Comparative Biochemistry and Physiology, Part A 166 (2013) 465–469

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Carotenoid supplementation during adulthood, but not development, decreases testis size in mallards

Michael W. Butler [a,b,*], Briette Karanfilian [a], Melissa Homsher [a], Kevin J. McGraw [b]

[a] Lafayette College, Department of Biology, Easton, PA, 18042-1778, USA
[b] Arizona State University, School of Life Sciences, Tempe, AZ, 85287-4501, USA

ARTICLE INFO

Article history:
Received 17 April 2013
Received in revised form 23 July 2013
Accepted 23 July 2013
Available online 31 July 2013

Keywords:
Anas platyrhynchos
Developmental plasticity
Dietary carotenoids
Lutein
Supplementation
Testes

ABSTRACT

Nutritional constraints on reproduction are well-characterized in female animals, but rarely have particular nutrients been linked to male reproductive investments. Carotenoid pigments promote egg-laying and fertility in several animals, and are displayed externally within secondary sex traits by males of many colorful species to attract mates, but it is unclear if or how carotenoids affect male primary sex traits. We manipulated carotenoid availability in the diet of male mallards (*Anas platyrhynchos*) during both development and adulthood to determine effects on size and carotenoid content of the testes. We found that developmental carotenoid manipulations did not affect testis size or carotenoid concentration, but that increased carotenoid dietary levels at adulthood resulted in more carotenoid-rich, but smaller, testes. This latter result was surprising, given positive correlations in mammals between testicle size and carotenoid concentration. We also found negative correlations between testis size and carotenoid concentration for individual ducks, regardless of dietary treatment. These results suggest that carotenoid deposition into testis tissue can reduce investment in gonad size (and thus overall sperm count), although the functional consequences of this relationship remain to be tested.

1. Introduction

Reproduction can be very costly to individuals, requiring the allocation of energy or specific nutrients toward the production and maintenance of offspring (Jönsson, 1997). Classically, these costs have been demonstrated in females, which allocate nutrients (Bortolotti et al., 2003; Thompson and Speake, 2003), immune cells and proteins (Boulinier and Staszewski, 2008; Gasparini et al., 2009), and hormones to their young. In contrast, investments made by males into reproduction have primarily focused on the development of secondary sexual characteristics, such as engaging in behaviors (e.g., defending territories; Booksmythe et al., 2011) or producing structures such as ornaments (Vergara et al., 2012) or armaments (Allen and Levinton, 2007), that may increase mating success. Comparatively little work has examined the costs of producing and maintaining male primary sexual characteristics, such as testis size or sperm production, mainly because of the much lower presumed costs incurred by males relative to females (Hayward and Gillooly, 2011).

Though much is known of the mechanistic pathways (e.g., hormonal, nutritional) that trigger testis maturation (e.g., Hötzel et al., 1995; Fernández et al., 2004), relatively few tests assessing the differential costs of testis size have been conducted. For example, testis size is positively correlated with adult body condition (i.e., size-corrected body mass) in dung beetles (Simmons and Kotiaho, 2002) and three species of small mammals (Schulte-Hostedde et al., 2005). Additionally, in commercially important ruminants, there are links between overall nutritional quality and testis size (Martin et al., 2010). However, the role of specific nutrients per se in generating variation in testis size has received comparatively far less attention, particularly in non-domesticated animals.

It is important to also consider how developmental versus current nutrition may affect male gonad development. Though most studies of animal reproduction hone in on current physiological state, there is ample evidence that nutritional conditions during development can constrain female reproductive investment in taxa ranging from insects (Barrett et al., 2009) to humans (Lummaa, 2003). There is similar evidence in males of a few species [e.g., walnut flies (*Rhagoletis juglandis*), Carsten-Conner et al., 2010; bulls (*Bos taurus*), Brito et al., 2007], but again we are in need of a better understanding of specific nutritional currencies for the ontogenetic and adult control of male primary sexual traits.

Carotenoid pigments are a class of lipid-soluble antioxidant nutrients that have been studied in the context of reproduction and mating (Hill and McGraw, 2006). Carotenoids are enriched in egg yolks (conferring them their yellow/orange appearance; Hipfner et al., 2010) and positively affect offspring health and development in several bird species (Berthouly et al., 2008; Romano et al., 2008). These pigments are also linked to primary sexual trait development in females (i.e., ovarian development; Zheng et al., 2012) and are common colorants of male secondary sexual traits (e.g. feathers, skin, scales; Hill and McGraw,

* Corresponding author at: Lafayette College, Department of Biology, Easton, PA, 18042-1778, USA. Tel.: +1 610 330 5762.
E-mail address: butlermw@lafayette.edu (M.W. Butler).

1095-6433/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpa.2013.07.024

2006), but still very little is known of possible links between carotenoid accumulation in the body and male gonad or sperm traits. Carotenoids are present in testes (Goodwin, 1950; Stahl et al., 1992; Rowe et al., 2012) and seminal fluid of several species (Rowe and McGraw, 2008), so it is plausible that variation in dietary intake of carotenoids drives differential carotenoid accumulation in these tissues and ultimately differential antioxidant action that permits optimal cell growth (either testis or spermatozoa) in these tissues that undergo high rates of cell division. The testis contains highly metabolically active tissue, and the generation of free radicals can be particularly detrimental to spermatogenesis (Kaur et al., 2006), allowing the antioxidant properties of carotenoids (Alonso-Alvarez et al., 2004) to play an important role in male reproductive biology. Lastly, because adult carotenoid physiology is affected by carotenoid availability in ovo (Koutsos et al., 2003) and during neonatal development (Blount et al., 2003), carotenoid availability early in life may play an important role in those components of gonad development that are linked to carotenoid physiology (Zheng et al., 2012).

To investigate how nutritional conditions during ontogeny and adulthood affect primary sexual traits in males, we manipulated dietary carotenoid access during development and/or adulthood and then determined testis size and carotenoid accumulation in adult male mallards (*Anas platyrhynchos*). This species has carotenoid-pigmented beak coloration (Butler et al., 2011) and frequently engages in forced extra-pair copulations (McKinney et al., 1983), resulting in mixed-paternity broods (Evarts and Williams, 1987), suggesting that sperm competition may be important. We predicted that carotenoid repletion during adulthood would increase testis carotenoid concentration, based on our prior work with this species showing that such a manipulation has effects on carotenoid concentration in plasma (Butler and McGraw, 2012a). We also predicted that dietary carotenoid supplementation of adults would increase testis size, based on a similar finding in rats (Taş et al., 2010). We did not have sufficient information to guide a directional prediction for the effect of developmental carotenoid access on testis traits, due to the paucity of ontogenetic studies in this area and due to inconsistent effects of neonatal carotenoid supplementation on adult traits in our prior research (Butler and McGraw, 2012a).

2. Materials and methods

2.1. Husbandry and experimental design

We acquired 42 one-day-old male mallard ducks (*A. platyrhynchos*; native North American breed) from Metzer Farms (Gonzales, CA, USA) in December 2010, and reared them in standard housing conditions (Butler and McGraw, 2009) until adulthood. As we have previously discussed the husbandry and experimental design of these birds in detail (Butler and McGraw, 2012a); here we provide only a brief review. Males were split into four groups using a 2 × 2 experimental design. Individuals were placed on either LOW (3 μg/g) or HIGH (25 μg/g) carotenoid diets from 2 to 49 days old (development: DEV) and during adulthood (ADULT: 17 to 20 weeks old; both N = 10 for birds receiving different diets during DEV and ADULT both N = 11 for birds receiving the same diets during DEV and ADULT). These two levels of dietary carotenoid content reflect the first and third quartile of carotenoid concentration found in wild duckling diets (Butler and McGraw, 2010), of which lutein is the predominant carotenoid. Diets consisted of a base duck chow (Mazuri Waterfowl Starter 0–7 weeks old, Mazuri Waterfowl Maintenance thereafter; Richmond, IN, USA) mixed with sunflower oil that contained ORO-GLO dry pigmenter (2% carotenoids by mass, predominately lutein; Kemin AgriFoods North America, Inc., Des Moines, IA, USA).

At adulthood (18 weeks old), individuals received immune challenges (i.e., phytohemagglutinin wing-web swelling test, keyhole-limpet hemocyanin injection) to assess immune function for a separate study, during which we collected blood samples 4 times over this 11-day period and determined average circulating carotenoid levels (see Butler and McGraw, 2012a). At the end of the study (three days later, when birds were 20 weeks old), we euthanized all individuals and collected both testes. All samples were stored at −80 °C until further analysis. At a later date, we measured the length, width, and depth of each testis to the nearest 0.1 mm and mass to the nearest 1 mg.

2.2. Carotenoid quantification

To quantify carotenoid concentration within testis tissue, we removed approximately 0.25 g of the mid-transverse section of a randomly selected testis for each bird (side was not nested within treatment; $\chi^2_3 = 1.06$, $P = 0.79$; right and left testes did not differ in carotenoid concentration: $F_{1,36} = 0.66$, $P = 0.42$). Using mid-transverse sections and sections from both ends of the testis from a subset of birds, we found that carotenoid concentration was significantly repeatable within individuals ($F_{3,8} = 12.85$, $P = 0.002$, $r = 0.80$), demonstrating that this method reliably captured testis carotenoid variation among birds. In the presence of 750 μL of 1:1 hexane:methyl tert-butyl ether, we ground the tissue with 6 stainless steel grinding balls in a 1.5 mL screw-cap Eppendorf tube in a grinding mill for 20 min at 30 Hz. We then centrifuged the samples for 3 min at 10,000 *g*, and transferred the carotenoid-containing supernatant to a new tube. We then added an additional 500 μL of 1:1 hexane: methyl tert-butyl ether to each testis sample and ground the tissue for 10 min at 30 Hz. The supernatant was combined with the previously transferred solution, dried under nitrogen, and stored at −80 °C. Each sample was then resuspended in 200 μL of mobile phase (42:42:16 methanol:acetonitrile:dichloromethane) and analyzed via high-performance liquid chromatography (McGraw et al., 2008) to calculate carotenoid concentration (μg of pigment per gram of testis). We found detectable amounts of lutein, zeaxanthin, and two unidentified xanthophylls. Concentrations of all carotenoids were highly positively correlated with each other (all $P < 0.0063$) and with total carotenoid concentration (all $P < 0.0002$), so we used total carotenoid concentration for all analyses. To calculate total carotenoid amount per testis, we multiplied total carotenoid concentration by testis mass.

2.3. Statistics

All statistics were performed using SAS v 9.2 (Cary, NC, USA), and testis mass was square-root transformed to meet assumptions of normality. All morphometrics were significantly repeatable (Lessells and Boag, 1987) between left and right testes (all $F_{41,43} > 7.76$, all $P < 0.001$, all $r > 0.77$), so we used average values within birds for all subsequent analyses. To test for effects of diet treatment, we used 2-way analyses of variance, with treatment as a class variable and testis length, depth, width, and mass as separate dependent variables. Differences between groups were tested using least-squares means. We also tested whether diet treatment affected carotenoid concentration and total carotenoid amount within testes.

3. Results

Carotenoid supplementation during development did not affect testis length, depth, width, or mass (all $F_{1,38} < 0.71$, all $P > 0.41$). However, carotenoid supplementation during adulthood resulted in testes that were shorter ($F_{1,38} = 5.00$, $P = 0.031$; Fig. 1) and less massive ($F_{1,38} = 4.96$, $P = 0.032$; Fig. 2), but not significantly different with respect to width or depth (both $F_{1,38} < 3.70$, both $P > 0.062$). Carotenoid supplementation during development did not interact with supplementation during adulthood to affect any metric of testis size (all $F_{1,38} < 0.30$, all $P > 0.59$).

Carotenoid supplementation during development did not affect testis carotenoid concentration ($F_{1,38} = 0.17$, $P = 0.68$), but birds that received carotenoid-supplemented diets during adulthood had testes that had a greater carotenoid concentration ($F_{1,38} = 57.85$, $P < 0.0001$; Fig. 3). However, diet during development or adulthood