# LONG-TERM EFFECT OF YOLK CAROTENOID LEVELS ON TESTIS SIZE IN A PRECOCIAL BIRD

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## ABSTRACT

Conditions experienced during prenatal development can have long-lasting organizational effects on offspring. Maternal carotenoids deposited in the eggs of birds and other oviparous species play an important role during fast embryonic growth and chick development through their antioxidant properties. However, the long-term consequences of variation in maternal carotenoid transfer for the offspring have been seldom considered. Since plasma carotenoid levels at adulthood are known to influence testis size and yolk carotenoid levels influence the ability to extract carotenoids later in life, we hypothesized that maternally transmitted carotenoids might influence gonad size at adulthood. Here, we showed that male Japanese quail (*Coturnix japonica*) originating from a carotenoid-enriched egg had smaller testes than control individuals at adulthood. This result shows that yolk carotenoids have long-term organizational effects. In addition, given that carotenoid intake at sexual maturity increases sperm quality and that a decreased testis size is associated with a lower sperm production, we propose that carotenoid exposure during embryo development might influence the trade-off between ejaculate size and sperm quality.

## INTRODUCTION

Conditions experienced during embryo development can have major organizational effects that can last until adulthood [1]. However, although numerous studies have documented short-term effects of prenatal conditions on offspring phenotype [2], the long-term consequences for fitness-related traits are still poorly understood.

Maternally transmitted antioxidants might influence embryonic developmental trajectory due to their capacity to scavenge the reactive oxygen species produced during development and the challenging period of rapid growth [3]. Several studies have examined the importance of these compounds during development using dietary carotenoid supplementation of laying females to indirectly manipulate yolk antioxidants levels (i.e. carotenoids) and have shown effects of this treatment on nestlings' condition or carotenoid levels just a few weeks after hatching [4,5,6]. However, these results must be considered cautiously since these effects may have been mediated by other effects of these dietary manipulations on maternal physiology and/or the differential allocation of other maternally transmitted compounds.

So far, only two studies have directly manipulated yolk carotenoid concentrations through *in ovo* injections, showing that these maternally transmitted compounds have the potential to influence chick growth, immunocompetence and antioxidation capacity [7,8,9]. The potential long-term effects of prenatal antioxidant exposure have so far only been examined in one study in which male barn swallows (*Hirundo rustica*) hatched from eggs injected with vitamin E arrived earlier at their breeding grounds than controls [10]. We thus need more studies where the long-term consequences of yolk antioxidant manipulations (through *in ovo* injections) are examined to determine the potential organizational effect of these maternally transmitted compounds.

Nutritional conditions during development and at adulthood have been shown to influence gonadal development in a variety of taxa (cockroach, [11]), humans [12], mallard [13]), but information on the importance of specific antioxidants (such as carotenoids) on gonadal growth is scarce. Carotenoids are present in both testes and seminal fluid and it has been proposed that these molecules might limit oxidative stress and allow optimal cell growth in testes [14], a tissue where the high rate of cell division might generate high levels of free radicals [15]. Given that variation in yolk carotenoid levels can influence the ability to extract or assimilate these compounds later in life [4] and that dietary carotenoid availability at adulthood influenced testes size [16], we hypothesized that maternally transmitted carotenoid may influence gonad size at adulthood. To test this hypothesis, we experimentally manipulated yolk lutein levels in Japanese quail eggs and measured the consequences of this treatment for the sons' testis size at adulthood.

## METHODS

Unincubated Japanese quail (*Coturnix japonicus*) eggs were collected from 55 females of our captive breeding population and injected with either 15 μg of carotenoids (FloraGLO Lutein 20%, Kemin Foods, Des Moines, Iowa) dissolved in 15μL of safflower oil or with only safflower oil as a control. Lutein was chosen since it is the most abundant carotenoid found in Japanese quail eggs and the dose injected represents approximately one standard deviation of the yolk carotenoid content in this species [17]. The overall hatching success was 41.1% (Control = 38.5%, Carotenoid = 44.1%) and comparable to previous studies in Japanese quail [18, 19]. See ESM for details on the incubating and rearing conditions. One year post-hatch, 48 males were randomly selected and euthanized (29 control and 19 birds from carotenoid-injected eggs, from 40 females and 430 eggs injected ((226 control-injected and 204 carotenoid-injected)). Both testes were collected and weighed to the nearest 1 mg. Tarsus length was measured to the nearest 0.1 mm.

### *Statistics*

Testis mass was significantly repeatable between the left and right sides ($F_{1, 45} = 23.45$, $P<0.001$), we thus used average values per bird for our analyses. In order to avoid pseudo-replication, we used the mean average testis mass of all male offspring per mother in our analyses since six mothers had more than one son. We ran general linear models to test whether the yolk carotenoid manipulation and bird size (tarsus length), as well as their interaction, affected testis size. All statistical analyses were run in R 3.01 (R Core Team 2013).

## RESULTS

Bigger birds had bigger testes ($F_{1,37} = 7.77$, $P = 0.008$). Furthermore, testis size was significantly affected by the carotenoid treatment ($F_{1,37} = 4.52$, $P = 0.04$) with males originating from carotenoid-injected eggs having smaller testes than males originating from control eggs (figure 1). The interaction effect between carotenoid treatment and tarsus length was non-significant ($F_{1, 36} = 0.030$, $P = 0.86$). Yolk carotenoid treatment did not influence body size at adulthood ($F_{1, 38} = 1.41$, $P = 0.24$).

## DISCUSSION

Here, we show that prenatal exposure to carotenoids has long-term effects on a primary sexual trait. Male Japanese quail originating from a carotenoid-injected egg had smaller testes than controls one year post-hatching. This result is in line with the idea that yolk carotenoids have organizational effects that last until adulthood, potentially due to their antioxidant properties [20, but see 21] and / or their effect on gene expression, cell proliferation, and cell–cell communication [22, 23].

Previous studies have indirectly manipulated yolk carotenoid levels through supplementation of laying females and showed that chick absorption and utilization of carotenoids, immunocompetence and plumage coloration were influenced by this dietary manipulation several weeks after hatching [4,5,24]. Our result confirms these long-lasting effects of yolk carotenoid levels using a direct *in ovo* manipulation of these maternally-transmitted compounds and by measuring a primary sexual trait several months after the end of the developmental phase. Given that only one carotenoid has been manipulated in our study, further work is needed to investigate if also other yolk carotenoids have long-term effects on fitness-related traits.

We propose three non-mutually exclusive hypotheses that might explain the reduction of testis size in males originating from carotenoid-enriched eggs. First, carotenoids may influence the trade-off between ejaculate size and sperm quality [25]. Recent studies have shown that an increased dietary intake of carotenoid leads to a reduction of testes size in mallards (*Anas platyrhynchos*) [13] and that testis size and testis carotenoid concentrations are negatively correlated in house finches (*Haemorhous mexicanus*) [16]. Given that testis size is positively associated with sperm production in birds [26], this strongly suggests that high carotenoid intake decreases ejaculate size in birds. However, studies in various taxa have also shown that an increased carotenoid intake at sexual maturity increases sperm quality [27, 28, 29], potentially through their antioxidant properties or the recycling of vitamin E molecules, a major actor of spermatozoa protection from oxidative damage [29]. Thus, carotenoids seem to stimulate the production of high-quality sperm, but in smaller amounts. Future studies should test this hypothesis by simultaneously measuring ejaculate size and sperm quality in individuals supplemented or not with carotenoid at different life stages (before or after hatching). Second, prenatal exposure to high levels of carotenoid may influence the trade-off between self-maintenance and survival toward a reduced reproductive investment during the first breeding event. However, this hypothesis seems unlikely since carotenoid supplementation has been shown to increase several components of reproductive investment in various species [30]. Finally, while we acknowledge the possibility that prenatal carotenoid exposure may have detrimental consequences for testes maturation and thus sperm production in our study, we believe that it is inconsistent with the accumulating evidence that carotenoid supplementation improves sperm quality [27, 29, but see 31]. In addition, the injected carotenoid dose was well within the natural range (Peluc *et al.* 2012) and yolk carotenoid levels after injection were not unnaturally high since females were fed with a low-carotenoid diet during the whole experiment.

To conclude, we show for the first time that yolk carotenoid levels have long-term effects on a primary sexual trait, strongly suggesting that maternally-transmitted antioxidants influence offspring fitness.

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**Ethics:** All procedures conform to the relevant regulatory standards and were conducted under licences provided by the Veterinary Office of the Canton of Zurich, Switzerland (195/2010; 14/2014; 156).

**Data accessibility:** Data are available from the Dryad repository: http:// doi:10.5061/dryad.523t4.

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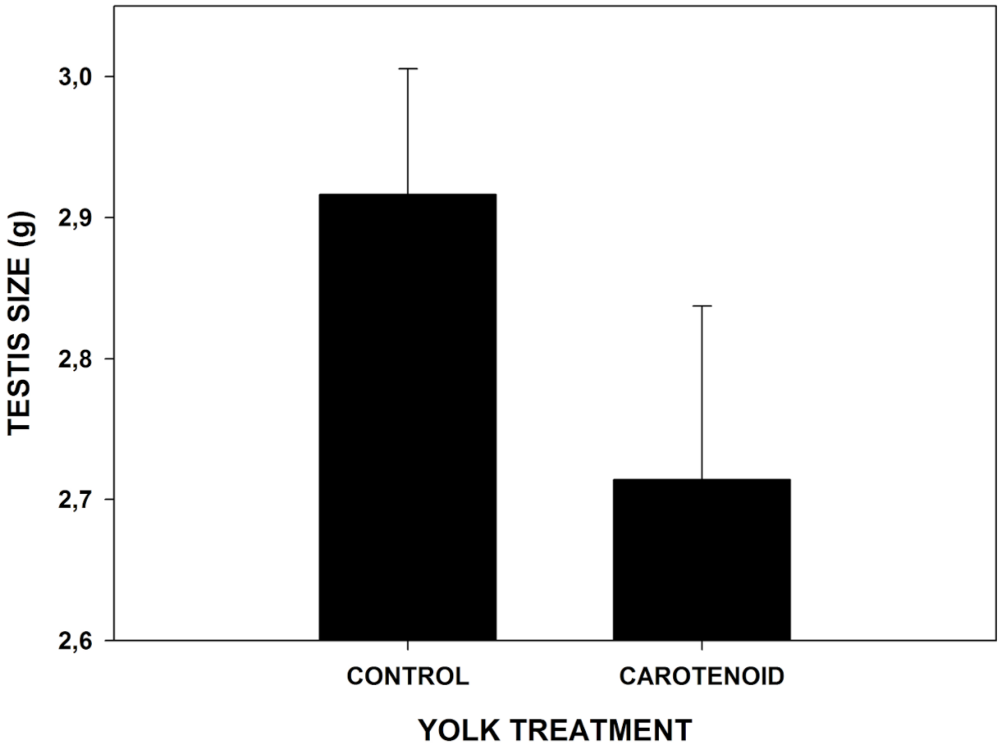
Figure 1: Effect of yolk carotenoid injection on testis size one year post-hatching. Means ±1 SE are shown.



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Eggs were artificially incubated for 14 days at a temperature of 37.6°C and 55% humidity and then at 37.6°C and 80% humidity for the last 3 days. Chicks were reared in mixed treatment cohorts of 40 chicks for 2 weeks and in cohorts of 20 chicks for three more weeks. At the age of 5 weeks, chicks were released into outdoor aviaries. Adults and chicks received *ad libitum* water and commercial game bird mix low in carotenoid content during the whole experiment.