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ORIGINAL ARTICLE

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Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird

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Abstract A salient feature of many secondary sexual characteristics in animals is that their expression is controlled by sex-steroid hormones. However, for only a few types of ornaments do we know the precise molecular mechanism by which androgens like testosterone (T) enhance trait production. We studied the red carotenoid-based beak of male zebra finches (*Taeniopygia guttata*), which serves as a sexually selected trait and is thought to be T dependent. In previous research, we demonstrated that the extent to which male finches produce lipoproteins in the bloodstream, which bind carotenoid pigments acquired from the diet and transport them to peripheral tissues, regulates the accumulation of carotenoids in the body and beak pigmentation. Here we show that T acts to upregulate lipoprotein production and allows male zebra finches to display flashy, sexually attractive coloration. Levels of circulating T in blood positively and significantly predicted lipoprotein profile (as measured by cholesterol levels), blood carotenoid concentration, and beak color. Exogenous T administration elevated cholesterol and carotenoid status as well as beak redness. Last, experimental inhibition of T (using the anti-androgen flutamide) downregulated lipoprotein production and carotenoid circulation and faded the beak. This androgen- and lipoprotein-mediated system represents one of the more detailed physiological mechanisms underlying the development of a sex-steroid-dependent trait in animals.

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Introduction

The development of gaudy sexually selected traits, like horns, antlers, songs, and dances, often comes under the control of sex-steroid hormones like androgens (reviewed in Folstad and Karter 1992; Andersson 1994). Given the recent surge of interest in how these ornaments function as honest indicators of mate quality to conspecifics (Johnstone 1995; Maynard-Smith and Harper 2003), an important challenge facing behavioral ecologists and endocrinologists alike has been to elucidate precisely how sex steroids mediate attractiveness at the physiological level. For traits like bird song, we know the specific neural pathways underlying song learning and output that are promoted by the presence of androgens and estrogens (reviewed in Schlinger 1997; Ball et al. 2002; also see Schlinger et al. 2001 for neuroendocrine and muscular control of wingsnap displays in manakins). For many other secondary sexual features, however, we understand little about the biochemical and molecular action of sex steroids that facilitate the acquisition of sexually attractive features.

The brilliant coloration of birds has become a model system for understanding how ornamental traits incur costs and reliably reveal the quality of signalers (reviewed in McGraw et al. 2002a). In many cases, their expression is also thought to be regulated by sex steroids (Ralph 1969; Kimball and Ligon 1999). Most of the flashy red, orange, and yellow colors in birds are created by the presence of carotenoid pigments (Fox and Vevers 1960; Brush 1978). Male zebra finches (*Taeniopygia guttata*), for example, enrich their beak with red carotenoids (McGraw et al. 2002b) and use bright coloration to attract mates (Burley and Coopersmith 1987; Blount et al. 2003). There are several lines of evidence suggesting that beak color is a testosterone (T)-dependent trait in *T. guttata*: (1) males have redder beaks than females (McGraw et al. 2003a), (2)

the beaks of castrated males fade, but redden when birds are then implanted with T (Cynx and Nottebohm 1992), and (3) masculinized females with sex-reversed gonads exhibit male-typical bill coloration (Adkins-Regan and Wade 2001). Other carotenoid-containing sexual colors in fish (e.g., guppies, *Poecilia reticulata*; Jayasooriya et al. 2002) and birds (e.g., house finches, *Carpodacus mexicanus*; Stoehr and Hill 2001) are also reportedly affected by T levels. Despite several investigations into the dietary restrictions and physiological underpinnings of carotenoid coloration in birds (reviewed in Hill 2002), we still do not know how T may modulate carotenoid accumulation and integumentary coloration in any species.

In previous studies, we found that dietary intake of food and pigments did not adequately explain variation in beak coloration among male zebra finches (McGraw et al. 2003a), but that levels of lipoproteins circulating through blood critically regulated carotenoid assimilation and beak pigmentation (McGraw and Parker 2005). Birds, like other vertebrates, bind carotenoids and other dietary lipids to lipoproteins, primarily high-density lipoproteins (HDL; Allen 1987), which transport them to peripheral tissues in the body (Trams 1969; Parker 1996). Based on literature showing the relationship between sex steroids and lipoproteins in humans (e.g., Brinton 1996; Büchter et al. 1999; Tan et al. 1999) and other animals, including birds (e.g., Kudzma et al. 1979), we suspected that T may control carotenoid coloration via a lipoprotein-mediated mechanism. Most notably, T has been shown to stimulate the efflux of cholesterol from cells onto HDL via transcriptional upregulation of an HDL scavenger receptor (class B1; Langer et al. 2002). Cholesterol is a critical component of the complex lipid- and protein-rich lipoprotein particle (Davis 1997) and strongly predicted carotenoid status and beak color in our previous work (McGraw and Parker 2005).

Therefore, we tested for relationships between T, lipoproteins, carotenoids, and bill color in male zebra finches. Zebra finches are opportunistic breeders and maintain their beak ornaments throughout their adult life (Zann 1996). In an initial correlational study, we drew blood and scored beak color to compare circulating levels of T with cholesterol concentration, carotenoid status, and beak ornamentation in prebreeding adult males. In follow-up experiments, we first implanted Silastic capsules filled with T into males to determine how elevated T levels affect lipoprotein and carotenoid profiles and beak redness. We then implanted a different set of males with capsules filled with flutamide, a nonsteroidal T-receptor blocker, to see if we could lower systemic cholesterol levels and consequently reduce carotenoid concentration in the blood and beak.

Methods

Correlational study

We measured plasma T, plasma cholesterol, plasma carotenoids, and beak color in a captive, nonbreeding population of 13 males (see McGraw et al. 2003a for housing conditions and diet). Bleeding one bird per day for 13 consecutive days, we collected 80–120 μl of blood from each male through the alar vein into heparinized microcapillary tubes and centrifuged the blood to remove and retain the plasma for cholesterol, carotenoid, and T analyses. Cholesterol levels must be determined from fresh, unfrozen plasma; so within 4 h of collection, we used commercially available kits (Polymedco, Inc., CHO-200 total cholesterol assay, Cortlandt Manor, NY) to quantify plasma cholesterol concentration from 10 μl aliquots of plasma (the rest was frozen for later carotenoid and T analyses). For details of this procedure, see McGraw and Parker (2005).

Plasma T titers were determined using radioimmunoassay (RIA) following the methods of Stoehr and Hill (2000). Thawed plasma (50 μl) was incubated with approximately 1,000 cpm of tritiated testosterone (NEN, Boston, MA) for 1 h before extraction with diethyl ether. Samples were then evaporated to dryness under a stream of nitrogen, resuspended in phosphate buffer, equilibrated at room temperature for 1 h, and then incubated in duplicate with tritiated testosterone and a testosterone antibody (Endocrine Sciences, Casablanca, CA) for 4 h at room temperature. Assay sensitivity was 10 pg/ml, intraassay variation averaged 11%, and average percentage recovery was 75%. The testosterone antibody used cross-reacts (44%) with dihydrotestosterone, thus making the assay technically a total androgen assay. High-performance liquid chromatography (HPLC) methods used to analyze plasma carotenoids and reflectance spectrophotometry procedures used to quantify beak coloration follow those in McGraw et al. (2003a).

Experiment I: T administration

We performed this experiment with a different captive finch population of 17 males. On the day before the experiment began, we drew blood from all males and scored beak color to determine preexperimental levels. We then randomly selected nine males to receive 10 mm Silastic capsule implants packed full with crystalline testosterone propionate (Sigma Chemical Co., St. Louis, MO) and sealed at both ends with 1 mm of Silastic adhesive. We chose an implant of this size based on previous work in this species (Bottjer and Hewer 1992; Balthazart et al. 1994). Birds were first given 50-μl injections of Lidocaine to numb the site of implantation, which was in the left-flank region, just above the hip and below the wing. We inserted implants subcutaneously, as far posterior as possible, and sealed the skin with surgical glue. Birds were returned to housing cages within 30 min. The same treatment was administered to the eight birds in the control group, except that they received empty implants.

Implants were left in for 8 weeks, at which time we scored beak color and sampled blood for cholesterol, carotenoid, and T analyses. Upon removing the implants, we found that 50–90% of T had dissolved from the capsules; in no case were any of the T-filled capsules empty.

We also measured body mass for all males before and after the experiment to examine the effect of elevated T on nutritional state.

Experiment II: T-receptor blocking

We performed this experiment on a third group of 13 males. Seven randomly chosen males received 10 mm Silastic implants filled with crystalline flutamide (Sigma Chemical Co.) for 6 weeks, whereas the remaining six males received empty implants. All other procedures follow those above in Experiment I, except that we did not measure T levels posttreatment since flutamide works not by lowering systemic T levels but by blocking T receptors.

Results

Correlational study

In three prior studies, we have shown that the concentration of carotenoids circulating through blood is significantly correlated with beak hue in male zebra finches (McGraw et al. 2003a; McGraw and Ardia 2003; McGraw and Parker 2005). We found this same pattern in our captive flock of 13 males (Pearson's correlation, r=−0.53, P=0.02; note that redder birds receive lower hue scores, so the correlation is negative). In addition, like our previous study on cholesterol in *T. guttata* (McGraw and Parker 2005), we found that cholesterol levels in plasma were predictive of plasma carotenoid concentration (r=0.6, P=0.007) and beak hue (although not significantly so, r=0.43, P=0.10). Here, we found that T titers in plasma were significantly correlated with all three of these parameters: plasma cholesterol (Fig. 1a), plasma carotenoids (Fig. 1b), and beak hue (Fig. 1c).

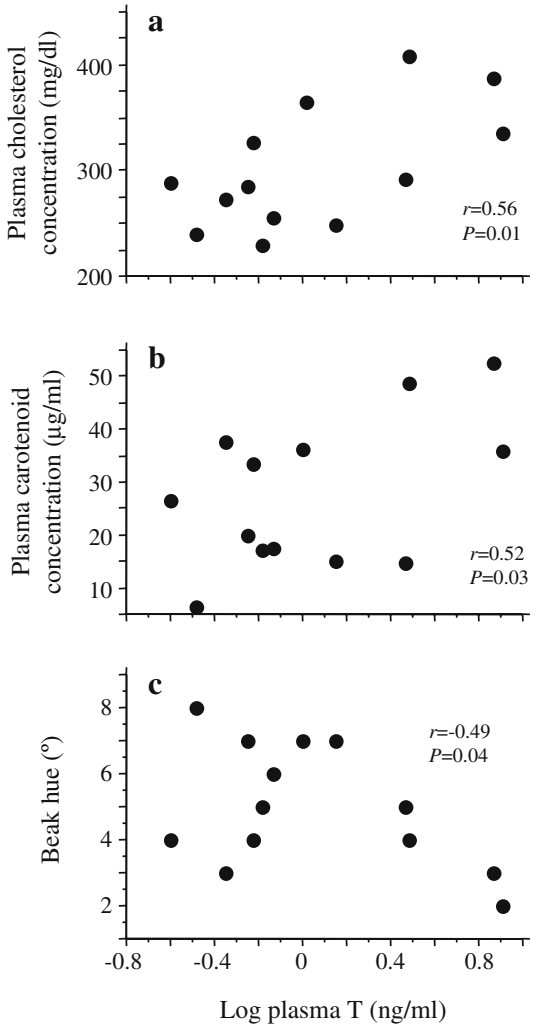

Fig. 1 Correlations between the levels of circulating T (log-transformed to meet the assumption of normality) and **a** plasma cholesterol concentration, **b** plasma carotenoid concentration, and **c** beak hue in captive male zebra finches (n=13 in each). Note in **c** that redder birds are assigned lower hue scores, based on the arbitrary assignment of degrees by the Colorshop software around a 360° color wheel (see McGraw et al. 2003a for details)

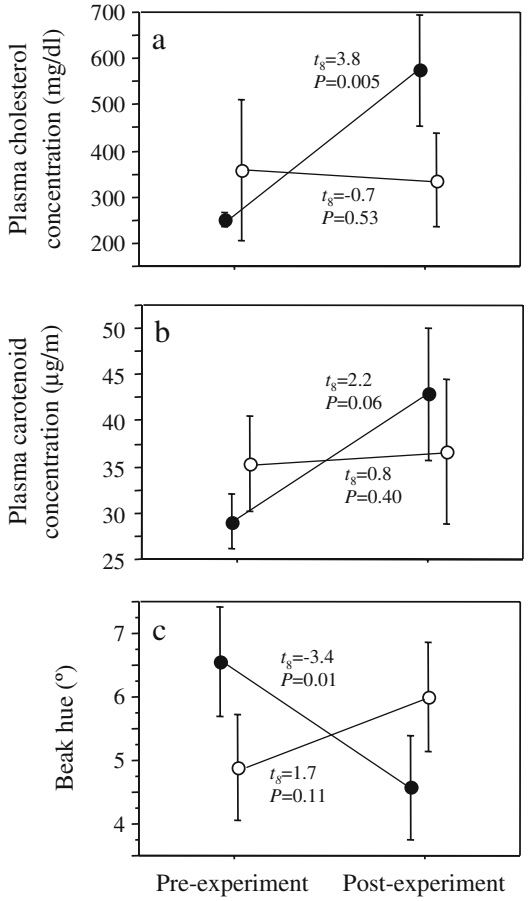

Fig. 2 Effect of 8-week exogenous T administration (via subcutaneous Silastic capsule implants) on **a** cholesterol circulation, **b** blood carotenoid status, and **c** carotenoid-based sexual coloration in male zebra finches. *Dark circles* denote T-implanted birds, whereas *open circles* signify sham controls. We used paired t tests to investigate changes in each of these response variables within treatment groups. Mean±SEM are shown; recall that n=9 for the T-administered group and n=8 for the control group in all panels

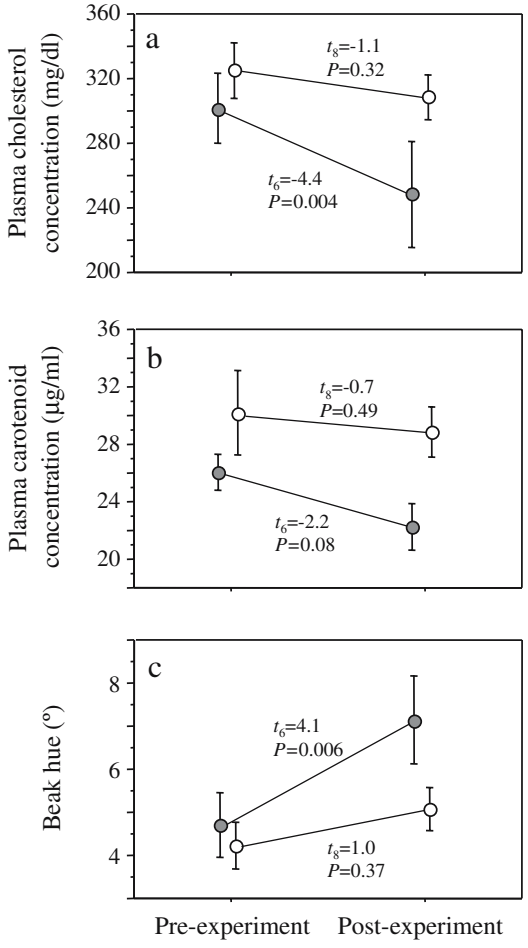

Fig. 3 Effect of 6-week flutamide treatment (an antiandrogen) on **a** plasma cholesterol levels, **b** plasma carotenoid concentration, and **c** beak hue in male zebra finches. Here, *gray circles* denote flutamide-implanted birds, and *open circles* signify controls. See Fig. 2 for more information on the analyses and plots; n=7 for the flutamide group and n=6 for the control group

Experiment I

Pretreatment plasma T concentrations ranged from 0.25 to 8.2 ng/ml, with a mean±SEM of 2.1±0.7 ng/ml (similar to that in Adkins-Regan et al. 1990). After 8 weeks of treatment, T implants elevated circulating levels by 81% (3.8±1.2 ng/ml) (again like those in Adkins-Regan et al. 1990). Before the experiment, T-implanted and control males did not differ in body mass, beak color, or levels of T, cholesterol, and carotenoids (unpaired t tests, all P>0.15). T implants, however, induced a significant, 130% increase in plasma cholesterol levels (Fig. 2a), a 53% increase in plasma carotenoid concentration (Fig. 2b), and an increase in beak redness of nearly 2 hue units (Fig. 2c). Birds with empty implants showed no significant changes in any of these measures (Fig. 2). There were no significant directional changes in body mass in either group (paired t tests, both P>0.3).

Experiment II

Again, treatment and control groups did not differ in body mass, beak color, or levels of T, cholesterol, and carotenoids in blood before the study, nor did they differ in body mass after the experiment (unpaired t tests, all P>0.2). As predicted, our 6-week flutamide treatment significantly lowered cholesterol levels (by 18%, Fig. 3a) and carotenoids levels (by 14%, Fig. 3b) and faded the beak (by over 2 hue units; Fig. 3c), whereas these measures did not change significantly in control males (Fig. 3).

Discussion

We tested the hypotheses that (1) beak color is a T-dependent trait in zebra finches and (2) the mechanism by which T affects carotenoid pigmentation is by the upregulation of lipoproteins that transport these lipid-soluble pigments through blood. We found correlational and experimental support for both hypotheses. First, beak color was correlated with circulating T levels in prebreeding captive adult males, was reddened by elevating systemic T levels, and faded when we administered a T blocker. To our knowledge, this is the third songbird species for which there is experimental evidence showing that T elevates male carotenoid-based ornamental coloration [American goldfinches (*Carduelis tristis*), Mundinger 1972; European starling (*Sturnus vulgaris*), Witschi 1961; but see Stoehr and Hill 2001 in house finches]. Other types of avian pigment-based ornaments, such as black melanic coloration (Ros 1999; Evans et al. 2000; Gonzalez et al. 2001) and hemoglobin-filled wattles and combs (Zuk et al. 1995), are similarly known to deepen in color when T levels are high.

Based on our previous work on the physiological mechanisms of sexual coloration in male zebra finches (McGraw et al. 2003a; McGraw and Parker 2005), we also sought to determine exactly how T endogenously controls beak redness. Several hypotheses have been advanced to explain the physiological demands of T as they relate to sexual signaling in animals (see more below), but specifically for carotenoid coloration, we were interested in the extent to which T affected lipoprotein profiles, since these particles bind carotenoids in blood circulation and are limiting agents for carotenoid accumulation and coloration in this species (McGraw and Parker 2005). Our findings here reveal that T can exert strong control over lipoprotein status to ultimately mediate carotenoid pigmentation. This occurs specifically via T-dependent regulation of cholesterol (see McGraw and Parker (2005) for justification of the role of cholesterol as an important component of lipoproteins). Plasma cholesterol levels were positively correlated with T and carotenoid concentrations in the blood and beak. Cholesterol and carotenoid levels were elevated in T-implanted birds but lowered in birds given a T-receptor inhibitor. This represents one of the most detailed physiological mechanisms underlying the expression of a sexually selected, androgen-dependent trait in animals.

The cellular mechanism by which T upregulates cholesterol in zebra finches is unclear from our results, but based on previous in vitro work with human cell lines, it is possible that it occurs by a similar process of reverse cholesterol transport (Langer et al. 2002). T upregulates scavenger receptor class B type 1 (SR-BI) in hepatocytes and macrophages, which consequently induces cholesterol efflux onto HDL (Mizutani et al. 2000). Other hormones like estradiol, adrenocorticotrophic hormone, and luteinizing hormone are also confirmed regulators of SR-BI gene expression (Landschulz et al. 1996; Rigotti et al. 1996). However, there is a second means by which T may increase systemic cholesterol levels—via the mobilization of fat stores. T raises basal metabolic rate in several animals, including colorful songbirds (e.g., house sparrows, *Passer domesticus*; Buchanan et al. 2001), and increases fat metabolism (Wingfield et al. 2001; Bhasin et al. 2003). Since fat is rich in cholesterol (Angel and Farkas 1974), high T titers may free once-stored cholesterol for incorporation into lipoproteins. Incidentally, adipose tissue also typically contains high concentrations of carotenoids (Negro et al. 2001; see McGraw et al. 2002b for evidence in zebra finches). Thus, in future work, it will be valuable to distinguish between these two potential T-dependent mechanisms for cholesterol modulation. We also cannot rule out the possibility that T is being reduced to dihydrotestosterone and that this derivative is exerting the potent receptor-mediated effects on lipid profile and color that we observed here.

In addition, regarding the relationship between cholesterol and T, it is important to note that cholesterol serves as the substrate for the production of steroid hormones like T (Nelson 2000). Thus, preliminarily, one might suspect an inverse causal relationship between cholesterol and T than the one elucidated here, namely, that cholesterol levels should affect T. However, there is good evidence that cholesterol availability per se does not limit T production, but instead that the activity of steroidogenic enzymes (e.g., cytochrome P450s) are rate-limiting for T synthesis (Black et al. 1994). In human clinical trials, for example, administration of cholesterol-lowering drugs has no effect on circulating T (Dobs et al. 2000; Ushiroyama et al. 2001). Ultimately, we anticipate that T unidirectionally influences cholesterol, and not vice versa, to control carotenoid-based sexual attractiveness in *T. guttata*.

An important challenge that remains in this line of work and others on androgen-mediated secondary sexual characteristics is identifying how and why T is costly to elevate or maintain. If these ornamental features are in fact reliable signals of an individual's value as a mate, then what factors prevent low-quality males from elevating T and developing brilliant colors? Much of the emphasis in the literature on the honesty-reinforcing mechanisms of T has been placed on the potential detrimental effects of T on the immune system (Folstad and Karter 1992). However, there are other ways that sex steroids may physiologically impair the bearers of exaggerated signals. In the case of carotenoid coloration, it is tempting to speculate about the fitness consequences of T-driven elevated cholesterol levels, given the body of work on the link between cholesterol and arteriosclerosis in humans (Gordon and Rifkind 1989; Campeau et al. 2005). Perhaps, only the highest quality male zebra finches can bear the health costs of high systemic cholesterol to acquire rich red beak coloration. Alternatively, T levels may come under social control and respond to the intensity of aggressive and submissive behaviors experienced by individuals (Zuk and Johnsen 2000; Setchell and Dixson 2001; Parker et al. 2002; McGraw et al. 2003b). We are currently testing both the physiological and social costs of elevated T and beak coloration in male zebra finches.

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