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Physiology & Behavior 87 (2006) 103 – 108

PHYSIOLOGY & BEHAVIOR

A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colorful songbird

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Received 5 July 2005; received in revised form 24 August 2005; accepted 5 September 2005

Abstract

Sexually selected traits like complex vocalizations or vibrant colors communicate reliable information about mate quality when they are costly to display. Although several general condition-dependent mechanisms underlying the acquisition of mating advertisements have been identified, we rarely know the precise physiological and molecular challenges that animals must meet to develop their sexual ornaments. The flashy pigment-based colors commonly displayed by birds are ideal candidates for investigating the pathways and demands of sexual-signal expression, because we know the biochemical currency with which the trait is produced. Carotenoid colors in birds, for example, are derived from pigments that are acquired from the diet and assimilated into feathers and bare parts. In previous work, we showed that variation in the sexually attractive red carotenoid-colored beak of male zebra finches *(Taeniopygia guttata*) was predicted not by the amount of food or pigments ingested, but by the levels of carotenoids that birds circulated in blood. Here we elucidate a novel physiological mechanism by which birds are able to accumulate high levels of carotenoids in the body and develop a colorful bill. Carotenoids are transported through the bloodstream bound to lipoproteins. We assayed a critical component of lipoprotein particles – cholesterol – and found that males with higher cholesterol levels circulated more carotenoids and displayed redder beaks. Experimental supplementation of dietary cholesterol elevated carotenoid levels in the blood and beak hue. Experimental reductions in blood cholesterol, using the human lipid-lowering agent atorvastatin, diminished blood carotenoids and faded the beak; carotenoid and cholesterol levels were restored, however, by subsequent addition of dietary cholesterol. These results suggest that the production of circulating lipoproteins critically regulates the development of a colorful sexually selected trait in zebra finches.

Keywords: Carotenoid pigmentation; Ornamental coloration; Sexual selection; *Taeniopygia guttata*; Zebra finch

1. Introduction

Honest advertisement models of sexual selection predict that traits conferring mating advantages must be costly to produce or maintain [1]. This results in a reliable signaling system because only the highest-quality individuals can incur the full costs of signal elaboration [2]. There are countless studies in which sexually selected features are argued to incur a physiological cost, such as a high energetic requirement or health maintenance [3,4]. In many of these instances, however, the exact biochemical or cellular demands that must be met in order to exaggerate sexual features are unknown.

To explicitly investigate the intrinsic control-agents of ornamental features, we must concentrate on systems in which we know the molecular components of a signal and can track the pathways down which these molecules travel. The carotenoid-based colors of animals are an ideal model. Many fishes and birds develop bright patches of red, orange, and yellow color using carotenoid pigments [5,6]. These carotenoid-derived colors are commonly used by male birds to attract or compete for female mates [7,8]. Vertebrates cannot synthesize carotenoids de novo, so they must ingest large amounts of carotenoid-enriched plant matter or herbivorous prey to become colorful [9,10]. However, once ingested, carotenoids must be extracted from food, delivered to peripheral locations in the body through the bloodstream, and

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doi:10.1016/j.physbeh.2005.09.001

taken up by colorful tissues for pigmentation [11]. Thus, beyond nutritional access, there is a series of physiological steps that may limit or enhance the expression of sexually attractive carotenoid coloration.

Adult male zebra finches *(Taeniopygia guttata)* display a bright red beak that is colored by carotenoids [12] and serves as a sexually selected trait [13,14]. In a previous study, we found that interindividual variation in beak coloration was not explained by levels of food and carotenoid intake, suggesting an important physiological component to trait expression [15]. Instead, it was the levels of carotenoids that birds accumulated in blood that significantly predicted the intensity of beak pigmentation [15]. Thus, we focused on carotenoid transport as a regulatory mechanism in this study.

After assimilating carotenoids from food in the small intestine, birds and other vertebrates bind carotenoids to lipoproteins that deliver lipids to peripheral tissues in the body [16,17]. Animals assemble lipoproteins in the liver and small intestine from a variety of lipids (e.g., triglycerides, phospholipids, cholesterol) and apolipoproteins [18]. There is a large body of literature on human nutrition that demonstrates the relationship between lipoprotein status and carotenoid concentration in the blood [19]. Under both observational and experimental conditions, people with higher levels of plasma lipoproteins are better able to take up carotenoids from the diet and circulate more carotenoids through blood [20,21]. Thus, we hypothesized that lipoprotein status in birds may be a critical control mechanism for the delivery of carotenoids to colorful tissues and ultimately the sexual attractiveness of males.

In this study, following previous work with humans [22–24], we monitored and manipulated a critical backbone component of the complex, lipid-rich plasma lipoprotein particle- cholesterol- to determine how lipoprotein profiles covary with carotenoid status in male zebra finches. In herbivores like seed-eating finches, cholesterol is absent from the diet and is instead manufactured endogenously [18]. Interestingly, in a study of chickens (*Gallus gallus domesticus*), the addition of cholesterol to the diet increased both plasma-lipoprotein and carotenoid levels [25]. First, we drew blood from non-breeding males to determine the relationship between plasma cholesterol, plasma carotenoids, and beak coloration. Next, we conducted separate experiments in which we manipulated cholesterol levels, either using dietary supplementation or drug administration (to lower systemic cholesterol), to test the degree to which plasma lipoprotein status mediates both carotenoid circulation and sexually attractive beak pigmentation.

2. Materials and methods

All males studied here were housed in small hardware cloth cages in an animal-approved indoor room on the campus of Cornell University (see [15] for more details). Birds were fed an ad libitum diet of water and Kaytee® Forti-Diet™ finch blend (Kaytee Products, Chilton, Wisconsin; see [15] for seed components).

2.1. Correlational study

We measured plasma cholesterol, plasma carotenoids, and beak color in two captive populations (#1 = 20 males, #2 = 14 males) at two different times (10 November and 28 December 2001, respectively). 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 in 1.5 mL screw-cap Eppendorf tubes for cholesterol and carotenoid 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 analyses). This assay employs heat-induced enzymatic hydrolysis and oxidation to form a colorful indicator of cholesterol that can be quantified with an absorbance spectrophotometer. In a fresh Eppendorf tube, we added 1 mL of cholesterol reagent to 10 μL plasma, vortexed the tube for 3 s, and incubated at 37 °C for 5 min. We then removed the tube from the water bath and transferred the contents to a clean cuvette to determine the absorbance of the solution at $\lambda = 500$ nm with an absorbance spectrophotometer (Bausch and Lomb Spectronic 1001). Total cholesterol concentration of samples was determined by comparison to external cholesterol standards provided by Polymedco. Inc. In pilot tests, this procedure was found to be highly repeatable (*sensu* [26]; $R_i = 0.99$, $F_{9,10} = 189.2$, $P < 0.0001$).

High-performance liquid chromatography (HPLC) methods used to analyze plasma carotenoids (in μg/mL) and reflectance spectrophotometry procedures used to quantify beak coloration (measured as hue) follow those in [15].

2.2. Experiment I: dietary cholesterol supplementation

We performed this experiment with captive finch population #2, along with 6 other males that we added to the group in January 2002. On the day before the experiment began, we drew blood from all males and scored beak color to determine pre-experimental levels. For the next three weeks, 10 randomly selected males were fed the baseline seed diet (as controls), whereas the other ten were fed seeds enriched with 2% powdered cholesterol (Sigma Chemical Co., St. Louis, MO). Cholesterol powder was first sprinkled onto the seeds and then thoroughly mixed in by shaking in a sealed Tupperware container. This experimental dosage and time-course follow those used previously to elevate blood cholesterol in chickens [25]. Pilot tests ($n = 5$ males) showed that this dose nearly doubles blood cholesterol levels in zebra finches over the course of 3 weeks (pre-treatment mean = 479 ± 171 mg/dL; post-treatment = 900 ± 119 mg/dL) and keeps them within the natural range of variation (pre-treatment range: 125–1300 mg/dL; post-treatment: 300–1300 mg/dL; also see Results). After the three weeks, we again sampled blood for cholesterol and carotenoid analyses and scored beak color. We also measured body mass for all males before and after the experiment to

examine the effect of cholesterol supplementation on the general nutritional state of males.

2.3. Experiment II: statin administration

We performed this experiment with finch population #1. Again, on the day before the experiment began, we drew blood from all males and scored beak color to determine pre-experimental levels. For 4 weeks, 10 randomly selected control males were again fed only seeds, whereas the other ten were fed seeds enriched with 0.012% atorvastatin. Atorvastatin is the active, synthetic ingredient in the lipid-lowering agent Lipitor® (Pfizer Inc., Groton, CT), which is an effective drug for lowering cholesterol levels in humans [27]. Atorvastatin, like other statins, reduces blood cholesterol by inhibiting the rate-limiting enzyme that catalyzes cholesterol biosynthesis [28]. Like the cholesterol diet in Experiment I, atorvastatin was delivered as a powder to finches and mixed with seeds. The dose and time-course of the drug treatment are derived from studies of statin use and cholesterol in chickens [29], except that we chose a more modest dose in our study (compared to 0.03% and 0.06% atorvastatin in [29]) because of the dramatic 60% reduction in plasma cholesterol that these authors observed from their treatment. Although even the higher atorvastatin doses used in [29] were not harmful to the birds (e.g., did not impair food intake or liver function), we still measured body mass before and after the study. After the five weeks, we again obtained a blood sample and scored bill color to determine the effect of statin treatment on plasma cholesterol and carotenoids and beak pigmentation. Also, to be sure that the drug was depressing carotenoid status via the predicted mechanism of cholesterol depletion, we extended our experiment by 2 weeks and fed half of the males in the statin-treated group supplemental cholesterol (as in Experiment I above) to see if we could halt or restore depressed cholesterol and carotenoid levels in these birds.

3. Results

3.1. Correlational study

In two earlier studies, we found a significant positive correlation between beak coloration and plasma-carotenoid concentration in separate captive populations of male zebra finches [15,30]. We confirmed this relationship in both groups of birds used in this study (population #1: $r=-0.47$, $n=20$, $P=0.03$; #2: $r=-0.54$, $n=14$, $P=0.04$). Also, the levels of all four main carotenoids found in blood (lutein, zeaxanthin, 2′,3′-anhydrolutein, and β -cryptoxanthin; [12]) were highly positively intercorrelated (all $r>0.77$, all $P<0.0007$).

In both groups of males, total carotenoid concentration in blood was significantly positively correlated with total plasma cholesterol concentration (Fig. 1A). Birds with higher cholesterol levels circulated more of all the main plasma carotenoids (Pearson correlation, $r>0.45$, $P<0.05$ for each of the four pigments). Cholesterol levels in blood also directly predicted beak color in both captive populations (Fig. 1B). These

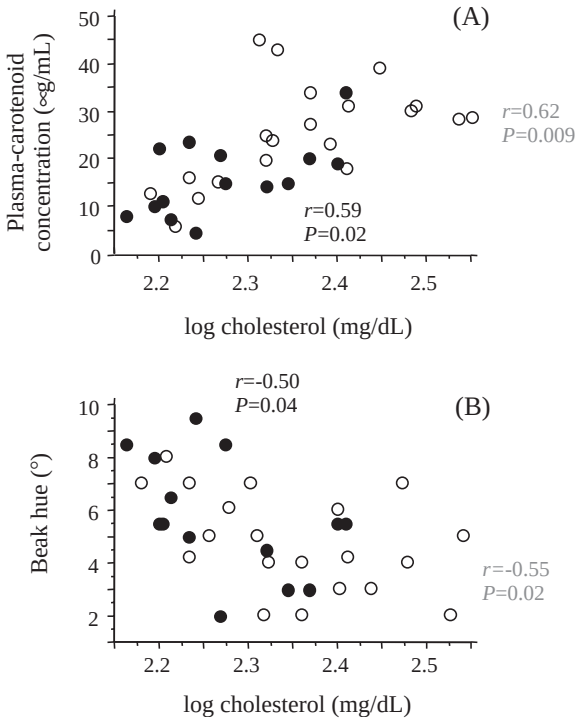

Fig. 1. Relationship between levels of cholesterol in blood (log-transformed to meet the assumption of normality) and (A) concentration of plasma carotenoids and (B) beak hue (as described in [30]; note that redder birds have lower hue scores) in male zebra finches. Open circles denote data for captive population #1 and black circles signify population #2.

relationships between cholesterol, carotenoids, and color held up not only in these analyses, but also when we compared these variables again in the same birds just prior to participating in their respective experiments (data not shown).

3.2. Experiment I

Treatment groups did not differ in beak hue, plasma-carotenoid concentration, or plasma-cholesterol levels before the study (unpaired t-tests, all $P>0.35$). After the experiment, cholesterol-supplemented (CS) birds circulated significantly more cholesterol in blood than controls (C); plasma cholesterol increased by 97% in CS males, whereas C birds showed a small but statistically non-significant 8% decline in cholesterol (Fig. 2A). CS males also circulated significantly more carotenoids through blood (a 75% increase; Fig. 2B) and acquired redder beaks (a shift of 1.75 hue units; Fig. 2C). As found in chickens [25], diet did not affect group differences or changes in body mass during the study (all $P>0.6$).

3.3. Experiment II

Atorvastatin-treated (AT) and control (C) groups did not differ significantly in beak hue, plasma-carotenoid concentration, or plasma-cholesterol levels before the experiment (unpaired t-tests, all $P>0.1$). Atorvastatin treatment reduced circulating cholesterol levels by 19% in AT males during the four-week experiment, whereas C finches showed a less than 1% increase in plasma cholesterol (Fig. 3A). Blood-carotenoid

levels also declined by 19% during this time in AT males, but increased (though not significantly) by 13% in controls (Fig. 3B). The beaks of AT finches also faded significantly (by 1.5 hue units), whereas C birds remained unchanged (Fig. 3C).

To determine whether we could prevent carotenoid depletion and beak fading in AT males, we administered a 2% dietary cholesterol supplement to five randomly selected AT birds for 2 weeks after the initial 4-week experiment. Though these birds were still receiving atorvastatin, cholesterol replacement effectively restored plasma-cholesterol levels, and in fact elevated them well above baseline (Fig. 3A). In turn, plasma-carotenoid levels recovered to above-baseline levels (18% above pre-experimental values) (Fig. 3B). Beak color reddened in the predicted direction, but not significantly so (Fig. 3C), perhaps due to the fact that beak tissue is replaced slowly and is likely to change color over a longer time-course (e.g., 4 weeks; [14,30]).

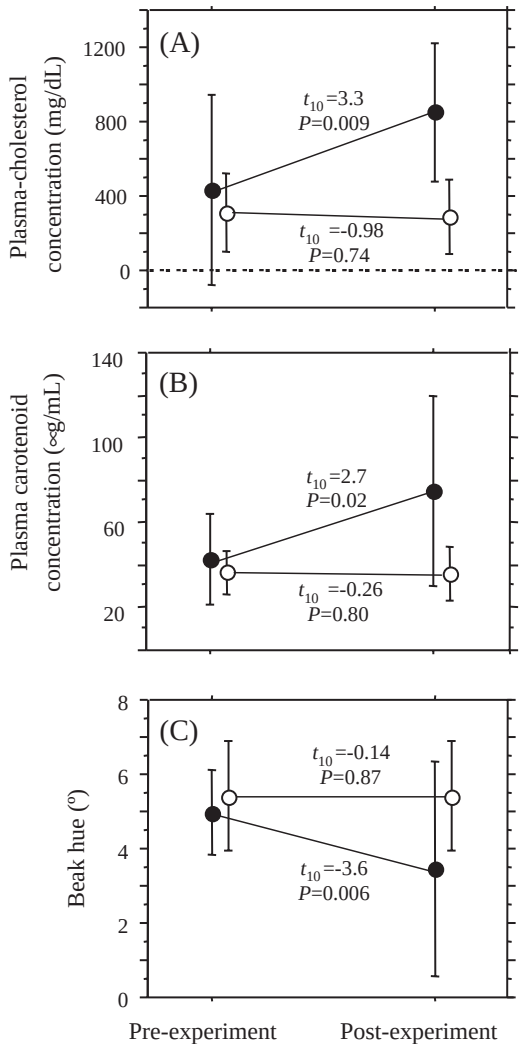

Fig. 2. Effect of cholesterol supplementation on (A) plasma-cholesterol levels, (B) plasma-carotenoid concentration, and (C) beak hue in male zebra finches. Dark circles=cholesterol-supplemented males; open circles=control males. We used paired *t*-tests to examine changes in each of these response variables within treatment groups. Mean ± s.d. shown; recall that $n=10$ males for both groups.

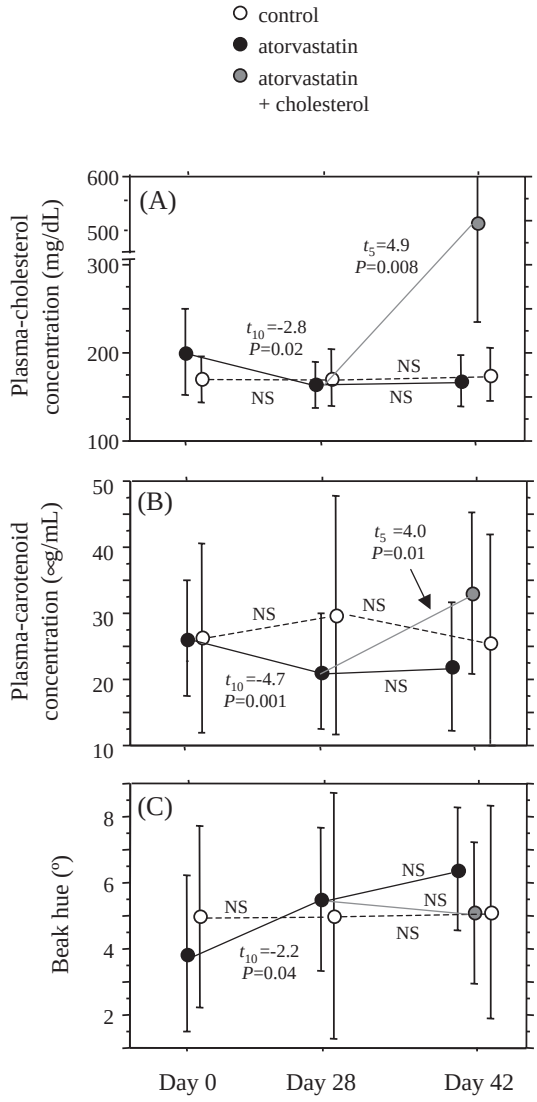

Fig. 3. Effect of atorvastatin treatment (a cholesterol-lowering drug) on (A) plasma-cholesterol levels, (B) plasma-carotenoid concentration, and (C) beak hue in male zebra finches. Blood was sampled and beaks were scored at three time points: (1) pre-experiment (day 0), (2) post-atorvastatin-treatment (day 28), and (3) after half of the atorvastatin-treated birds received cholesterol replacement (through the diet) for two subsequent weeks (day 42). Again, means ± s.d. are shown, $n=10$ males per group, and paired *t*-tests were used to examine changes in each of these response variables within treatment groups.

As found in chickens [29], body mass did not differ or change significantly among any of the groups at any of the three sampling points during the 6-week study (all $P>0.05$). Incidentally, these researchers also noted that the blood plasma of statin-treated chickens was pale in comparison to the yellow-pigmented appearance of controls [R. Elkin, pers. comm.].

4. Discussion

We investigated the extent to which cholesterol – an important component of lipoproteins in animals – mediates carotenoid status in the bloodstream and the expression of carotenoid-based beak coloration in male zebra finches. In humans, lipoproteins are touted as molecules that regulate the uptake and transport of lipids like carotenoids in the body

[31,32], but this idea has never before been applied to the regulatory mechanisms of ornamental carotenoid coloration in animals. The only relevant study of which we are aware in birds showed that a mutant strain of chicken (Wisconsin hypoalpha mutant, or WHAM) that is 90% deficient in plasma high-density lipoprotein (HDL) develops white, carotenoid-lacking skin and beaks as opposed to the usual carotenoid-rich yellow color [33]. Here, we found that blood cholesterol levels were highly positively correlated with both plasma-carotenoid concentration and beak pigmentation. Our experimental studies also revealed that (a) blood carotenoids and beak color can be elevated with dietary cholesterol supplements, and (b) administration of a drug that inhibits cholesterol biosynthesis can lower blood carotenoid levels and fade beak color. Collectively, our results suggest that the availability of circulating lipoproteins regulates the development of a colorful sexually selected trait in zebra finches.

Two critical assumptions of this study, like others in humans on carotenoid transport, are that (1) blood cholesterol is a reliable proxy for lipoprotein concentration and (2) experimental manipulations of cholesterol effectively alter lipid delivery by lipoproteins. Lipoproteins are complex lipid- and protein-containing particles, and it is not always clear which of the components should be measured or manipulated to deduce lipid-transport capabilities [18]. There are two lines of evidence that support these assumptions, however. First, as indicated above, when multiple components of the lipid status of humans are considered, blood cholesterol levels are among the best predictors of blood-carotenoid concentration [22–24]. Second, in WHAM chickens that lack carotenoids in blood, it was found that blood cholesterol levels in HDL (high-density lipoprotein, the main lipoprotein carrier of carotenoids in birds; [16,34]) were diminished by over 99% [33], whereas synthesis of the principle HDL protein, apoA-I, was unaffected [35]. Thus, it likely is something unique about cholesterol in lipoproteins that factors critically into the control of carotenoids and color in zebra finches.

We hypothesize that the degree to which animals can manufacture lipoproteins may be a costly physiological process and helps keep carotenoid-based sexual colors honest. From the results of our study, we cannot infer whether the rate-limiting step for lipoprotein production is cholesterol biosynthesis or construction of the lipid-protein emulsion. However, the specific proteins, not the lipids, that are embedded into the surface monolayer largely function in assembling the lipoprotein [18], which again points to a lipid- and cholesterol-specific control mechanism here. Cholesterol is a molecule that serves several valuable physiological functions in animals, including as a structural component of cell membranes and as substrate for bile-acid and steroid synthesis [36]. Its levels are tightly regulated internally with respect to an animal's nutritional state [18,37]. Enzyme activity (HMG-CoA reductase) is rate-limiting for cholesterol biosynthesis and energy is required to drive these reactions [38]. Stressed animals with higher glucocorticoid levels show depressed HMG-CoA activity and lower cholesterol levels [39]. Last, the publicized link between cholesterol and arteriosclerosis also makes it an attractive molecule when considering honesty reinforcement of this signal; though we are still learning about the health consequences of elevated cholesterol in birds [40], one could envision the differential health-related consequences of cholesterol elevation, such that only the highest-quality individuals can bear the survival costs of elevating cholesterol to acquire rich red beak coloration. In sum, there are several possible physiological mechanisms and trade-offs through which cholesterol is difficult or expensive to elevate for maximal ornament expression.

Although our study has exposed a new physiological mechanism controlling carotenoid-based ornamental coloration, by no means should it be considered the only one. Past studies of the governing mechanisms and signaling function of carotenoid colors in animals have emphasized the role that dietary carotenoid access plays in mediating trait expression [9,10] as well as the role of 'nutritional or health state', as measured by factors such as feather growth, food intake, or parasite burden [41–44]. What our study adds to these is a more specific pathway to focus on for uncovering the series of challenges that animals face to becoming maximally colorful. In fact, many of these previously touted mechanisms for carotenoid coloration may feed through lipoprotein status. For example, reduced food intake (independent of carotenoid intake) impairs carotenoid transport in American goldfinches *(Carduelis tristis*; [45]), and parasitic infections like coccidiosis in chickens are known to disrupt lipoprotein circulation [34]. Now that we have a cursory understanding of the important components of carotenoid use in colorful animals, the task will be to more carefully document how general phenotypic and nutritional/health manipulations explicitly alter these steps along the pathway toward carotenoid accumulation and sexual attractiveness.

Acknowledgments

We thank M. Bacon of Cornell's Office of Sponsored Programs and D. Owens of Pfizer Inc. for assistance with acquiring atorvastatin, E. Adkins-Regan for use of her finch colonies and input on experimental design, T. van Deusen, D. Sheils, and P. Smith for animal care, V. Johnson at Polymedco Inc. for help with cholesterol assays, E. Mackillop for administering dietary cholesterol, and D. DeNardo, G. Walsberg, and an anonymous referee for helpful comments on the manuscript. KJM was financially supported by a graduate STAR fellowship from the Environmental Protection Agency and by the School of Life Sciences and College of Liberal Arts and Sciences at Arizona State University. All procedures reported here were approved by the Institutional Animal Care and Use Committee at Cornell University (protocol # 99-89).

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