g. [3]), favouring the activation of antioxidant mechanisms and repair processes independent of food intake. The adaptive function of this programming and the impact that it may exert on the life history and fitness merit future research.

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