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Dietary mineral content influences the expression of melanin-based ornamental coloration

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Many animals develop bold patches of black or brown coloration that are derived from melanin pigments and serve as sexual or social signals. At present, there is much debate among behavioral ecologists over whether melanin-based color signals are costly to produce. Studies that have manipulated crude aspects of nutrition (i.e., total food intake) or health have generally found melanin-based plumage ornaments to be less responsive to such factors than other types of extravagant color (e.g., carotenoid or structural based). However, a recently advanced hypothesis argues that limited minerals in the diet, such as calcium (Ca), zinc (Zn), and iron (Fe), may serve to increase melanin pigment production and maintain signal honesty. Here, I experimentally tested whether variation in the calcium content of the diet affects the color and extent of melanin-based plumage in male zebra finches (*Taeniopygia guttata*). Calcium supplementation increased the size, but not darkness, of the black breast plumage patch in fledgling and adult males; however, sexually selected, carotenoid-based red beak coloration was not affected by the diet manipulation. These results are the first to support the idea that acquisition of minerals from the diet is a unique, limiting factor for the expression of ornamental melanin coloration in animals. *Key words:* calcium, honest signaling, plumage pigmentation, *Taeniopygia guttata*, zebra finch. *[Behav Ecol 18:137–142 (2007)]*

The striking colors of birds have emerged as model systems for studying the information content and value of social and sexual signals in animals (Hill and McGraw 2006a,b). Colors provide ideal subjects for investigating the costs and benefits of signal elaboration because, easier than most sexual traits, we can "reverse engineer" the molecules and processes involved in constructing a color. Pigments and microstructures are the 2 fundamental mechanisms via which animals produce colors, and because of the different factors that go into pigmenting tissue with different molecules or structurally arranging tissue components to scatter light, a main theme in recent work on the control and function of bird colors is that different types of color may be used to communicate different information about the quality of the bearer (e.g., Gray 1996; Owens and Hartley 1999; McGraw and Hill 2000; Senar et al. 2003).

Carotenoid-, melanin-, and structural-based colors are the most common colors used as social or sexual signals by vertebrates and thus have received much of the attention by behavioral ecologists of late. Carotenoid pigments are derived from food and play important health roles (Lozano 1994; Olson and Owens 1998; Hill 1999; Møller et al. 2000), and it is clear from several studies in several bird species that carotenoid-based colors typically reveal the nutritional and immunological state of individuals (reviewed in Hill 2006). The honesty-reinforcing mechanisms of ornamental structural colors have historically received comparatively less attention, but at present, the evidence suggests that they also are consistently sensitive to nutrition and health factors (e.g., Keyser and Hill 1999; Doucet 2002; McGraw et al. 2002; Doucet and Montgomerie 2003a,b; Hill et al. 2005; Siefferman and Hill 2005; Costa and Macedo 2006).

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In contrast, there has been much less support for, and more controversy over, the notion that melanin-based ornamental colors are dependent on nutrition and health (Jawor and Breitwisch 2003). In a number of bird species, manipulations of debilitating endoparasites (McGraw and Hill 2000), calorie intake (Buchanan et al. 2001; McGraw et al. 2002; Siefferman and Hill 2005), and protein content (Gonzalez et al. 1999) at the time of feather growth have had no effect on the color or extent of melanic plumage coloration. However, in other correlational (Veiga and Puerta 1996; Parker et al. 2003) and experimental (Griffith 2000; Fitze and Richner 2002) studies, results are consistent with the notion that melanic colors are condition dependent. In these latter studies, however, particular nutrition- or health-related factors have not been tracked explicitly at the time of molt, so clearly more work is needed to understand, if in fact some melanin colors are sensitive to health or nutrition, exactly what dietary or immunological components underlie such patterns.

A recent hypothesis was advanced that offered a potential micronutrient basis to the production of melanin pigments and coloration (McGraw 2003; Niecke et al. 2003; West and Packer 2003). Studies of domestic animal production and in vitro biochemical reactions separately indicate that the accumulation of melanin in cells and tissues (such as the pelage of cattle and cats) can be influenced by the presence of transition metals—minerals like calcium (Ca), zinc (Zn), and iron (Fe) (reviewed in McGraw 2003). Niecke et al. (1999, 2003) showed that melanic regions of plumage in white-tailed eagles (*Haliaeetus albicilla*) and barn owls (*Tyto alba*) contain more Ca and Zn than unpigmented feathers. However, to date, no studies have analyzed the relationship between dietary mineral content and ornamental melanin coloration in birds.

I experimentally tested the effect of a dietary mineral supplementation during the period of feather growth on the development of melanin-based plumage pigmentation in male zebra finches (*Taeniopygia guttata*). Zebra finches display a patch of eumelanin-rich (McGraw and Wakamatsu 2004) black feathers on the breast that varies in size among males

and is absent in females (Zann 1996). I chose to manipulate calcium in this study because 1) it is required in the highest dietary amount among all minerals in the avian body (Klasing 1998) and 2) zebra finches are granivores, and grains usually contain much less calcium (0.02–0.1%) than is required for both growth of young birds (up to 1.2%) and maintenance in adults (>0.2%) (Klasing 1998). Because of this life-stage difference in calcium requirements, I separately examined the relationship between minerals and melanin pigmentation in young (fledgling) and adult birds.

METHODS

Diet manipulation

I studied 20 individually housed adult male and 22 individually housed fledgling male zebra finches from a large captive colony of outbred birds at Cornell University in Ithaca, New York (see McGraw, Gregory et al. 2003 for more housing details). All individuals were fed a base, ad libitum diet of tap water and Kaytee Forti-Diet finch blend (Kaytee Products, Chilton, WI). Oystershells and cuttlebone, which are common calcium supplements given to captive birds, were withheld from the diet of adult finches for 1 month prior to the study and throughout the course of the diet experiments on both adults and fledglings. The fledglings under study likely received calcium during their nestling period because their parents had access to oystershells and cuttlebone for breeding purposes.

Feeding experiments on both the adults and fledglings lasted 2 months, which was sufficient time for all birds to grow their breast feathers. Diet supplementation of fledglings began on day 40, when they reached independence. I randomly split the adults into 10 control (C) and 10 experimental birds. For the fledgling study, I bred 16 sets of parents in a single bout to produce 43 offspring, but because zebra finch fledglings are phenotypically unsexable at independence, I randomly split them into 2 groups and separated siblings into different treatment groups whenever possible. At the end of the study, this assignment left me with 10 C and 12 experimental fledgling males. The only information available on the origin and history of adults, which were acquired from a variety of local pet stores 6 months earlier, was that all were at least 9 months old because all had adult beak coloration at the time of purchase (which takes 3 months to fully develop; Zann 1996).

Experimental birds were fed food-grade calcium carbonate powder (Prince Agri Products Inc., Quincy, IL), which I spread homogenously throughout the seeds by shaking the mixture vigorously in a sealed Tupperware container. Calcium carbonate was chosen for use because it is a natural form (e.g., in the shells of bivalves and bird eggs) and, unlike some other sources, it has 100% calcium bioavailability (Soares 1995). Data provided by the manufacturer indicated that the fortified seed diet given to zebra finches (see McGraw, Gregory et al. 2003 for more details) contained 0.65% calcium (Brue R, personal communication). I then supplemented $CaCO_3$ powder in the experimental group to achieve a total dietary calcium level of 1.2%—the upper limit of the natural range of dietary calcium requirements in birds (Klasing 1998). Food was replenished on a bidaily basis (for both treatment groups) to keep the seed and supplement fresh. Body mass was measured before and after the experiments, as a way of accounting for other physiological changes that might have occurred due to calcium supplementation.

Scoring coloration

Captive adult zebra finches do not exhibit a periodic molt, so to systematically study development of melanic plumage in adults, I was forced to pluck black breast feathers (mean number of black feathers plucked = 29.7 ± 1.8) prior to supplementation and examine changes in melanization of regrown feathers. Plucking was done blind with respect to treatment, to ensure that there was no treatment bias. To allow individuals to grow patches larger than they previously displayed if they were capable (i.e., if induced by the calcium treatment), at this time I also plucked the neighboring gray (above the patch) or white (below the patch) feathers that surrounded the black patch on all sides. Because of inherent differences in the size of the black breast patch, the number of surrounding breast feathers plucked necessarily differed across individuals (mean number of nonblack feathers plucked = 33.7 ± 2.5), and this number was recorded and analyzed statistically (see Results). I was able to study plumage development in a more natural situation for fledgling males, who have no black breast feathers in the juvenal plumage but do in their definitive basic plumage.

Before (for adults only) and after calcium supplementation, birds were digitally photographed against a gray board under standardized lighting and at a standard distance (*sensu* McGraw et al. 2002). In all, 4 photos were taken per bird per measurement period: 2 of the breast, for determining melanin coloration, and 2 of the beak, for determining the effect of this treatment on an ornament—the sexually selected, red, carotenoid-containing beak (Burley and Coopersmith 1987; Blount et al. 2003)—that I predicted should not be specifically sensitive to dietary calcium content. Included in each photo was a size and color standard against which I could calibrate all photos for any slight variations in light capture and from which I could measure area of melanin pigmentation. Images were imported into Adobe Photoshop CS (v. 8.0) at a resolution of 2048 × 1536 pixels. The body region of interest was selected with the lasso tool, and I used RGB values from the Histogram palette to calculate hue (the variable parameter of interest for the beak, calculated in degrees around a 360° color wheel, with red set at 0°) and brightness (the variable parameter of interest for the breast, scored as a percent relative to absolute black and white) scores using the Color Picker function. Number of pixels occupied by the black breast was determined with the Histogram palette to quantify patch size. Analyses of duplicate photos from the beak and breast demonstrated that these color measures were highly repeatable (beak hue: $R_i = 0.95$, $F = 42.7$, $P < 0.0001$; breast brightness: $R_i = 0.97$, $F = 68.1$, $P < 0.0001$; breast patch size: $R_i = 0.88$, $F = 15.7$, $P < 0.0001$; sensu Lessells and Boag 1987). I used the average values from the 2 photographs of each bird in statistical analyses. As secondary measures of patch size, I counted the number of breast feathers that contained black pigment (for fledglings) and the percentage of total feathers plucked that grew back in black (for adults). Breast patch size is strongly dependent on the number of black breast feathers in male zebra finches ($r = 0.74$, $n = 20$, $P < 0.0001$), which might argue for not measuring both characteristics in this study, but elucidating a calcium-dependent shift in the number of black-pigmented feathers would be important for understanding the cellular or molecular mechanism via which minerals could increase the area of a plumage region covered with eumelanin (see Discussion).

Mineral content of feathers

In 3 pooled samples of feathers from both treatment groups at the end of the adult experiment only, we also tested whether our dietary supplement raised calcium levels in feathers (*sensu* Niecke et al. 1999, 2003). These analyses were conducted using inductively coupled plasma atomic emission spectrometry in the Department of Horticulture at Cornell University,

following previous methods performed on bird feathers, including those of zebra finches (Dauwe et al. 2000, 2002). Trimmed feather portions were rinsed twice with Micro-90 detergent (3 drops per 100 ml distilled water) and dried overnight at 85 °C prior to microwave heating digestion (Blust et al. 1988) and analysis at 184 nm (minimum detection limit = 0.05 ppm). We confirmed a physiological effect of the calcium treatment by determining that black feathers from calcium-supplemented (CS) birds contained 7 times more calcium, on average, than did those of C birds (CS = 12485 ± 4406 ppm; C = 1810 ± 164 ppm).

Statistical analysis

Parametric statistics were used when data fit assumptions of normality and homoscedascity or when variables could be transformed to meet these assumptions; otherwise, nonparametric tests were employed. In the adult feeding experiment, I used ANOVA (or Mann-Whitney *U* tests) to examine treatment effects on pre- and postexperimental morphological variables, as well as changes in these variables that occurred as a result of the experiment. Because fledglings lacked both the red beak and the black breast patch at the start of the experiment, I was only able to analyze (using ANOVA or Mann-Whitney tests) differences in color that existed between treatment groups after the feeding experiment. In all cases, means ± standard error are presented.

RESULTS

Adult calcium-supplementation experiment

Prior to the experiment, CS and C adult males did not differ significantly in melanin-based breast patch darkness or size, or in the hue of the carotenoid-based beak (Table 1). There also were no preexperimental differences between the treatment groups in the number of black feathers or nonblack feathers plucked from the breast (Table 1). After the experiment, CS and C males also did not differ in any measure of carotenoid or melanin pigmentation (Table 1). However, the change in breast patch size that occurred during the experiment did differ significantly between treatment groups ($F_{1,18} = 3.2$, $P = 0.03$); the patches of CS males increased in size significantly more (by 180%) than did those of C males (Figure 1a). I also calculated the percentage of change in the number of black feathers for each individual during the experiment, which accounts for the unstandardized number of nonblack feathers plucked from each bird and found that this differed significantly between CS and C males as well ($F_{1,18} = 3.9$, $P = 0.01$); supplemented birds increased in the number of black feathers grown by 13%, whereas Cs decreased in black feathers regrown by 8% (Figure 1b). I found no significant treatment differences in the changes in beak hue ($F_{1,18} = 0.0003$, $P = 0.99$) or breast patch brightness ($F_{1,18} = 0.003$, $P = 0.95$) that occurred during the experiment (Figure 1c,d). There also were no significant differences in preexperimental (Table 1), postexperimental (Table 1), or the change in body mass ($F_{1,18} = 0.04$, $P = 0.85$) between CS and C males.

Fledgling calcium-supplementation experiment

CS and C fledglings did not differ in body mass prior to the study ($F_{1,20} = 0.57$, $P = 0.51$), nor did they differ in postexperimental body mass or change in mass (both $F_{1,20} < 0.5$, both $P > 0.44$). Calcium supplementation had no significant effect on the darkness of black breast feathers grown by fledglings ($F_{1,20} = 0.06$, $P = 0.82$; Figure 2c) or beak redness ($F_{1,20} = 1.7$, $P = 0.20$; Figure 2d). However, CS males grew significantly more black breast feathers (41% more) than did C males ($F_{1,20} = 5.4$, $P = 0.03$; Figure 2b) and tended to grow larger areas of black color (31% larger) on the breast ($F_{1,20} = 3.7$, $P = 0.08$; Figure 2a).

DISCUSSION

The specific condition-dependent factors underlying melanin-based color signals are much debated (Griffith et al. 2006). Prior manipulations of exogenous factors, like reproductive effort (Griffith 2000) or ectoparasite load (Fitze and Richner 2002), demonstrate that some melanic colors can be sensitive to certain environmental perturbations, but it is difficult to discern from such coarse manipulations what specific dietary, health, or physiological parameters mediate such sensitivity. Here I uncover micronutritional dependence of melanin-based coloration in a sexually dichromatic passerine. Dietary calcium manipulations increased the size of the black breast patch in male zebra finches. This was true both when adults were regrowing black feathers and when fledglings first developed their adult plumage, consistent with both activational and organizational control of melanin color (*sensu* Strasser and Schwabl 2004; Eising et al. 2005) by this dietary mineral. The fact that the carotenoid-based beak of males was not affected by this treatment indicates that this mineral-driven mechanism is unique to melanin ornamentation.

These results beg the question of how calcium might influence melanin pigmentation at the cellular or molecular level.

Table 1

Comparisons of morphological variables between CS and C adult males before and after the adult feeding experiment

Time of measurement	Trait	Calcium enriched	Control	$F_{1,18}$	U	P
Preexperimental	Melanin-based patch darkness (%)	49.9 ± 3.3	52.6 ± 7.5		54	0.89
	Melanin-based patch size (cm^2)	0.84 ± 0.06	0.98 ± 0.18	0.92		0.35
	Number of black feathers in patch	30.1 ± 2.3	29.0 ± 3.1	0.07		0.79
	Number of nonblack feathers plucked	33.3 ± 3.3	34.6 ± 4.3	0.05		0.82
	Carotenoid-based beak hue (°)	7.8 ± 1.0	9.6 ± 1.4	1.1		0.30
	Body mass (g)	12.7 ± 0.2	13.1 ± 0.2	1.5		0.23
Postexperimental	Melanin-based patch darkness (%)	53.8 ± 3.2	56.7 ± 7.9		56	0.81
	Melanin-based patch size (cm^2)	0.98 ± 0.07	1.03 ± 0.14	0.19		0.67
	Number of black feathers in patch	32.8 ± 2.8	27.4 ± 4.3	1.1		0.30
	Carotenoid-based beak hue (°)	8.6 ± 0.9	10.5 ± 1.6	1.5		0.23
	Body mass (g)	12.3 ± 0.2	12.6 ± 0.2	1.2		0.29

Parametric tests (ANOVA, *F* reported) were used when data met assumptions of normality and homoscedascity; otherwise I used nonparametric Mann-Whitney *U* tests.

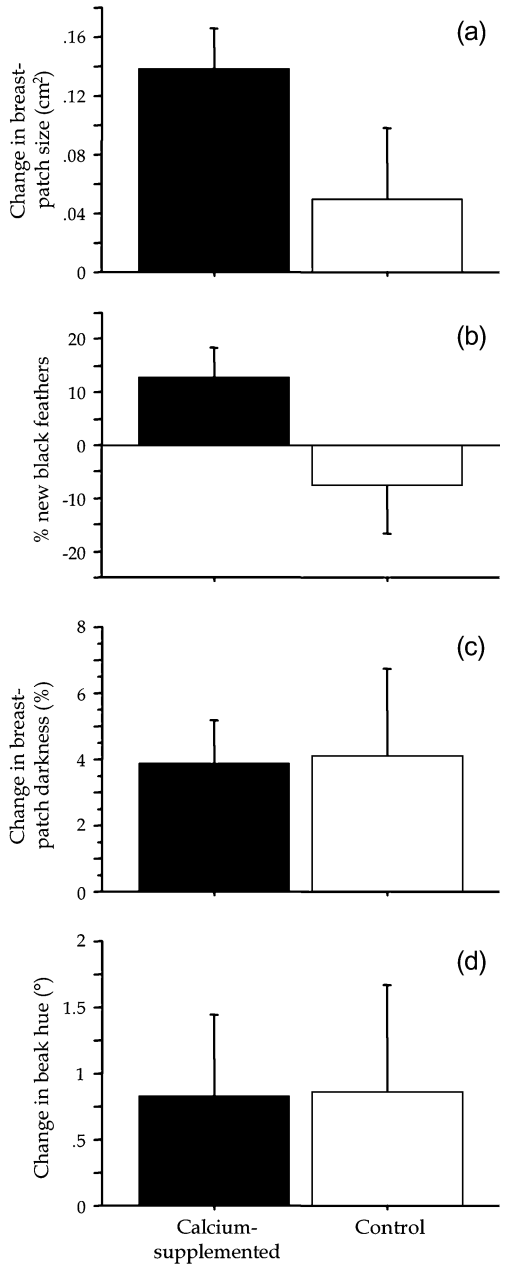

Figure 1
Effect of dietary calcium supplementation on changes in (a) the size of the black breast patch, (b) the percentage of new black feathers grown, (c) the darkness of the black breast patch, and (d) the hue of the red beak in adult male zebra finches. Recall that differences in (a), (c), and (d) should not exactly equate numerically to the population-level post- and preexperimental differences reported for each treatment group (in Table 1) because the data here account for individual-level changes in values.

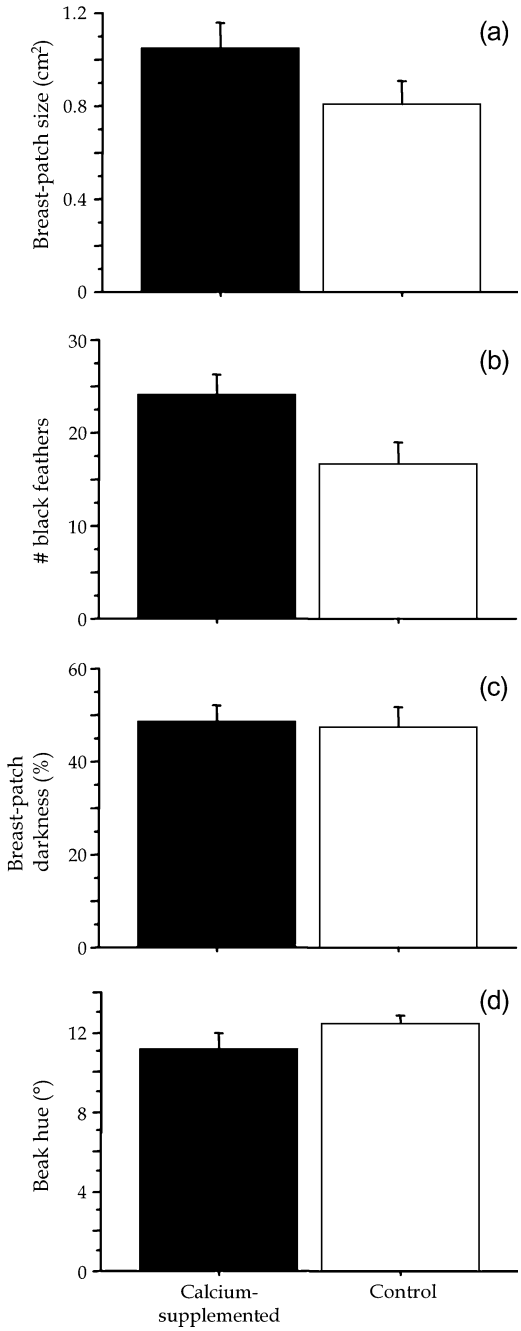

Figure 2
Effect of dietary calcium supplementation on (a) the size of the black breast patch, (b) the number of black breast feathers grown, (c) the darkness of the black breast patch, and (d) the hue of the red beak in fledgling male zebra finches.

Animals endogenously synthesize melanin from amino acids (e.g., tyrosine, phenylalanine) using the enzyme tyrosinase, and pigment synthesis is completed within organelles (melanosomes) that are housed in dedicated epidermal cells known as melanocytes (Prota 1992). The proliferation of melanocytes in feather follicles is one cellular mechanism for increasing melanin pigmentation (Ito 2003). The biochemical literature also suggests that dietary minerals can enhance melanin production by 1) accelerating the molecular rearrangement of indole intermediates (e.g., dopachrome) in the melanin cascade (e.g., Napolitano, Chioccara et al. 1985; Palumbo et al. 1987; Gallas et al. 1999; Di Donato et al. 2002), 2) permitting oxidative polymerization of these intermediates to melanin (Napolitano, Corradini, and Prota 1985; Pezzella et al. 1996), and 3) upregulating tyrosinase activity via a Ca-mediated cyclic adenosine monophosphate pathway (Buffey et al. 1993). The fact that my results show that Ca affected the number of feathers pigmented with black melanin suggests that Ca promoted the proliferation of or melanogenesis in melanocytes from more feather follicles. Despite the numerous studies on the factors that affect melanin coloration in birds (reviewed in

McGraw 2006), attention has rarely been paid to the feather-specific mechanism by which a melanin-based badge increases or decreases in size. Clearly, more studies that aim to understand the honesty-reinforcing mechanisms of avian melanin coloration should consider whether extrinsic or intrinsic manipulations influence pigment density or distribution on or among feathers.

Though dietary mineral content clearly influenced melanization in this study, it should by no means be viewed as the lone factor underlying variation in this signal type. There is a large literature on how hormonal (e.g., testosterone, luteinizing hormone; reviewed in Ralph 1969; McGraw 2006), genetic (reviewed in Roulin 2004; Mundy 2006), and social (Korzan et al. 2000; McGraw, Dale et al. 2003; Tibbetts and Dale 2004) processes shape melanin color expression in animals. In fact, in zebra finches, melanin colors and patterns are known to have a large heritable component (Burley and Bartels 1990; Zann 1996). Under some conditions, amino acid availability can also influence melanin colors and patterns (Grau et al. 1989; Yu et al. 2001; Poston et al. 2005), although it has not yet been demonstrated to alter relevant signaling variation in an avian melanic trait. Because my study was conducted on a domesticated species, for which little is known of the function of the sexually dichromatic melanic trait, efforts now should be dedicated to determining how relatively important dietary mineral availability is, compared with these other factors, in a free-ranging bird species that displays a confirmed sexual or social melanin-based signal and for which we can better elucidate natural variance in dietary calcium limitations. It will also be useful to build on this first test of the "mineral-acquisition" hypothesis for melanin ornamentation by considering whether: 1) transition metals other than Ca (e.g., Zn, Fe) can regulate melanin color signals, 2) minerals might also be traded-off against other functions to help maintain the honesty of the control mechanism, and 3) phaeomelanin-containing color signals (e.g., brown, rufous) are equally as sensitive to dietary mineral intake as was eumelanin-based ornamentation here.

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