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Seeing red: behavioral evidence of trichromatic color vision in strepsirrhine primates

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Among primates, catarrhines (Old World monkeys and apes) and certain platyrrhines (New World monkeys) possess trichromatic color vision, which might confer important evolutionary advantages, particularly during foraging. Recently, a polymorphism has been shown to shift the spectral sensitivity of the X-linked opsin protein in certain strepsirrhines (e.g., Malagasy lemurs); however, its behavioral significance remains unknown. We assign genotypes at the X-linked variant to 45 lemurs, representing 4 species, and test if the genetic capacity for trichromacy impacts foraging performance, particularly under green camouflage conditions in which red detection can be advantageous. We confirm polymorphism at the critical site in sifakas and ruffed lemurs and fail to find this polymorphism in collared lemurs and ring-tailed lemurs. We show that this polymorphism may be linked to "behavioral trichromacy" in heterozygous ruffed lemurs but find no comparable evidence in a single heterozygous sifaka. Despite their putative dichromatic vision, female collared lemurs were surprisingly efficient at retrieving both red and green food items under camouflage conditions. Thus, species-specific feeding ecologies may be as important as trichromacy in influencing foraging behavior. Although the lemur opsin polymorphism produced measurable behavioral effects in at least one species, the ruffed lemur, these effects were modest, consistent with the modest shift in spectral sensitivity. Additionally, the magnitude of these effects varied across individuals of the same genotype, emphasizing the need for combined genetic and behavioral studies of trichromatic vision. We conclude that trichromacy may be only one of several routes toward increased foraging efficiency in visually complex environments. *Key words:* dichromacy, feeding ecology, lemurs, opsin gene polymorphism, trichromacy. *[Behav Ecol 20:1–12 (2009)]*

Among eutherian mammals, trichromatic color vision is unique to the order Primates, reflecting the presence of several types of cone photopigments in the eye and a specialized neural mechanism for integrating the output from these cones (Bowmaker 1998). Traditionally, trichromacy—the ability to discriminate hues along the human visible color spectrum (Nathans et al. 1986)—was thought to have arisen only in the anthropoid lineage (monkeys and apes: Jacobs 1993), but recent molecular evidence places at least one additional origin of allelic trichromacy much earlier, in the strepsirrhine lineage (lemurs, lorises, and galagos; Tan and Li 1999). Because strepsirrhines lack a fovea centralis (Rohen and Castenho 1967), show a relative paucity of cones (Peichl et al. 2001), and have reduced visual acuity compared with anthropoids (Jacobs 1995), some researchers have suggested that they may lack the neuronal preconditions necessary to support color vision (Jacobs et al. 1999; Zeki 1999; Gegenfurtner and Kiper 2003). If so, even strepsirrhines that produce 3 differentiated opsin proteins would fail to exhibit behavioral correlates of trichromatic vision. Other researchers (Tan and Li 1999; Tan et al. 2005), however, have reasoned in favor of functional trichromacy in strepsirrhines, citing the ubiquitous presence among primates of the parvocellular system involved in mediating red–green color opponency (Casagrande and Kaas 1994) and the shift in spectral sensitivity that is often associated with opsin gene polymorphism (Jacobs et al. 2002). By this argument, strepsirrhines that produce 3 spectrally differentiated opsin proteins should behave more like routine trichromatic primates than should their dichromatic conspecifics.

We used foraging tasks to differentiate between these alternative predictions and present the first behavioral test of the functional significance of allelic trichromacy in strepsirrhines. Specifically, we ask if the genetic capacity for trichromacy translates into "behavioral trichromacy," defined as the ability to distinguish red from green. We assess this discriminatory ability in genetic trichromats, primarily by testing for a "foraging advantage" over dichromats, defined as more efficient retrieval of red food items presented against a green background.

Dichromatic versus trichromatic color vision in primates owes to the presence of different opsin genes coding for different classes of cone photopigments expressed in the eye (Jacobs et al. 1996). Most primates possess the autosomal short (S) cone, but only catarrhines (Old World monkeys and apes) and one genus of platyrrhines (New World monkeys), the howling monkey (*Alouatta* spp), show routine trichromacy, resulting from the combination of the S cone gene with 2 anciently duplicated X-linked genes that code for the middle (M) and long (L) photopigments (Jacobs and Deegan 1999). Other platyrrhines have only one opsin gene on their X chromosome, but this gene is known to be functionally variable in several species. Within these latter species, a series of polymorphic nonsynonymous substitutions in the coding sequence causes a detectable shift in wavelength absorbance that mimics the effect of the L cone in routine trichromats. Accordingly, males and homozygous females are dichromatic, whereas heterozygous females are trichromatic (Mollon et al. 1984; Tovee et al. 1992).

Recently, Tan and Li (1999) identified a similar opsin gene polymorphism in 2 strepsirrhine species: the Coquerel's sifaka (*Propithecus verreauxi coquereli*) and the red ruffed lemur

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(*Varecia variegata rubra*). This polymorphism is a functionally variable, nonsynonymous substitution in exon 5 of the X-linked opsin gene that changes residue 285 of the protein from an alanine to a threonine and significantly shifts its resulting spectral sensitivity. This shift is modest compared with that observed in platyrrhines or between opsin genes in catarrhines (Tan and Li 1999; Jacobs et al. 2002), and the evolutionary and behavioral significance of this polymorphism is not clear. Nevertheless, given that these 2 strepsirrhine species last shared a common ancestor approximately 40–45 million years ago (Yoder and Yang 2004), either the functional variant has been persistently polymorphic for a surprisingly long time in evolutionary history or it has arisen and been maintained independently multiple times in this lineage. Either scenario is consistent with nonneutral evolution at the polymorphic site, suggesting that the polymorphism is not only behaviorally functional but also possibly of adaptive importance (as argued for platyrrhines: Surridge and Mundy 2002).

One of the main hypotheses favoring the evolution of trichromatic color vision is the facilitated visual detection of ripe fruit by frugivores (e.g., Allen 1879; Mollon 1989; Bowmaker et al. 1991). The changing color of ripening fruit, from green to yellow, orange, or red, correlates with changing glucose content, thereby directly advertising nutritious value to those animals that perceive the color gradient (Schaefer et al. 2004; Riba-Hernandez et al. 2005). Nevertheless, whereas trichromatic frugivores sometimes enjoy a significant advantage over dichromatic conspecifics in detecting reddish food against a green or camouflaged background (Caine and Mundy 2000; Smith et al. 2003), they do not always show the expected advantage in detecting red foods (Caine et al. 2003; Dominy, Garber, et al. 2003). Moreover, trichromacy is not limited to frugivores. Consequently, some researchers more recently link the evolution of trichromacy to the visual detection of young, red leaves by folivores (Dominy and Lucas 2001; Dominy, Garber, et al. 2003; Lucas et al. 2003). Compared with mature leaves, young leaves are less tough; are richer in proteins, free amino acids, and nonstructural carbohydrates; and may contain fewer repellents, such as alkaloids or tannins (Milton 1979; Glander 1981; Dominy, Svenning, and Li, 2003). Moreover, compared with the more frugivorous, dichromatic New World primates, trichromatic Old World primates incorporate relatively higher proportions of leaves in their diets (Robinson and Janson 1986; Terborgh 1986).

The common thread shared by both of these hypotheses is the central role of foraging in the evolution of primate color vision. If correct, variation in the ability to detect food under certain conditions will be mediated by differences between the sensory systems of dichromatic and trichromatic strepsirrhines. Such differences would be expressed behaviorally and would be measurable as part of the animals' foraging activity. Using behavioral bioassays that model the detection of red and green food against either a control (tan) or an experimental (green) background, we compare the foraging behavior of dichromats and potential trichromats belonging to 2 species shown to possess the opsin gene polymorphism, the Coquerel's sifaka and the ruffed lemur (a single species that includes 2 subspecies: the red ruffed lemur and the black-and-white ruffed lemur, *Varecia variegata variegata*). If possession of the opsin gene polymorphism produces functional trichromatic color vision, that is, behavioral trichromacy, we would expect to observe an advantage for detecting red food in the experimental condition by heterozygous female sifakas and ruffed lemurs.

We also compare the performance of trichromatic sifakas and ruffed lemurs against the performance of 2 species believed to be routinely dichromatic, the ring-tailed lemur (*Lemur catta*) and the collared lemur (*Eulemur fulvus collaris*, a subspecies of brown lemur: see Jacobs and Deegan 1993). If trichromatic color vision is the major factor influencing foraging efficiency within our bioassay, then trichromatic sifakas and ruffed lemurs also should enjoy the same foraging advantage (involving red on green) over members of the dichromatic species. Nevertheless, as implied by the variable roles attributed to frugivory and folivory in the evolution of color vision, performance differences across species also might relate to differences in feeding ecology or dietary specialization (sifakas are folivores: Campbell et al. 2000; ruffed lemurs are frugivores: Britt 1998; and ring-tailed and collared lemurs are both generalists: Mittermeier et al. 2006) and possibly to activity pattern (collared lemurs are predominantly cathemeral: Mittermeier et al. 2006; the other 3 species are primarily diurnal: Richard and Dewar 1991). If feeding ecology or activity pattern is a better predictor of performance than is color vision, consistent species differences could arise independently of opsin gene polymorphism, suggesting that any foraging advantages conferred by trichromacy might also be attained via alternative evolutionary routes.

MATERIALS AND METHODS

Subjects and housing

The subjects were 58 strepsirrhine primates (35 females and 23 males) housed at the Duke Lemur Center in Durham, NC (Table 1). Of these subjects, we genotyped 45 individuals and behaviorally tested 40 individuals. Animals that free range most of the year in large forested enclosures (3–7 ha) were genotyped only. The subjects used in the behavioral bioassays were housed in groups of 2–6 animals within enclosures of roughly 5–15 m^2 at the base and up to 5 m in height that included appropriate environmental enrichment. We conducted our study from November 2005 to June 2006 and performed the behavioral trials in the subjects' habitual open-air enclosures. The sifakas were provisioned with leafy greens, nuts, and Leaf-Eater Primate Diet mini-biscuits; the ruffed, ring-tailed, and collared lemurs were fed fruits, vegetables, and Monkey Diet™. Water was freely available. To encourage interest in the task, we delayed the usual 0800 h morning feeding until after we had completed testing (at around 1100 h).

Animal handling and genotyping

Blood sampling

For the purposes of genotyping, we obtained whole blood (1 cc) by femoral venipuncture of manually restrained or anesthetized subjects (procedural details are provided in Williams et al. 2003). Our original aim had been to genotype the sifakas and ruffed lemurs only; however, we ultimately genotyped members ($n = 45$) of all subject species for the following reasons. First, females are underrepresented in the relevant electroretinogram (ERG) flicker photometry studies of strepsirrhine primates (e.g., Jacobs and Deegan 1993, 2003b). Second, although some ERG data exist for ring-tailed and brown lemurs (Jacobs and Deegan 1993, 2003b), to our knowledge only one male brown lemur and one male ring-tailed lemur have been genotyped at the functionally polymorphic site (Tan and Li 1999). Third, the red-biased performance of female collared lemurs (see Results) led us to question their dichromatic status. The dearth of data on these species left open the possibility that the functional opsin polymorphism may be present in additional species, including those previously considered to be monomorphically dichromatic.

DNA extraction, sequencing, and genotyping

We extracted genomic DNA from 100 μL of whole blood using the DNeasy DNA Extraction Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. We polymerase chain

Table 1

Demographics and opsin genotypes, including middle (M) and long (L) photopigments, of strepsirrhine subjects, with ruffed lemurs separated by subspecies

Subjects (Duke Lemur Center #) by species or subspecies	Sex	Age at testing (years)	Residue (opsin)		Color vision
			alanine (M)	threonine (L)	
Coquerel's sifaka (*Propithecus verreauxi coquereli*, $n = 16$)					
6825	Female	2	+	+	Trichromatic
6538, 6727[a], 6743, 6770, 6828, 6850	Female	13, 8, 7, 6, 2, 1	−	+	Dichromatic
6518[a,b] 6583[a,b]	Male	13, 12	+	−	Dichromatic
6608[b], 6650, 6747, 6797, 6823[b]	Male	12, 11, 7, 4, 2	−	+	Dichromatic
6807, 6827	Male	3, 2	NA	NA	Presumed dichromatic
Red ruffed lemur (*Varecia variegata rubra*, $n = 12$)					
6377, 6424, 6633, 6838	Female	16, 15, 11, 2	+	+	Trichromatic
6205[b], 6802	Female	≈21, 4	+	−	Dichromatic
6311, 6378[b], 6839	Female	17, 16, 2	−	+	Dichromatic
6206[b]	Male	≈21	−	+	Dichromatic
6240, 6684	Male	18, 10	NA	NA	Presumed dichromatic
Black-and-white ruffed lemur (*Varecia variegata variegata*, $n = 3$)					
2560, 6415[b]	Female	30, 15	+	−	Dichromatic
5604	Male	25	+	−	Dichromatic
Ring-tailed lemur (*Lemur catta*, $n = 18$)					
5984[b], 6159, 6229[b], 6276[b], 6709, 6711[b], 6761[b], 6796[b], 6830[b], 6831[b], 6832[b]	Female	21, 19, 18, 17, 9, 9, 6, 4, 2, 2, 2	+	−	Dichromatic
6857, 6865	Female	1, 1	NA	NA	Presumed dichromatic
6440, 6622	Male	15, 11	+	−	Dichromatic
6862, 6485, 6534	Male	23, 14, 13	NA	NA	Presumed dichromatic
Collared lemur (*Eulemur fulvus collaris*, $n = 9$)					
5982, 6305, 6362[b]	Female	21, 17, 16	+	−	Dichromatic
6145	Female	19	NA	NA	Presumed dichromatic
6225, 6433	Male	18, 15	+	−	Dichromatic
5973, 6389, 6586	Male	21, 16, 12	NA	NA	Presumed dichromatic

Estimated ages are provided for 2 wild-caught individuals; all other animals were captive born, so their ages were known; animals for which no genetic data were obtained are presumed dichromatic.

[a] Indicates an animal studied by Jacobs et al. (2002).

[b] Indicates an animal for which no behavioral data were obtained, owing to the subject's death, sickness, biting tendencies, or timidity.

reaction (PCR) amplified the DNA sequence immediately flanking the functionally important single nucleotide polymorphism (SNP) in M/L opsin exon 5, using either the primer combination reported by Jacobs et al. (2002) (forward primer: 5′-gtggcaaagcagcagaaagag-3′; reverse primer: 5′-ctgccggttcataaagacgtagataat-3′) or a set of novel primers designed in the same region (forward primer: 5′-ggtgatggtc-ctcgcatact-3′; reverse primer: 5′-tgccggttcataaagacgta-3′). Reaction mixtures contained 17.65 μL sterile water, 2.5 μL 10× PCR buffer (Invitrogen, Carlsbad, CA), 1.25 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM deoxynucleoside triphosphates (Invitrogen), 1.25 μL each of 10 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA), and 0.5 μL Platinum Taq (Invitrogen). We used the following PCR conditions: 1) one cycle for 2 min at 95 °C; 2) 39 cycles for 40 s at 95 °C, 30 s at 52 °C, and 1 min at 68 °C; and 3) 5 min at 72 °C for the final extension. We sequenced the resulting product in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3730xl DNA Analyzer (Applied Biosystems).

We assigned individuals as heterozygous (A/G) for the SNP variant if the sequencing read for that site showed overlapping, equivalent signals for both A and G. We assigned individuals as homozygous if the resulting DNA sequence showed a valid signal for only one of the 2 possible variants at the polymorphic site. To confirm the genotyping call, we genotyped most individuals at least twice from separate initial PCR amplifications and inspected the sequence reads in both directions. All sequence inspection was carried out using the program Sequencher 4.5 (GeneCodes, Ann Arbor, MI). We performed all the genotyping subsequent to behavioral testing and blind to the bioassay results.

Foraging task and reflectance data

Task and apparatus

Our task for modeling different visual foraging situations or environments involved red and green food items dispersed in a textural medium that provided either a neutral tan background, against which both food colors stood out ("control" condition; Figure 1A), or a green background intended to model a foliar condition, against which green food became camouflaged ("experimental" condition; Figure 1B). As in Caine and Mundy (2000), we used Kix® cereal (General Mills, Minneapolis, MN), dyed red or green with food coloring. Kix® cereal is relatively odorless and unsweetened. It was favored by sifakas, ruffed lemurs, and collared lemurs, but not by ring-tailed lemurs, for which we instead used unscented Noyes Precision Sucrose Pellets™. We randomly dispersed 5 red and 5 green food items on each of 2 metal trays (30 × 60 cm) that we had previously covered with sawdust. For our control condition, we used natural sawdust; for our experimental condition, we used sawdust dyed green, which models foliar camouflage by matching the green food.

Reflectance properties of the stimuli and background

To evaluate the effectiveness of our experimental and camouflage conditions, independently of the human visual system, we characterized the spectral properties of the items used in our

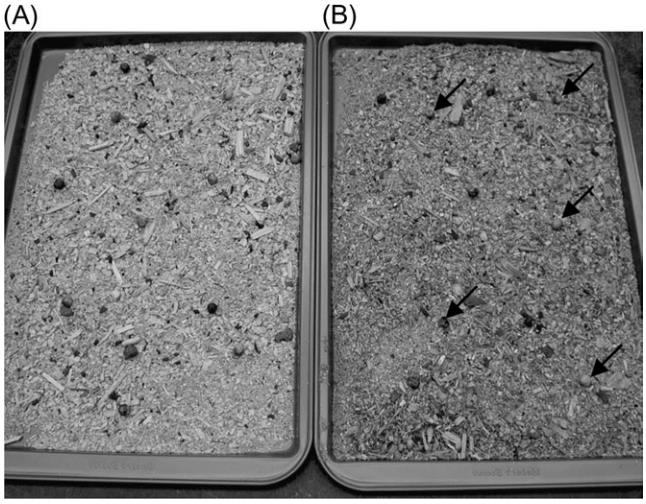

Figure 1
Foraging tasks presented to strepsirrhine primates, showing red and green food dispersed in natural (tan) or green-tinted sawdust, representing the (A) control and (B) experimental conditions, respectively. Arrows point to camouflaged, green food in (B).

foraging trials. The spectral properties of the background and colored food items were measured under laboratory conditions at Duke University.

In the laboratory, we used an Ocean Optics USB2000 spectroradiometer, a Xenon-flash (Ocean Optics PX-2, Dunedin, FL) as a light source and a reflection/backscattering probe attached to a bifurcated fiber optic (ultraviolet/visible) to measure spectral reflectance. The probe was attached to a micromanipulator and was held perpendicular (90° ± 5°) to the surface of interest (e.g., colored food and sawdust), and its stainless steel ferrule was housed in a hollow clear plastic sheet that contacted the object of interest at a constant distance of 0.75 cm from the surface (for discussion of the potential problems when angle and distance are not kept constant across reflectance measurements, see Endler 1990 and Fleishman et al. 2006). Readings were taken from a circular area with a diameter of approximately 7 mm. All lights inside the room were turned off prior to taking each measurement, ensuring that the Xenon-flash was the only source of illumination. Also, dark current and white standard (WS-1, Ocean Optics) measurements were taken before measuring each sample. We took reflectance measurements from 20 randomly selected food items from each of the 4 categories (i.e., red cereal, green cereal, red sucrose pellets, and green sucrose pellets) used in the foraging trials.

To characterize the spectral properties of the background, we took measurements at 20 distinct locations for each of the backgrounds; a tray containing natural sawdust (control condition) and a tray containing sawdust dyed green (experimental condition). We scanned the same trays that were used for the foraging trails. In each tray, the amount, depth, and coloration of the sawdust were the same as those presented to the animals. We took measurements following an "X" pattern, taking a reading every 7 cm. Measurements were taken following the same protocol as the one used for measurements of food items, as described above.

We determined reflectance by dividing the value at each wavelength for the colored food or background by the value at the same wavelength for the WS-1. Data were initially collected over the 300-to 800-nm range and were later interpolated to 2-nm intervals. We reduced the number of data points for each spectrum by calculating the median value at 10-nm intervals from 400 to 700 nm.

Representative spectral properties of the food items and backgrounds are shown in Figure 2. The shape of the spectrum for the tan background is broad, without any obvious peak (Figure 2A, C). Against this tan background, the shape of the spectrum for green food items is disproportionately richer between 520 and 570 nm, with a peak at approximately 550 nm, whereas the shape of the spectrum for red food items is disproportionately richer in long wavelengths, between 610 and 660 nm. By contrast, the spectral shapes for the green background and green food items are essentially the same (Figure 2B, D): Although there are minor differences in brightness, in both cases, the spectral shape is disproportionately richer between 520 and 570 nm, with a peak at

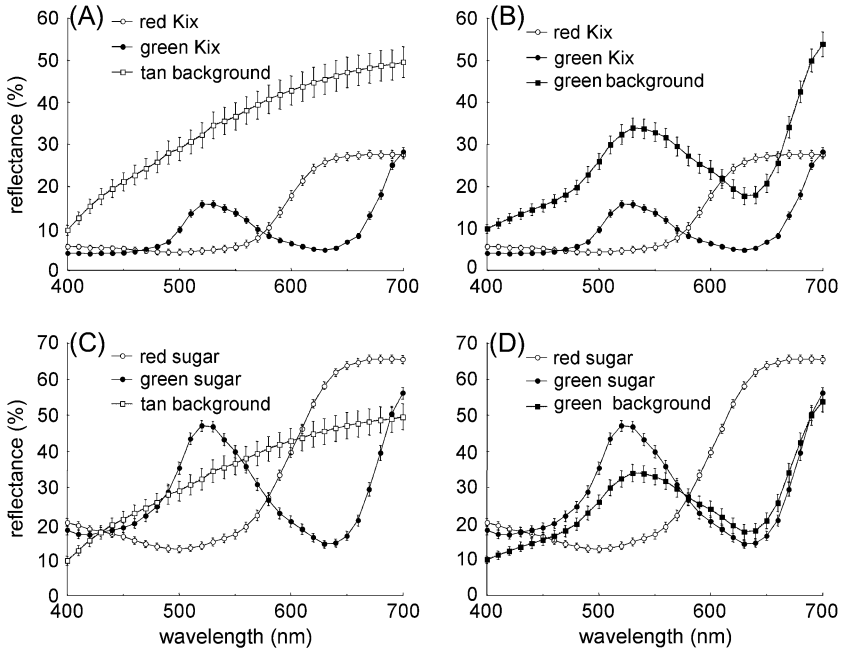

Figure 2
Representative reflectance curves for the red and green food items, including Kix® cereal (A, B) and sugar pellets (C, D) presented against the control, tan (A, C) and experimental, green (B, D) backgrounds.

approximately 550 nm. Against this green background, the spectral shape of the red food items is disproportionately richer in long wavelengths, between 610 and 660 nm and is relatively darker.

Our experiments were conducted under natural light conditions (i.e., full spectrum). Therefore, these reflectance measurements (Figure 2) show that, for a visually oriented animal foraging under full spectrum light conditions, our behavioral experiments presented 2 distinct conditions. First, under the control condition, the spectral properties (i.e., shape and brightness) of both food items stand out easily against the tan background. There is no overlap between the spectra, which should increase the probability of detecting any colored food item against the background. Second, under experimental conditions, the spectral properties of the red food items stand out easily against the green background, owing to differences in both shape and intensity, whereas the green food items are nearly a perfect match to the background, exhibiting similar spectral quality and only minor differences in brightness. Under these latter conditions, the overall conspicuousness of green food items is reduced, whereas the conspicuousness of the red food items is expected to be high for trichromats, but not dichromats.

Behavioral testing

Data collection procedures

Testing of the subjects occurred in the presence of all group members. We first habituated each subject to the apparatus and procedures: typically, one experimenter coaxed the target animal to the tray, whereas a second experimenter distracted the other group members. Habituated animals voluntarily approached the tray and retrieved the food uninterrupted. The animals that did not meet habituation criteria were excluded from behavioral trials, resulting in $n = 40$ subjects. During testing, we presented each animal first the control tray, then the experimental tray, separated by a 2-min break between trials. We used a handheld computer (Psion "Workabout," Noldus Information Technology, Leesburg, VA) to record the color and time of each food item chosen. We repeated this set of testing twice more, for a total of 6 trials per animal (3 control and 3 experimental), conducted over the course of 1–2 days. We terminated a trial either after the subject had retrieved all available food or after 5 min had elapsed.

Performance measures

Our expectation for trichromats is that red items against a green background would stand out, whereas green items would be camouflaged. Accordingly, the signature of behavioral trichromacy should be revealed in the efficiency (i.e., speed, order, and probability) with which red food items are retrieved (see below) and should be revealed in both between-subject and within-subject comparisons. Between subjects, trichromats should select red food with greater efficiency than should dichromats in the experimental condition, but not the control condition. Within subjects, trichromats should select red food with greater efficiency than their own selection of green food in the experimental condition and with greater or equal efficiency than their own selection of red food in the control condition.

As the animals typically retrieved all the food, the total number of colored food items retrieved was uninformative. Moreover, the color of the food item first selected made use of only a small portion of the data. Consequently, we used the following 3 measures of foraging performance: 1) interval latency to choose red versus green food, 2) rank order of red food choices, and 3) proportion of red food choices in equal probability situations. These measures (described below) handled 1) the potentially subtle foraging advantages that might be evidenced by non–food-deprived animals under noncompetitive situations and in close proximity to the food (see Caine and Mundy 2000) and 2) the changing probabilities of colored food selection within trials on removal of each food item.

Interval latency to choose red versus green food

If the eye is drawn to a particular locale, we reasoned that nearby food items might have a greater likelihood of detection, regardless of their color or that of the background; however, the time interval between food choices might differ depending on the level of camouflage. Thus, following each food item selected per trial, we calculated the latency to select the next red versus green food item.

Rank order of red color choices

We also expected trichromats to choose the 5 red food items before the 5 green food items within the experimental (but not control) trials. Assigning red food choices a number according to this rank order (i.e., 1, 2, 3, 4, and 5, respectively) and tallying them produces a low number (i.e., 15). By contrast, if green food items were universally chosen before red food items, the tally for red food items (i.e., 6, 7, 8, 9, and 10, respectively) would be high (i.e., 40). We assigned numbers to the 5 red food choices made per trial (such that low tallies reflected a visual bias toward seeing red, medium tallies reflected no visual bias, and high tallies reflected a visual bias toward seeing green). We then summed those numbers across the 3 replicate trials per individual in each condition, producing a range of "rank orders" from 45 to 120.

Proportion of red food choices in equal probability situations

Because all food items were retrieved in every trial, we evaluated differences in the proportion of colored food items selected under equal probability situations by extracting from the data set all choices made whenever the likelihood of selecting red versus green food was equal (e.g., on the first choice, if one of each colored food item had been taken, if 2 each had been taken, etc.).

Analyses

Initial analyses of the overall data set showed that our foraging bioassay was robust, in that individuals performed consistently across sequential trials and the experimental condition produced the desired camouflage effect (Supplementary materials). We therefore proceeded to assess foraging performance in the following 3 steps: 1) As a first test of behavioral trichromacy, we collapsed all species and compared the performance of all genetic dichromats against all genetic trichromats. 2) Because factors other than visual status, especially species-specific feeding ecologies, may also explain foraging performance, we next tested for species differences, irrespective of visual status. 3) Lastly, as a finer grain analysis of behavioral trichromacy and possible sex differences, we compared the performance of all sex and/or visual status subgroups (i.e., dichromatic males, dichromatic females, and trichromatic females) within species.

For the tables presenting the genotyping results (Tables 1 and 2), we separate the 2 subspecies of ruffed lemurs; thereafter, in all behavioral analyses (detailed below) and illustrations, we collapsed these 2 subspecies. For analyses of variance (ANOVAs) conducted per test condition, we resolved significant interaction effects in planned between- or within-subjects comparisons using F tests for simple effects (Sokal and Rohlf 1981). We used t-tests for within-subjects comparisons across test conditions. In the few cases that variances were nonhomogeneous, we performed our analyses on log-transformed data; however, for ease of interpretation, we present the nontransformed data in all figures.

Performance of dichromats and trichromats across species
Using the interval latency data set across species, we compared the performance of all dichromats versus all trichromats per test condition using 2-factor ANOVAs (food color × visual status). We expect the foraging behavior of both visual groups to be comparable in the control condition, but to differ in the experimental condition, specifically with trichromats showing the fastest retrieval of red food against a green background.

Performance by species
Again using the interval latency measure, we assessed species differences in foraging performance for both foods combined using a 2-factor ANOVA (test condition × species).

If differences in feeding ecology or activity pattern were equally important as trichromatic vision in influencing foraging behavior, then regardless of color vision status, we would expect to see significant species differences in foraging latencies under either test condition.

Performance by sex and/or visual status within species
To assess behavioral trichromacy and sex differences within species, we ran a series of analyses using all 3 performance measures:

Using the latency measure, we ran 2-factor ANOVAs (food color × sex and/or visual group) for between- and within-subjects comparisons within test conditions and *t*-tests for within-subjects comparison between test conditions. Relative to green food, red food would be conspicuous to trichromats in the experimental condition only. Thus, within species, we expect trichromats in the experimental condition to select red food faster than dichromats, faster than they select green food, and faster than they select red food in the control condition.

For the rank-order test, we performed 2 within-subject analyses. First, we asked whether trichromats or dichromats as a group (within species) shifted to lower rank-order tallies in the experimental condition relative to the control condition. We expect the distribution of tallies for trichromats in the experimental condition to be shifted toward smaller rank orders relative to their distribution of tallies in the control condition, whereas the distribution of tallies for dichromats should not change between conditions. We tested these predictions using the Wilcoxon matched-pairs test. Second, we asked whether individual trichromats selected red items sooner in the experimental condition than expected by chance. We produced rank-order tallies from 10 000 simulated trials in which red and green items were chosen at random (using the R platform, R Statistical Computing Foundation, Vienna, Austria). We then assigned *P* values to our experimental results based on the probability of observing each result in the random chance scenario.

For within-species analyses of red food choices in equal probability situations, we compared each individual's and each subgroup's frequencies of red versus green food selected within experimental conditions, using *G* tests (Sokal and Rohlf 1981). Under the hypothesis of behavioral trichromatism, genetic trichromats in the experimental condition should select red items significantly more often than green items when both items are equally available. By contrast, no preferences are expected for trichromats in the control condition or for dichromats in either condition.

RESULTS

Genotyping

We assigned genotypes to 14 sifakas, 13 ruffed lemurs, 13 ring-tailed lemurs, and 5 collared lemurs (Tables 1 and 2). Only one of the 7 genotyped female sifakas was heterozygous for the functional site in M/L opsin gene exon 5, limiting our analyses of the behavioral consequences of trichromacy in this species. Four of the 9 genotyped female red ruffed lemurs were scored as heterozygotes for the functional M/L opsin SNP. The male sifakas included individuals carrying both variants of the SNP. By contrast, all 3 genotyped black-and-white ruffed lemurs, all genotyped ring-tailed lemurs, and all genotyped collared lemurs were either homozygous or hemizygous for a single variant (coding for the M wavelength alanine residue).

Performance of dichromats and trichromats across species

When considering all lemurs tested behaviorally ($n = 40$), foraging performance did not differ by visual status in the control condition. When food was presented against a neutral background, we found a main effect of food color, such that all lemurs retrieved red food faster than green food ($F_{1,38} = 5.671$, $P < 0.025$); however, as anticipated, there was no significant interaction between food color and visual status ($F_{1,38} = 0.414$, not significant [NS]; Figure 3A). Thus, compared with one another, dichromats and trichromats were equally adept at (or equally biased in) retrieving these different food items.

By contrast, foraging performance differed significantly by visual status in the experimental condition. When food was presented against a green background, the same main effect of

Table 2
Summary of genotyping results for all subjects, with ruffed lemurs separated by subspecies

Species	Total alleles sampled	Alleles M	Alleles L	Individuals sampled		P_{MM}/P_{M*} (%)	P_{ML} (females only) (%)	P_{LL}/P_{L*} (%)
Coquerel's sifaka	21	3	18	Female	7	0	14.3	85.7
				Male	7	28.6	—	71.4
Red ruffed lemur	19	8	11	Female	9	22.2	44.4	33.3
				Male	1	0	—	100
Black-and-white ruffed lemur	5	5	0	Female	2	100	0	0
				Male	1	100	—	0
Ring-tailed lemur	24	24	0	Female	11	100	0	0
				Male	2	100	0	0
Collared lemur	8	8	0	Female	3	100	0	0
				Male	2	100	0	0

P_{MM}/P_{M*} refers to the percentage of individuals that are homozygous or hemizygous for the medium wavelength opsin; P_{ML} refers to individuals heterozygous for the medium and long wavelength opsins; and P_{LL}/P_{L*} refers to individuals that are homozygous or hemizygous for the long wavelength opsin.

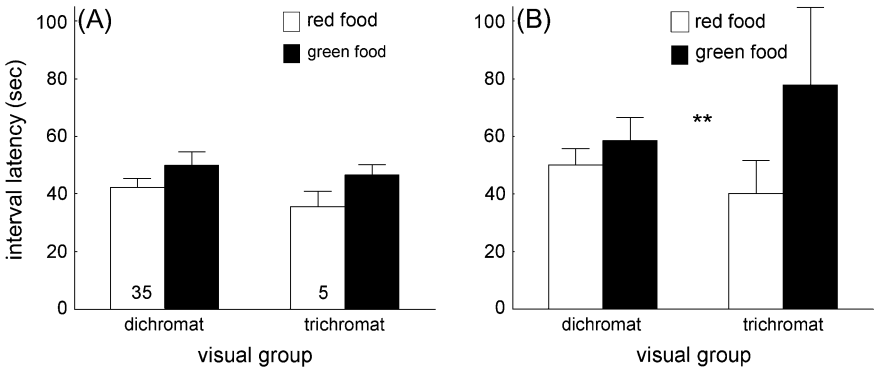

Figure 3
Interval latencies (mean ± standard error of mean in seconds) for retrieving red (open bars) and green (filled bars) food in the (A) control and (B) experimental conditions by all strepsirrhine primates tested, separated by visual status (trichromat or dichromat), with sample sizes for each visual group provided in (A). (ANOVA interaction effect between food color and lemur visual status: $^{**}P < 0.025$.)

food color maintained ($F_{1,38} = 5.861$, $P < 0.025$); however, a significant interaction between food color and visual status emerged ($F_{1,38} = 5.464$, $P < 0.025$; Figure 3B). In particular, latencies for retrieving red food versus green food were more dissimilar for trichromats than for dichromats (F tests for simple effects did not further resolve this interaction). Given that the camouflage condition prolonged food retrieval for all animals (Supplementary materials), trichromats appeared to better maintain efficient retrieval of red food than did dichromats, as predicted under the hypothesis of behavioral trichromacy. Interestingly, however, dichromats appeared to better maintain efficient retrieval of green food items than did trichromats.

Performance by species

We found significant species differences in foraging efficiency ($F_{3,36} = 11.010$, $P < 0.001$), with sifakas showing the longest interval latencies and collared lemurs showing the shortest interval latencies in both test conditions (Figure 4). Thus, a routinely dichromatic species (that also has a generalist diet and is active cathemerally) was the most efficient forager overall. Although we detected no interaction between species and food color ($F_{3,36} = 1.487$, NS), background color mattered more for some species than for others ($F_{3,36} = 6.291$, $P < 0.005$). In particular, interval latencies increased dramatically in the camouflage condition for sifakas only (F test for simple effects: $F_{3,36} = 24.087$, $P < 0.001$; Figure 4), the sole folivore we tested.

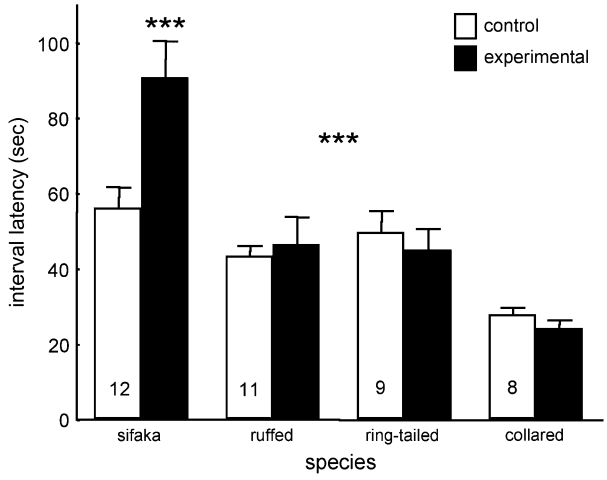

Figure 4
Interval latencies (mean ± standard error of mean in seconds) for retrieving food by different strepsirrhine species in the control (open bars) and experimental (filled bars) conditions, with sample sizes provided for each species. (ANOVA main effect of species and F test for simple effect of control vs. experimental condition: $^{***}P < 0.001$.)

Performance by sex and/or visual status within species

Interval latency to choose red versus green food

The interval latencies associated with red and green food choices against tan and green backgrounds are illustrated per species and per sex and/or visual group in Figure 5. In the control condition (for which we had no strong predictions, as both colors should have been conspicuous to all lemurs), only ruffed lemurs showed significant differences in their interval latencies within trials, with faster retrieval of red food relative to green food, evidenced both by dichromatic ($F_{2,8} = 14.117$, $P < 0.01$) and trichromatic ($F_{2,8} = 7.950$, $P < 0.05$) females (Figure 5A). Thus, female ruffed lemurs may have a preference for red foods overall.

In the experimental condition, 2 of our 3 predictions of behavioral trichromacy were variably met among the ruffed lemurs. In particular, only genetically trichromatic females retrieved red food faster than green food under camouflage conditions ($F_{2,8} = 6.799$, $P < 0.05$; Figure 5B). These females also showed a nonsignificant tendency to choose red food faster in the experimental than control condition ($t_3 = 2.941$, $P < 0.06$, NS; Figure 5). They were not, however, significantly faster than dichromats at retrieving red food. Given an overall female preference for red food (Figure 5A), these data show that only trichromatic females are able to exercise this preference under camouflage conditions (Figure 5B).

Although we could not perform similar analyses for the sole trichromatic sifaka, she showed no evidence of the predicted foraging advantage in the experimental condition: as depicted in Figure 4, she and the other sifakas required more foraging time under camouflage than control conditions (Figure 5). Interestingly, like trichromatic ruffed lemurs, dichromatic female collared lemurs also chose red food faster than green food under camouflage conditions ($F_{1,6} = 27.963$, $P < 0.01$; Figure 5B); however, they did not retrieve red food faster in the experimental than in the control condition ($t_3 = 2.260$, NS; Figure 5). Lastly, ring-tailed lemurs showed no performance differences between the sexes or test conditions (Figure 5).

Rank order of red color choices

As anticipated for the control condition, the vast majority (97.7%) of lemurs showed no color preference in the order of food items selected (Figure 6A); the sole exception was a putatively dichromatic, female ruffed lemur (#6839, Table 1)

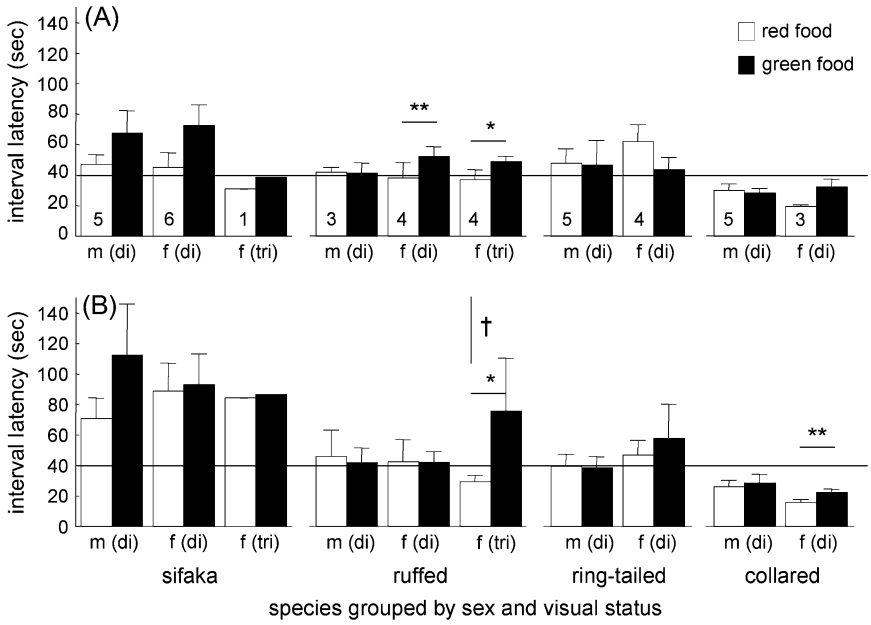

Figure 5
Interval latencies (mean ± standard error of mean in seconds) for retrieving red (open bars) and green (filled bars) food in the (A) control and (B) experimental conditions by strepsirrhine primates, grouped by species. Results are shown for male dichromats (m di), female dichromats (f di), and female trichromats (f tri), with sample sizes for each subgroup provided in (A). The horizontal line at 40 s represents, approximately, the mean performance of trichromatic ruffed lemurs retrieving red food in the control condition. (F test for simple effect of red vs. green food: $^*P < 0.05$ and $^{**}P < 0.01$; t-test for control vs. experimental condition: $\dagger P = 0.06$.)

that selected almost all the red food before choosing any green food ($P < 0.01$). There were also no significant differences between species in this measure (Kruskal–Wallis ANOVA: $H_{3,40} = 1.372$, NS).

Likewise, in the experimental condition, there was no significant difference between species in the order in which colored food items were selected ($H_{3,40} = 4.488$, NS). Although no species subgroup (males, dichromatic females, or trichromatic females) showed any difference in rank orders between the 2 test conditions, several animals shifted in the direction of a red food bias (Figure 6B), including 3 of the 4 trichromatic ruffed lemurs, although the magnitude of their shift was not significant (Wilcoxon matched pair: $Z_4 = 1.461$, NS). Interestingly, we observed a considerable degree of variability in the rank-order results among these latter individuals, suggesting that genetic trichromacy may not translate to behavioral

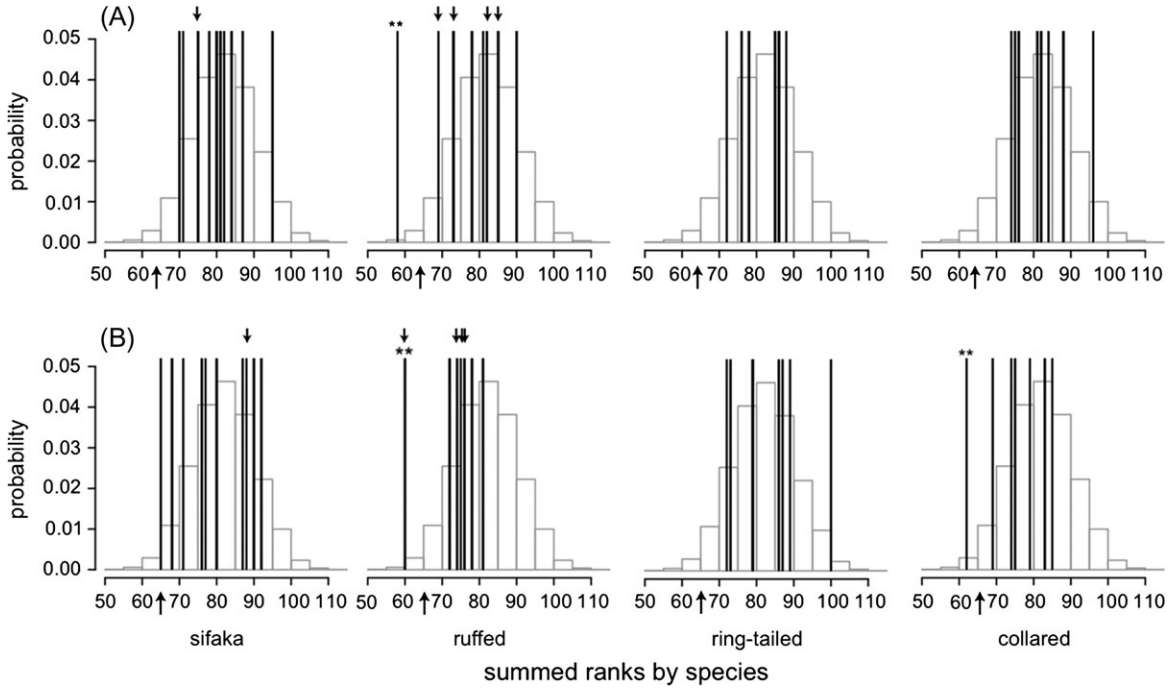

Figure 6
Rank-order tallies for the red food items selected by individual members (solid black lines) of different strepsirrhine species in the (A) control and (B) experimental conditions, compared against histograms of randomly generated rank orders (open bars). Area under the histogram represents probability and integrates to 1. Arrows above the black lines indicate trichromatic individuals that, in the experimental condition, are expected to show low sums or red-biased rank tallies (to the left on the x axis), with arrows below each histogram demarcating the point at which random red food choices become statistically unlikely ($^{**}P < 0.01$).

trichromacy in a simple manner and that the X-linked variant that confers trichromacy may be incompletely penetrant for this trait. More specifically, only 2 animals behaved in a manner consistent with behavioral trichromacy, in that significantly more of their early choices under camouflage conditions comprised red food than green food ($P < 0.01$; Figure 6B). One was a female ruffed lemur that was genotyped as heterozygous for the functional SNP in the X-linked opsin gene, consistent with the possibility that she possesses trichromatic vision (#6633, Table 1). The other, however, was a dichromatic female collared lemur (#5982, Table 1), identifying this species as potentially important for continued study.

Proportion of red color choices in equal probability situations

Among all species and subgroups tested, only male sifakas and female ruffed lemurs chose red food with significantly higher probability (i.e., they "preferred" red) when both red and green food items were equally available. Male sifakas showed a significant preference for red food in both the control ($G_1 = 4.14$, $P < 0.05$; Figure 7A) and experimental ($G_1 = 4.19$, $P < 0.05$; Figure 7B) conditions. By contrast, female ruffed lemurs showed no color preference in the control condition but showed a significant preference for red food in the experimental condition that was expressed most strongly among trichromats (dichromats: $G_1 = 5.68$, $P < 0.025$; trichromats: $G_1 = 6.64$, $P < 0.01$; Figure 7B). Indeed, the significant red food preference that emerged in dichromatic ruffed lemurs during the experimental condition was driven by the behavior of a single female (#6839 in Table 1; heterogeneity $G_4 = 11.73$, $P < 0.05$) that also showed unusually red-biased behavior in the rank-order analysis. Exclusion of this female from the analysis eliminated the significant preference for red food by dichromatic females ($G_1 = 1.06$, NS). By contrast, the 4 trichromatic ruffed lemurs were homogeneous in their bias for red food during experimental trials (heterogeneity $G_3 = 0.84$, NS), suggesting that, despite some individual variation (Figure 6B), the observed color preference may be a general quality of trichromatic female ruffed lemurs, rather than a characteristic of certain individuals (Figure 7B). Thus, in agreement with the interval latency metric, trichromatic ruffed lemurs selected red food with significantly greater probability over green food in the experimental condition, as predicted by behavioral trichromacy.

DISCUSSION

We tested whether polymorphism at a single locus in the opsin gene of strepsirrhine primates alters foraging behavior. Specifically, we asked whether individuals possessing the genetic capacity for trichromacy show behavioral trichromacy, as revealed by improved detection of red food under green camouflage conditions. We found strong evidence that genetic trichromacy alters foraging behavior but less consistent evidence that behavioral trichromacy necessarily produces the foraging advantages expected under current models of the evolution of trichromatic vision. In particular, some or all heterozygous female ruffed lemurs tended to select red food presented against a green background faster, earlier, and more often (under equal probability conditions) than they did green food against a green background or red food against a tan background. Specifically, in cross-species comparisons, trichromats were more efficient at retrieving red food than were dichromats (Figure 3B). Within species, trichromatic ruffed lemurs retrieved red food faster than green food (Figure 5B) and expressed the strongest preference for red food (Figure 7B) under camouflage conditions. Lastly, with respect to individual performance, significantly more of the early choices by a trichromatic ruffed lemur under camouflage conditions comprised red food than green food (Figure 6B). The same biases were generally absent in conspecific males and conspecific homozygous females, as well as in other species tested. These data provide the first behavioral evidence that allelic trichromacy in strepsirrhines translates to a sensory capacity for red–green color discrimination.

Although the female ruffed lemurs in our study often performed like behavioral trichromats, they did not retrieve red items significantly faster overall than did their dichromatic conspecifics (and their detection of green items was comparatively slower). Nonetheless, if any foraging advantage conferred by trichromacy is relatively modest, our sample size for trichromatic ruffed lemurs may have been too small to detect it. The possibility of a modest advantage is supported by

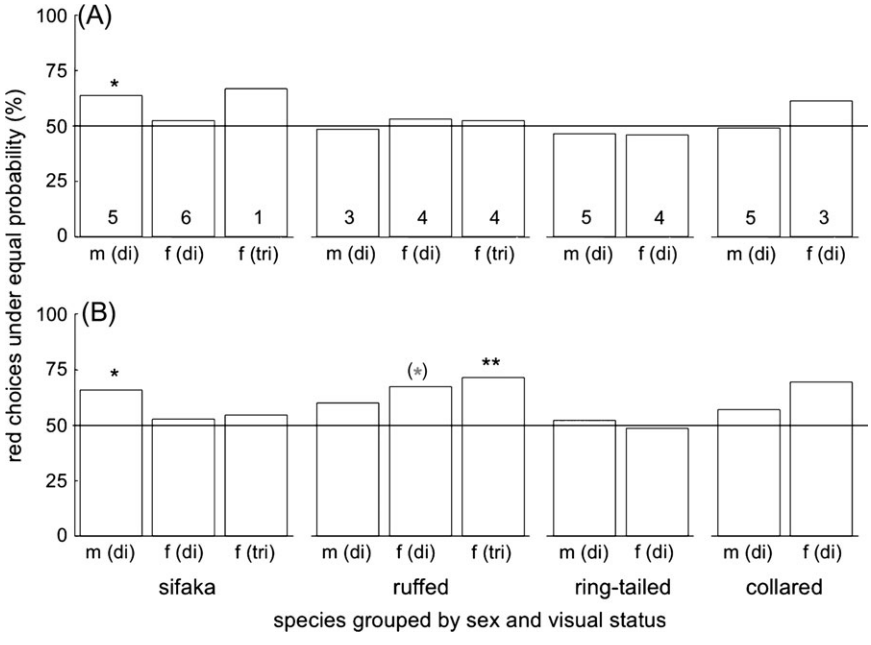

Figure 7
Percentage of red food choices made under equal probability conditions in the (A) control and (B) experimental conditions by strepsirrhine primates, grouped by species. Results are shown for male dichromats (m di), female dichromats (f di), and female trichromats (f tri), with sample sizes for each subgroup provided in (A). The horizontal line represents chance performance. Values above the line indicate preferences for selecting red food, as opposed to green food, under equal probability conditions (G test: $^*P < 0.05$ and $^{**}P < 0.01$; gray symbol within parenthesis reflects significance that disappears after excluding a single subject).

the small shift in spectral sensitivity conferred by the strepsirrhine opsin gene polymorphism, relative to the greater divergence between different opsin genes in catarrhines. In Coquerel's sifakas, for example, the spectral sensitivity of M- and L-cones is about 545 and 558 nm, respectively (Jacobs et al. 2002). These sensitivities yield a spectral separation between these 2 cones of approximately 13 nm in strepsirrhines, compared with 27 nm in catarrhines. As a similarly reduced spectral separation exists in the ruffed lemur, the trichromatic phenotype of strepsirrhine primates is probably more similar to human anomalous trichromacy (in which altered visual pigments result in an impairment, rather than loss, of trichromacy) than to normal catarrhine trichromacy (Jacobs and Deegan 2003a). Compared with Old World species, including humans, some trichromatic New World primates also have reduced spectral shifts between opsins and also show reduced ability to visually distinguish red–green color differences (Caine et al. 2003; Dominy, Garber, et al. 2003; Riba-Hernandez et al. 2004). For instance, using a comparable task presented to Geoffroy's marmosets (*Callithrix geoffroyi*), previous researchers found no foraging advantage for trichromatic females over dichromatic conspecifics (Caine et al. 2003).

Interestingly, we found no evidence of behavioral trichromacy in the sole heterozygous female sifaka. Although a sample size of one precludes generalization, it may be the case that not all species utilize red–green color perception equally or garner the same benefits from trichromacy. Perhaps trichromacy is functionally more important in the frugivorous ruffed lemurs than in the primarily folivorous sifakas, but larger samples will be required to draw such conclusions. Alternatively, as has been shown for heterozygosity at the opsin polymorphism and spectral sensitivity in ERG data (Jacobs et al. 2002), it may be the case that heterozygosity for the opsin gene polymorphism does not always predict behavioral trichromatism. Hence, the interindividual variation in performance we sometimes observed among the trichromatic ruffed lemurs may be due to incomplete penetrance of the opsin polymorphism. These latter possibilities highlight the need for behavioral testing whenever assessing or interpreting the functional significance of visual sensitivities.

That being said, the degree to which differences in visual discrimination are detected likely also depends on the nature of the foraging task. In our case, as a necessary first step, we presented subjects with food objects at close range, in a noncompetitive environment. The modest foraging advantages we observed in our trichromatic females were thus detected using a task with potentially low power to reveal differences in foraging behavior between visual groups. In follow-up studies, we suspect that stronger differences in visually guided performance may arise if tasks require greater travel distance (e.g., Caine and Mundy 2000) or incorporate social competition (Drea 2006). Nonetheless, this same task revealed strong differences in foraging behavior among the 4 lemur species. For instance, across species, the sole folivore showed the longest interval latencies to retrieve food. Such species-specific findings might suggest that variation in natural foraging ecologies, responsiveness to the task, or activity patterns may play an important role in foraging efficiency, irrespective of opsin trichromacy or dichromacy.

In addition, female collared lemurs showed a consistent preference for red-colored food, despite being homozygous at the known functional site in exon 5 of the X-linked opsin gene. These results may point to a trade-off between the foraging advantages associated with chromatic and achromatic vision (Livingstone and Hubel 1988; Mollon 1989). How species or populations respond to this trade-off is a subject of much debate (for a recent discussion, see Osorio et al. 2004). It is possible that the differences in visual ecology and food preferences between the species we examined might have favored the evolution of chromatic versus achromatic mechanisms to discriminate between potential food items. A dichromatic system would allow folivores, for example, to rely on shape and texture. Nevertheless, depending on reflectance spectra and light intensity, dichromats also may have some ability to detect red against green, as shown in modeling studies (Osorio et al. 2004). Indeed, under certain conditions associated with detecting objects under camouflaged or foliar conditions, dichromats outperform trichromats (Morgan et al. 1992; Smith et al. 2003). Likewise, under the camouflage conditions of our study, dichromats appeared to better maintain efficient retrieval of green food items than did trichromats. Assuming that such a trade-off exists, it is possible that both dichromatism and trichromatism could be maintained within a species, with each visual group being able to differentially exploit different foraging strategies.

Because both male and female collared lemur subjects were generally older than the subjects of our other species (Table 1) and performance on the foraging task improved with age (Supplementary materials), we cannot distinguish between an experiential effect and an effect of activity pattern (i.e., cathemerality, see Kirk 2006) on the mean performance of this species. Nevertheless, neither of these possibilities explains why, with the exception of trichromatic ruffed lemurs, only collared lemurs showed a sex difference in foraging on red and green food. One possibility is that a second functionally important polymorphism may be present on the X chromosome of this species, as is the case in many New World primates. To date, limited ERG flicker photometry has been conducted on female brown lemurs (Jacobs and Deegan 1993). We suggest that collared lemurs would be an excellent candidate species both for additional ERG analysis and for more extensive molecular genetic analysis of X-linked sequence variation.

Also related to species differences, and as an alternative to foraging pressures, color vision in anthropoid primates may have been driven by sexual selection and/or social communication, particularly involving male detection of female reproductive state (Dixson 1983; Liman and Innan 2003; Zhang and Webb 2003) or blushing (Changizi et al. 2006). Although the use of red coloration in primate social communication follows the predictions of the preexisting bias hypothesis (Fernandez and Morris 2007), the routine dichromacy of many male primates presents a drawback for this theory, as applied broadly across the primate order. More specifically, although sexual skin swellings have been best characterized in catarrhine females (e.g., Nunn 1999), strepsirrhine females also display cyclical swellings accompanied by changes in the color of genital skin (Drea and Weil 2008); yet, male strepsirrhines are routinely dichromatic.

In closing, although we found evidence for behavioral trichromacy in at least one lemur species, the ruffed lemur, it remains unclear what evolutionary advantage this trait might confer. That the genetic capacity for trichromacy exists in at least 2 strepsirrhines species, despite their different diets and the long divergence time between them, suggests that a general foraging-related mechanism for the evolution of primate color vision may have greater explanatory power than an argument based solely on frugivory or folivory. More parsimoniously, color vision may have evolved because it confers advantages related to detecting multiple meaningful environmental signals.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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