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Opsin Genes and Visual Ecology in a Nocturnal Folivorous Lemur

Carrie C. Veilleux · Rachel L. Jacobs · Molly E. Cummings · Edward E. Louis · Deborah A. Bolnick

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Abstract Primate color vision has traditionally been examined in the context of diurnal activity, but recent genetic and ecological studies suggest that color vision plays a role in nocturnal primate behavior and ecology as well. In this study, we united molecular analyses of cone visual pigment (opsin) genes with visual modeling analyses of food items to explore the evolution of color vision in the folivorous woolly lemur (genus *Avahi*). Previous studies have shown that leaf quality, e.g., protein content, leaf toughness, and protein/toughness ratio, is significantly correlated with green-red and blue-yellow chromatic differences, suggesting a potential role of color in leaf discrimination in *Avahi*, and, consequently, a potential adaptive advantage to color vision in this taxon. Phylogenetic selection tests determined that the strength of selection on the *SWS1* opsin gene to retain blue-sensitive SWS cones did not significantly differ in *Avahi* compared to day-active primates. Genotyping of the *M/LWS* opsin gene in 60 individuals from nine species found that the 558-nm-sensitive (red-sensitive) allele is conserved across all *Avahi.* Finally, we measured spectral reflectance from five species of young leaves

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C. C. Veilleux (✉)
Department of Anthropology, University of Texas at Austin, Austin, Texas 78712, USA
e-mail: carrie.veilleux@utexas.edu

R. L. Jacobs
Interdepartmental Doctoral Program in Anthropological Sciences, Stony Brook University, Stony Brook, New York 11794, USA

M. E. Cummings
Section of Integrative Biology, University of Texas at Austin, Austin, Texas 78712, USA

E. E. Louis
Molecular Genetics, Omaha's Henry Doorly Zoo and Aquarium, Omaha, Nebraska 68107, USA

D. A. Bolnick
Department of Anthropology and Population Research Center, University of Texas at Austin, Austin, Texas 78712, USA

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consumed by *Avahi* and background foliage in Ranomafana National Park and modeled performance of possible S and M/L pigment pairs for detecting these food items under different nocturnal illuminations (e.g. twilight, moonlight). We found that the observed cone pigment pair in *Avahi* was optimally tuned for color-based detection of young green leaves in all nocturnal light environments, suggesting a potential adaptive role of nocturnal color vision in selection for dichromacy in this genus.

Keywords Color vision · Foraging · Lemurs · Sensory ecology

Introduction

Compared to other mammalian orders, primates are highly visually oriented and exhibit several derived visual adaptations, such as increased acuity (Kirk and Kay 2004; Veilleux and Kirk 2009), relatively large eyes (Ross and Kirk 2007), greater orbital convergence for binocularity (Heesy 2008), and greater diversity of color vision abilities (Jacobs 2009; Surridge *et al*. 2003). Foraging tasks, habitat preference, and social signaling have all been proposed as potential factors influencing the evolution and diversity of visual abilities and anatomy in primates (Barton 1998; Mollon 1989; Regan *et al*. 1998; Ross and Kirk 2007; Yamashita *et al*. 2005; Veilleux and Lewis 2011). Although nocturnal environments offer more limited opportunities for visual function, a growing body of evidence suggests that vision can also be important for primates active at night. Several experimental and observational studies have determined that vision plays an important role in close-range prey detection in nocturnal strepsirrhines (Bearder *et al*. 2002; Nekaris 2005; Piep *et al*. 2008; Siemers *et al*. 2007). Similarly, visual performance in platform experiments does not significantly differ between nocturnal owl monkeys (genus *Aotus*) at night and sympatric diurnal species in daylight (Bicca-Marques and Garber 2004). Some researchers have even suggested that vision may be involved in aspects of nocturnal social communication or species recognition (Bearder *et al*. 2006).

Although ecological factors influencing the diversity of haplorhine visual abilities have been well studied (Mollon 1989; Surridge *et al*. 2003; Walls 1942), researchers have only recently begun identifying the extent of variation present and the ecological factors influencing vision in strepsirrhines. Actual estimates of strepsirrhine visual acuity are limited to six strepsirrhines (Veilleux and Kirk 2009). Yet despite these relatively limited data, studies of acuity and comparative visual anatomy suggest that activity pattern and diet have both influenced the evolution of visual resolution in strepsirrhines (Kirk 2006; Ross and Kirk 2007; Veilleux and Kirk 2009). Color vision abilities also appear highly variable among strepsirrhines. Most mammals have two types of retinal cones (short wavelength-sensitive S and medium/long wavelength-sensitive M/L), permitting dichromatic color vision between shorter (violets/blues) and longer (greens/yellows/reds) wavelengths of light (Jacobs 2009). Whereas dichromacy is retained in many lemurs, all lorisiforms have lost S cones (cone monochromacy) due to deleterious mutations in the *SWS1* opsin gene (Jacobs 2013; Kawamura and Kubotera 2004). Recent studies have also detected polymorphic trichromacy in several lemur species (Tan and Li 1999; Veilleux and Bolnick 2009). In these species, two or more alleles producing cone pigments with different spectral sensitivities are present at the X-linked *M/LWS* opsin locus (Surridge *et al*. 2003; Tan and Li 1999; Veilleux and Bolnick 2009). Consequently,

heterozygous females express both green- and red-sensitive M/L cones and can distinguish between greens and reds, whereas homozygous females and males are dichromats (Jacobs *et al*. 2002).

The distribution of color vision types across lemurs is not fully understood (Fig. 1), but does not appear to be closely tied to particular activity patterns. Polymorphic trichromacy has been identified in both diurnal and cathemeral species (Bradley *et al*. 2009; Kamilar *et al*. 2013; Tan and Li 1999; Veilleux and Bolnick 2009). Intriguingly, there also appears to be population-level variation in the presence of trichromacy in some species (Bradley *et al*. 2009). Although some researchers have hypothesized that all nocturnal primates should experience relaxed selection on the *SWS1* opsin gene leading to eventual S cone loss (Tan *et al*. 2005), recent genetic studies have detected signatures of purifying selection to maintain SWS cones and dichromacy in many nocturnal lemur species (Perry *et al*. 2007; Veilleux *et al*. 2013). In fact, S cone loss has been identified in only three nocturnal lemur lineages: *Allocebus trichotis, Cheirogaleus medius,* and *Phaner* (Jacobs 2013; Peichl *et al*. 2004; Veilleux *et al*. 2013).

In addition to variation in types of color vision, lemurs exhibit some variability in the peak spectral sensitivities (λ_{max}) of their M/L and S cones (Fig. 1). All lorisiforms, by contrast, appear to share the same M/L λ_{max} (Tan and Li 1999). To date, two M/L visual pigments have been identified in lemurs (543-nm-sensitive and 558-nm-sensitive) caused by a single nucleotide polymorphism in amino acid site 285 (Tan and Li 1999). Although evidence suggests that λ_{max} values of S cones vary between lemur genera, actual estimates of λ_{max} differ with measurement method. Studies

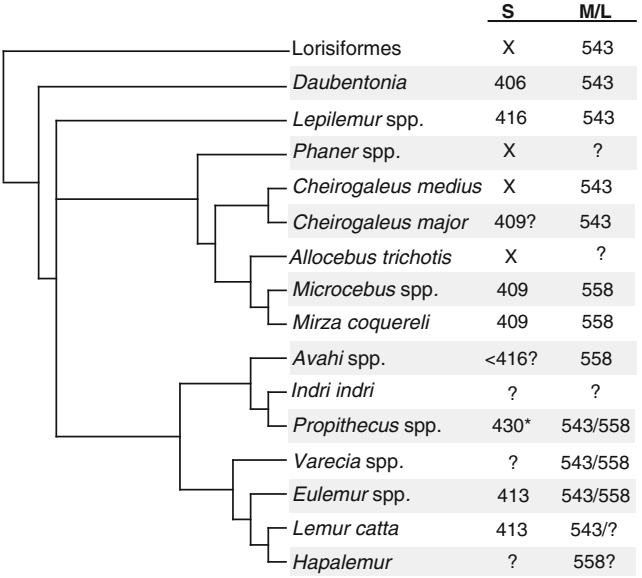


Fig. 1 Phylogeny and spectral tuning of cone visual pigments in strepsirrhines. "X" represents loss of the visual pigment and "?" reflects current uncertainty regarding pigment presence or spectral tuning. Note: the presence of S cones in *Cheirogaleus major* is currently unclear (Veilleux *et al*. 2013), while the presence/absence of polymorphic trichromacy in all *Lemur catta* and *Hapalemur* is also uncertain. (*) Spectral tuning for *Propithecus* is measured physiologically. Data sources for SWS: Carvalho *et al*. 2012; Jacobs *et al*. 2002; Kawamura and Kubotera 2004; Peichl *et al*. 2004; Veilleux *et al*. 2013. Data sources for M/LWS: Kamilar *et al*. 2013; Tan and Li 1999; Veilleux and Bolnick 2009; Veilleux unpublished data (*Phaner*).

expressing S opsin pigments or comparing amino acids present at *SWS1* opsin gene spectral tuning sites suggest a range of 406–416 nm in lemurs (Carvalho *et al*. 2012; Veilleux *et al*. 2013). In contrast, physiological estimates using electroretinography (ERG) identified S λ_{max} at 430 nm and 437 nm in indriids and lemurids (Jacobs and Deegan 1993; Jacobs *et al*. 2002). Thus, S λ_{max} for *Eulemur fulvus* has been estimated at both 413 nm (pigment expression: Carvalho *et al*. 2012) and 437 nm (ERG: Jacobs and Deegan 1993). This difference may reflect filtering effects of the cornea and lens, leading to S λ_{max} overestimation (Carvalho *et al*. 2012). Although diurnal haplorhines and sciurids exhibit short wavelength-filtering lenses (Kirk and Kay 2004), it is not clear whether diurnal/cathemeral lemurs share this trait.

Despite the relatively limited range of lemur S and M/L λ_{max}, species and genera vary in the combination of pigments they possess (Fig. 1). Although it is possible that this variation is a product of genetic drift or phylogenetic inertia, it could also reflect ecologically driven spectral tuning for selectively important visual tasks. For instance, a recent study of aye-ayes (*Daubentonia madagascariensis*) hypothesized that aye-aye cones are spectrally tuned to detect preferred foods in twilight or full moonlight (Melin *et al*. 2012).

In this study, we sought to explore color vision and visual ecology in the woolly lemur (genus *Avahi*), a 0.5- to 1-kg folivore from the dry deciduous forest and rain forest of Madagascar (Ganzhorn 1988; Ganzhorn *et al*. 1985; Thalmann 2001). Of nocturnal lemurs, *Avahi* offers an interesting case study for the visual ecology of color. Feeding studies suggest that *Avahi* are discriminate folivores, preferring leaves high in protein and sometimes discriminating based on secondary compounds (Ganzhorn 1988; Norscia *et al*. 2012). Protein content; secondary compounds, e.g., phenols, tannins; leaf toughness; and protein/toughness ratio all significantly correlate with green-red and blue-yellow chromatic differences (Dominy and Lucas 2004), suggesting a potential role of color in leaf discrimination in *Avahi*. In particular, the protein/toughness ratio is a measure of leaf quality and is important for leaf selection in other primate folivores (Lucas *et al*. 2003). We thus hypothesize that *Avahi* may be using color to detect and discriminate these preferred leaves. Our hypothesis is bolstered by the results of a recent molecular study that detected selection to maintain *SWS1* opsin gene function, and therefore color vision, across *Avahi* (Veilleux *et al*. 2013). Moreover, some researchers have hypothesized that *Avahi* has secondarily transitioned to a nocturnal lifestyle, evolving from a diurnal or cathemeral ancestor with polymorphic trichromatic color vision (Ganzhorn *et al*. 1985; Griffin *et al*. 2012; Roos *et al*. 2004; Veilleux and Bolnick 2009). Thus, the evolution of color vision in *Avahi* could offer an interesting comparison to that in other secondarily nocturnal primates, such as *Aotus* and tarsiers.

We united molecular and ecological approaches to address three questions about the ecology and evolution of color vision in *Avahi*. First, is there evidence of change in selection on the *SWS1* opsin gene through the evolution of the lineage *Avahi*? This question is particularly relevant to hypotheses of secondary nocturnality. In secondarily nocturnal haplorhines, relaxed selection on the *SWS1* gene led to S cone loss in *Aotus*, while purifying selection resulted in S cone retention in tarsiers (Jacobs 2013; Kawamura and Kubotera 2004). Although a recent study identified purifying selection acting to retain dichromacy in *Avahi* and other nocturnal lemurs (Veilleux *et al*. 2013), that study primarily compared phylogenetic signatures of selection on *SWS1* in the context of other extant nocturnal lemur lineages. Here, we used phylogenetic tests

to specifically estimate the strength of purifying selection on *SWS1* in the last common ancestor (LCA) of *Avahi* compared to day-active lemurs and other day-active primates. If color vision has remained selectively important throughout the evolutionary history of *Avahi* (such as for detecting preferred leaves), we predict that there will be no significant difference in the strength of selection estimated for *Avahi* compared to that on the rest of the day-active tree.

Second, is there evidence of intraspecific or interspecific variation in predicted M/L spectral tuning among *Avahi*? Current understanding of M/L spectral sensitivity in *Avahi* is based on a sample of only three *A. laniger* females of undescribed provenience, all exhibiting the 558-nm-sensitive allele (Tan *et al*. 2005). However, there is some suggestion that the last common lemurid-indriid ancestor may have exhibited polymorphic trichromacy (Roos *et al*. 2004; Veilleux & Bolnick 2009). In this study, we expand the available sample to include 60 individuals from populations of nine species of *Avahi* across Madagascar to explore whether there is variation in M/L λ_{max} between or within *Avahi* spp. This broad sampling also permits us to explore potential environmental effects on variation in M/L λ_{max} of *Avahi*, as have been hypothesized for tarsiers (Melin *et al*. 2013; Moritz and Dominy 2010).

Third, what are the optimal cone spectral sensitivities for detecting foods consumed by *Avahi* under different illuminations? In this study, we explore the effectiveness of different combinations of M/L and S cone λ_{max} for detecting foods of *Avahi* against a mature leaf background under twilight and nocturnal light environments. Optimizing cone spectral tuning to preferred foods should improve foraging efficiency by shortening visual search time and enhancing discrimination of the most nutritionally rewarding food items. Similar optimal pigment modeling techniques have been used to examine spectral tuning and diet in platyrrhines and catarrhines (Regan *et al*. 1998; Sumner and Mollon 2000). We predict that if color vision is important, the pigments observed in *Avahi* living in a rain forest environment will outperform other possible lemur pigments in chromatic (color-based) detection of food species consumed by *Avahi*. We also predict that observed pigments of *Avahi* will have greater detection performance for plant species comprising a larger proportion of the diet. By examining target detection using both chromatic and luminance vision (Cummings 2004), the results of this model permit us to explore potential effects of color, luminance, and light environments (twilight, moonlight) on foraging ecology in *Avahi.*

Methods

Selection on the *SWS1* Opsin Gene

We examined the history of selection to maintain dichromacy in *Avahi* by analyzing the *SWS1* opsin gene in lineages of *Avahi* and day-active (cathemeral and diurnal) primates. We assembled a data set of complete *SWS1* exon sequences from GenBank for 4 *Avahi* spp. (*A. cleesei, A. laniger, A. occidentalis, A. peyrierasi*), 12 other primates (3 lemurs, 2 platyrrhines, 8 catarrhines), and 2 cathemeral mammals as outgroups (*Felis catus*, *Bos taurus*). Because the indriid and lemurid–indriid LCA activity patterns are uncertain, we ran an additional analysis with a diurnal tree shrew

(*Tupaia belangeri*) outgroup, for which only a partial *SWS1* gene was available from Ensembl. We aligned nucleotide sequences with ClustalW in MEGA version 5.1 (Tamura *et al*. 2011). We constructed a *SWS1* gene phylogenetic tree using bootstrap maximum likelihood (ML) and Bayesian techniques. We constructed the ML tree in MEGA using the HKY+G model of sequence evolution estimated by ModelTest 3.6 (Posada and Crandall 1998). For this tree, we employed a heuristic search using the nearest-neighbor-interchange method and generated 500 bootstrap replicate ML trees. We constructed the Bayesian tree in MrBayes v.3.2.1 (Ronquist *et al*. 2012) using the HKY+G model of sequence evolution.

We used the ML bootstrap tree for codon-based selection tests in the codeml program in PAML version 4.5 (Yang 2007). These tests compared estimates of the ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions ($d_N/d_S = \omega$) for each branch of the tree under different models of evolution using likelihood ratio tests (LRTs). In these analyses, ω represents the type and magnitude of selection acting on the gene, with $\omega < 1$ indicating purifying selection to retain function and remove deleterious mutations, $\omega = 1$ indicating neutral evolution, and $\omega > 1$ indicating positive selection for increased diversity (Yang 2007). We used branch model tests to evaluate whether selection for maintaining *SWS1* functionality varies between nocturnal *Avahi* and day-active primate lineages. The null model assumes a single ω parameter characterizes all branches within the tree. In the alternative two-ratio models, we designated certain branches of interest as "foreground" branches that are permitted to have a different ω than all of the other branches of the tree. We tested two alternative models: one designating just the LCA branch of *Avahi* as foreground and the other designating all *Avahi* + the *Avahi* LCA as foreground. The fit of each alternative model to the data was compared to the fit of the null model using LRTs, where the LRT statistic was computed as 2*log likelihood difference (2ΔLnL) between the models and tested against the χ^2 distribution, with one degree of freedom (the difference between the null model and the alternative model in number of parameters) (Yang 2007).

M/LWS Opsin Genotyping and Spectral Tuning

We examined the *M/LWS* opsin gene in 60 *Avahi* (50 females, 10 males) from 9 species (Table I). Samples were collected from wild individuals across Madagascar. Genomic DNA was extracted from tissue samples using a standard phenol-chloroform isoamyl alcohol extraction protocol at the Conservation Genetics Laboratory at Omaha's Henry Doorly Zoo and Aquarium (OHDZA, Omaha, NE). All immobilizations, handling, sample collections, and export/import protocols adhered to and were approved by the OHDZA's Institutional Animal Care and Use Committee (IACUC), Convention on International Trade in Endangered Species regulations, US Fish & Wildlife Services, and laws of the wildlife and government of Madagascar.

Previous work has identified amino acid site 285 in exon 5 as the primary site involved in lemur M/L cone spectral tuning (Tan and Li 1999; Veilleux and Bolnick 2009). We used published primers (Jacobs *et al*. 2002) to sequence exon 5 and genotype all individuals. We performed polymerase chain reactions (PCRs) in 25-μl reactions containing 2–3 μl of DNA template, 0.625 μl of each 20 μM primer, and 22.5 μl *Taq* Mastermix or Platinum *Taq* Mastermix (Invitrogen/Life Technologies, Grand Island, NY). The PCR conditions were: 1) an initial 2-min hold at 94°C; 2) 40 cycles of 30 s at

Table I Species, collection site, sample size, and *M/LWS* allele

Species	Site (habitat[a])	N (F, M)	M/LWS
Avahi betsileo	Fandriana (r)	4, 0	558 nm
A. cleesei	Tsingy de Bemaraha (d)	2, 1	558 nm
A. laniger	Mantadia (r)	3, 4	558 nm
A. laniger	Mananara-Nord (r)	7, 0	558 nm
A. meridonalis	Andohahela (r)	7, 0	558 nm
A. mooreorum	Masoala (r)	5, 0	558 nm
A. occidentalis	Mariarano (Mahajunga) (d)	3, 4	558 nm
A. occidentalis	Ankarafantsika (d)	4, 0	558 nm
A. peyrierasi	Talatakely-RNP (r)	2, 1	558 nm
A. peyrierasi	Vohiparara-RNP (r)	4, 0	558 nm
A. ramanantsoavanai	Vevembe (r)	5, 0	558 nm
A. unicolor	Antafondro (s)	2, 0	558 nm
A. unicolor	Ampasindava (s)	2, 0	558 nm

[a] Habitat categories: rain forest (r), open canopy dry deciduous forest (d), Sambirano forest (s)— transitional between rain forest and deciduous forest.

94°C, 30 s at 56.6°C, and 1 min at 72°C; and 3) a final hold for 6 min at 72°C. PCR products were purified using magnetic beads (Thermo Fisher Scientific, Waltham, MA) and sequenced at the University of Texas at Austin DNA Sequencing Facility. We analyzed sequences using Sequencher v.5.0.1 (Gene Codes, Ann Arbor, MI).

Visual Ecology of Foods Consumed by *Avahi*

Field Site Samples of food items consumed by *Avahi* were collected in Ranomafana National Park (RNP), which occupies *ca*. 43,500 ha of continuous montane rain forest in southeastern Madagascar (E47°18 –47°37 , S21°02 –21°25). Although previous research identified the *Avahi* sp. present in Ranomafana as *Avahi laniger*, a recent taxonomic revision has reclassified Ranomafana *Avahi* as *A. peyrierasi* (Andriantompohavana *et al*. 2007). One of 12 species of lemurs found in RNP, the dietary composition of *Avahi peyrierasi* at this site has previously been described (Faulkner and Lehman 2006; Harcourt 1991).

Data Collection and Reflectance Measurement We assembled a list of leaf food species consumed by *Avahi* from previous feeding ecology studies at RNP (Online Resource 1). In November 2013, we worked with a local botanist to collect samples of young and mature leaves from as many of these species as possible. Five species had young leaves flushing during the collection period. These five comprise a combined 61.2% of feeding time in the Harcourt (1991) study and 71.6% of feeding time in the Faulkner and Lehman (2006) study. One of these species (*Harungana madagascariensis*) was the predominant species consumed in both studies (42.15% and 48.51%, respectively). The next most common plant species we collected were *Erythroxylum* sp. (1.64–12.52%), followed by *Gaetnera* sp. at 8.26% (only in Harcourt's study), *Dombeya pubescens* (5.85–6.61%), and *Canthium* sp. (2.48–4.72%). We measured reflectance spectra from at least three mature and four young

leaves per species. *Erythroxylum* is not distinguished locally by species, but is distinguished as having a large and small leaf "morph." Each has its own young and mature leaves. Because *Erythroxylum* was not distinguished by leaf morph type in the previous studies, we analyzed each young leaf morph separately.

We recorded the reflectance spectra of sample leaves on the day they were collected (within 5 h of collection) using a USB2000+UV–VIS Miniature Fiber Optic Spectrometer (Ocean Optics, Dunedin, FL). We recorded all measurements under standardized lighting conditions using a PX-2 Pulsed Xenon Light Source (Ocean Optics) and relative to a diffuse reflectance standard (WS-1, Ocean Optics). The reflection probe was maintained at a fixed angle (45°) and distance (5 mm) from each sample using a probe holder (RPH-1; Ocean Optics). During the measurement periods, we recalibrated the spectrometer frequently to minimize drift. We took two or three independent measurements from the top and bottom of each leaf.

Modeling Optimal Visual Pigments To estimate the optimal visual pigment pair for detecting foods consumed by *Avahi* in nocturnal and twilight environments, we calculated foraging target detection performances of 61 M/L and 61 S model absorbance curves. Model absorbance curves were calculated using A1 algorithm templates (Govardovskii *et al*. 2000) and varying the λ_{max} at 1-nm intervals for S (400–460 nm) and M/L (520–580 nm) and normalized to the maximum absorption peak (λ_{max}). We evaluated the performance of each pair of model M/L and S absorbance spectra in discriminating differences in two foraging targets (young leaf against background mature leaves) using estimates of luminance and chromatic contrast pathways (similar to Cummings 2004) across different irradiance, $I(\lambda)$, measurements. The spectral or chromatic feature, S, of each target was evaluated as the difference in quantum catch (Q_c) between M/L and S cone classes ($S_t = Q_{sws} - Q_{lws}$), where $Q_c = \int_{\lambda=400}^{700} I(\lambda)R_t(\lambda)A_c(\lambda)d\lambda$, with $A(\lambda)$ representing the normalized absorptance spectrum for a specific cone class, c; and $R_t(\lambda)$ representing the reflectance spectrum of a given foraging target. Spectral contrast was then evaluated as $\Delta S = S_{t1} - S_{t2}$.

We evaluated the luminance or brightness feature, L, of each target in two ways — including and excluding S cone contribution— because the importance of S cones in luminance vision in mammals is not fully established (Chatterjee and Callaway 2002; Ripamonti *et al*. 2009). Previous work suggested that S cones do not contribute to luminance, but more recent studies have found evidence of some S cone contribution (Chatterjee and Callaway 2002; Li and De Vries 2006), although the contribution may depend on the presence of a background radiance that excites M/L cones (Ripamonti *et al*. 2009). Because the degree of S cone contribution to luminance vision in *Avahi* is uncertain, we evaluated L as both the summation of quantum catch for M/L and S cone classes, ($L_t = Q_{sws} + Q_{lws}$) and as a single (M/L cone class) channel ($L_t = Q_{lws}$) and then evaluated brightness contrast as the difference between targets ($DL = L_{t1} - L_{t2}$) for each possible luminance pathway.

For foraging targets, we calculated mean "young leaf" and "mature leaf" reflectance spectra for each species. For *Erythroxylum*, we separately analyzed the big morph young leaf and small morph young leaf against a combined morph mature leaf background. We modeled detection performance under four irradiance spectra (Fig. 2). Three spectra represent nocturnal conditions (gibbous moonlight in the understory of a closed canopy rain forest, full moonlight and no moon in the understory of an open canopy dry deciduous forest) previously measured in Madagascar by two of the authors (Veilleux

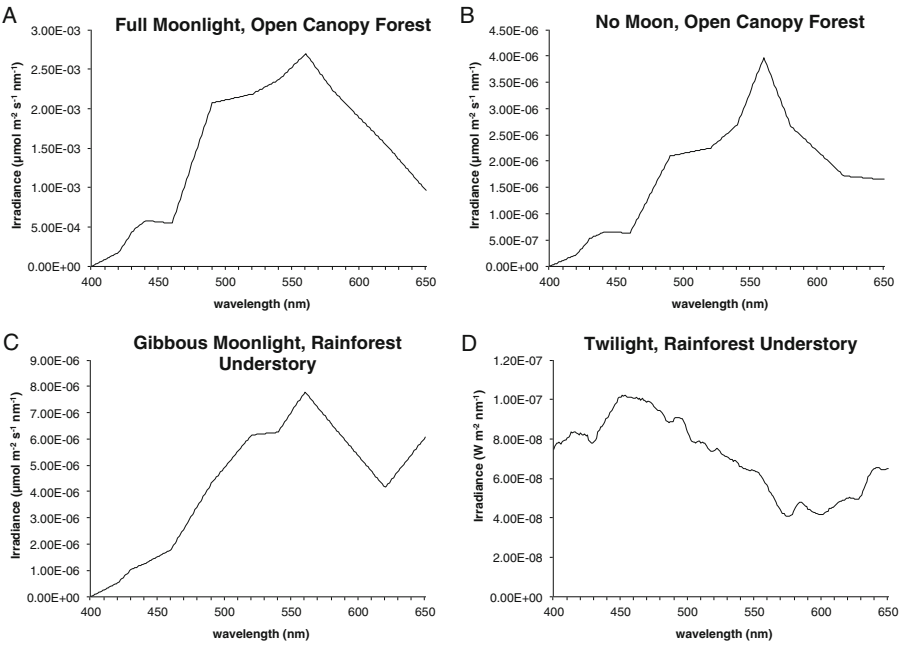


Fig. 2 Irradiance spectra for modeling analyses. **(a–c)** Published nocturnal spectra from Malagasy forests (Veilleux and Cummings 2012), measured using an IL 1700 research radiometer and a PMC271C detector (International Light) with 12 narrow bandpass filters (Newport Oriel Corporation). Open canopy forest spectra from dry deciduous forest at Kirindy Mitea National Park during dry season (July–September 2009). Closed canopy rainforest measurement from Ranomafana National Park (October 2009). **(a)** Mean full moonlight irradiance from open locations (33–50% canopy openness, $N = 19$) in dry deciduous forest during a clear night at high lunar altitude (>70°). **(b)** Mean irradiance from open locations (40–50% canopy openness, $N = 37$) in a dry deciduous forest during a clear night sky with no moon present. **(c)** Mean gibbous moonlight (70–90% full) from understory of a closed canopy rainforest (15–22% canopy openness, $N = 9$) at low lunar altitude (22–40°). (d) Twilight irradiance from understory of closed canopy rain forest at Kianjavato in southeastern Madagascar using an OL770 spectroradiometer (Gooch & Housego) during a clear sky at 17:40 h on June 27, 2012, solar altitude at 1°.

and Cummings 2012). Because the overall shape of nocturnal irradiance spectra does not vary between the two habitats (Veilleux and Cummings 2012), the open canopy spectra can serve as proxies for light environments higher in the rainforest canopy where canopy openness is greater. We also used a rain forest twilight irradiance spectrum collected on a clear evening at 17:40 h (solar altitude at 1°) in the Sangasanga forest (Kianjavato) in southeastern Madagascar that was graciously provided by Steig Johnson, Sheila Holmes, Nate Dominy, and Amanda Melin. We restricted the target detection analyses to the tops of the young and mature leaves because our irradiance spectra were only taken in downwelling light (detector pointed upward). These irradiance spectra do not represent the light environments in which leaf bottoms are viewed. Our analyses thus make the assumption that *Avahi* is looking down at the leaves during detection.

Statistical Analyses We compared the performances of observed M/L and S visual pigment pairs of *Avahi* for different classes of targets, light environments, and target detection styles (chromatic, luminance) to the optimal performing pigment pair for each condition. The observed M/L pigment λ_{max} was that identified in our *M/LWS* opsin genotyping analysis (exon 5, tuning site 285). Determining the appropriate S

visual pigment λ_{max} for these comparisons was more difficult because no physiological tests or opsin expression studies of S cones of *Avahi* have been conducted. Using *SWS1* opsin gene tuning sites, Veilleux and colleagues found that *Avahi* differed from *Lepilemur* (estimated at 416 nm) at one tuning site with a residue suggested to shift λ_{max} to shorter wavelengths (Veilleux *et al*. 2013), and thus predicted that the S visual pigment of *Avahi* has $\lambda_{max} < 416$ nm. An additional concern for determining observed S λ_{max} is the effects of filtering from the cornea and lens (Carvalho *et al*. 2012). However, because no data are available on cornea and lens filtering effects in nocturnal lemurs, we followed Melin and colleagues' study of visual ecology of *Daubentonia* (Melin *et al*. 2012) using the pigment expression-based S λ_{max} and chose 416 nm for statistical analyses. We also determined performance for 430 nm, the S λ_{max} of *Propithecus coquereli* (Jacobs *et al*. 2002) as a possible ancestral day-active condition.

We used linear mixed-effects models (LMMs) to explore whether there were any overarching effects of young leaf color, light environment, and detection on the chromatic and luminance detection performances of the observed pigment pair of *Avahi*. Because it is currently not known at what light levels nocturnal lemur cones can function, we restricted these analyses to performance data under twilight and full moonlight open canopy conditions. Cathemeral horses and diurnal humans can make color discriminations in some moonlight levels (Roth *et al*. 2008), whereas nocturnal mice cones can function at dim moonlight levels (Umino *et al*. 2008). As such, cones in nocturnally adapted *Avahi* should also be able to function in at least full moonlight and possibly other conditions. We implemented the LMMs in R version 2.15.2 (R Core Team 2012) using the *lme4* package (Bates *et al*. 2012). We tested the effects of target young leaf color ("red," "green"), light environment (twilight, moonlight), and detection type (chromatic, luminance with S cones and without S cones) on the dependent variable target detection performance. Young leaves were categorized as "green" if peak reflectance was *ca*. 550 nm and as "red" if peak reflectance was >600 nm. For all models, we designated leaf species as a random effect to control for performance differences among species. We computed *P* values for fixed effects using the *languageR* package (Baayen 2011). We also used LMMs to compare the detection performance of alternate visual pigments of *Avahi* (M/L: 543 vs. 558 nm, S: 416 vs. 430 nm). For these analyses, we used the *anova* function to compare the fit of two alternative models to the data: one that includes allele type as a fixed factor and one that excludes allele type.

Results

Selection on *SWS1* Opsin Gene

Selection Analyses SWS1 opsin gene trees constructed using Bayesian and ML bootstrap methods are very similar, differing only in branching patterns among the hominoids (Fig. 3). The ML bootstrap tree found a trichotomy between the *Nomascus, Pongo*, and (*Pan* + *Homo*) branches. The Bayesian tree found a sister relationship between the *Pongo* and (*Pan* + *Homo*) branches, but the posterior probability for the clade is only 57/100. We report the selection results based on the ML bootstrap tree but results using the Bayesian tree are comparable.

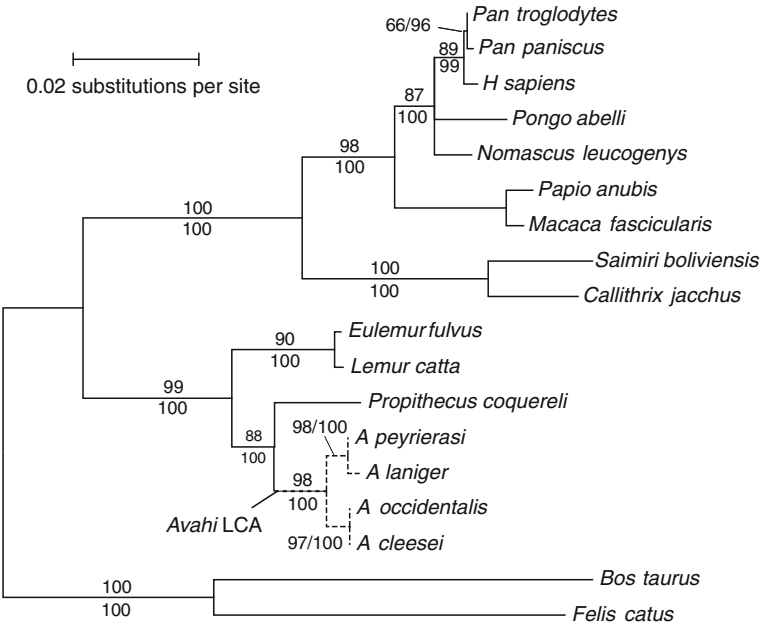


Fig. 3 *SWS1* gene tree for selection tests including cathemeral mammals as outgroups. Numbers above and below the branches represent the ML bootstrap values and Bayesian posterior probabilities, respectively. Dashed lines depict the branches used in branch models tests: *Avahi* LCA only and all *Avahi* (including the *Avahi* LCA).

Using branch model tests, we examined the history of selection on the *SWS1* opsin gene in *Avahi* compared to diurnal and cathemeral mammals. With the cathemeral mammal outgroups, results of the branch model tests suggest that the strength of selection to maintain *SWS1* opsin gene functionality did not significantly change through *Avahi*'s evolution, including during the postulated transition to nocturnality. The null model (which assumes a single ω across all branches) found ω = 0.24, consistent with purifying selection on *SWS1* across the tree. When ω was permitted to differ between the *Avahi* LCA branch (Fig. 3) and all other branches of the tree, the two ω values were estimated to be identical ($\omega_1 = 0.24$, $\omega_0 = 0.24$, respectively). Not surprisingly this *Avahi* LCA two-ratio model does not fit the data better than the null model (LRT: $2\Delta\text{LnL} = 0.0096$, df = 1, $P = 0.922$). A two-ratio model comparing all branches of *Avahi* to all other branches of the tree ($\omega_1 = 0.25$, $\omega_0 = 0.24$, respectively) also does not fit the data better than the null model (LRT: $2\Delta\text{LnL} = 0.0016$, df = 1, $P = 0.968$). The results exhibit the same pattern when the partial diurnal sequence for *Tupaia* is included.

M/L Cone Spectral Tuning

We found no evidence of intraspecific or interspecific variation in *M/LWS* exon 5 tuning sites in the 60 individuals of *Avahi* sampled (Table I). Regardless of habitat type or species, all *Avahi* exhibit the same 558-nm-sensitive allele (residue threonine at amino acid position 285).

Reflectance and Detection of Foods Consumed by *Avahi*

Reflectance Spectra The spectral reflectances of *Avahi*-consumed young leaves at RNP can be divided into two major types similar to previous findings in a rainforest in Uganda (Sumner and Mollon 2000). In the first type, the young leaf spectrum exhibits peak reflectance at *ca*. 550 nm ("green"). Although these "green" young leaves have a similar peak reflectance as mature leaves, the young leaves exhibit much greater total reflectance (Fig. 4b, c). This "green" young leaf characterizes *Canthium, Gaetnera*, and the small leaf morph of *Erythroxylum* (Fig. 4b, c). In the second type, the young leaf spectrum has greater reflectance at longer wavelengths (>600 nm: "red") compared to mature leaves. This "red" young leaf is found in *Harungana*, *Dombeya,* and the big leaf morph of *Erythroxylum* (Fig. 4a, b). While all three "red" young leaf species exhibit greatest reflectance at longer wavelengths, *Harungana* and *Dombeya* differ from the *Erythroxylum* big leaf morph in having overall greater reflectance at other wavelengths as well.

Performance of Observed Visual Pigments of Avahi *in Target Detection* We modeled the optimal S and M/L cone pair to detect each young leaf target against the mature background (Fig. 4d) separately for 1) chromatic detection, 2) luminance detection with S cone contribution (S cone+), and 3) luminance detection without S cone contribution (S cone–). Across all light environments, the performance of the observed visual pigments of *Avahi* (S: 416 nm, M/L: 558 nm) generally appears related to target leaf color (Fig. 4d, also Online Resource 2). The *Avahi* pigment pair exhibits near perfect performance in detecting young "green" leaves using chromatic cues (mean performance = 97.9% ± SD 2.2%). Pigments of *Avahi* perform slightly less well in detecting young "green" leaves using S cone+ luminance contrasts (mean performance = 87.6% ± SD 3.1%), but perform better when S cone contribution is excluded (mean performance = 98.1% ± SD 2.1%). Interestingly, pigments of *Avahi* appear much less optimized for detecting young "red" leaves (Fig. 4d). Overall, chromatic, S cone+ and S cone– luminance performances are *ca.* 32%, *ca*. 17%, and *ca*. 20% greater, respectively, for "green" compared to "red" young leaf targets (Online Resource 2). Mean chromatic performance for "red" young leaves is 65% (± SD 10.8%), while mean luminance performance is 70.9% (± SD 7.7%).

We further quantified the effects of leaf color, target detection type, and light environment on the performance of the observed pigment pair of *Avahi* using LMMs. When S cone contribution is included in luminance, leaf color ($P = 0.0001$) and target detection type ($P = 0.029$) have significant effects on performance. There are also significant color*light environment ($P = 0.011$) and color*detection*light ($P = 0.026$) interactions. These results suggest that performance of the observed pigment pair of *Avahi* is substantially lower for "red" targets, for luminance detection, and for red targets in twilight (Online Resource 3). Consequently, performance is higher for "green" targets and for chromatic detection. To understand better the three-way interaction, we also ran separate LMMs for "green" and "red" leaf targets. For young "green" leaves, performance is significantly influenced only by detection type ($P < 0.0001$), suggesting that the pigment pair of *Avahi* is better at chromatic detection than luminance detection. For young "red" leaves, both light environment ($P = 0.018$) and a light*detection interaction are significant ($P = 0.027$). Thus for "red" targets, chromatic performance decreases in twilight, while luminance detection increases. When S cone

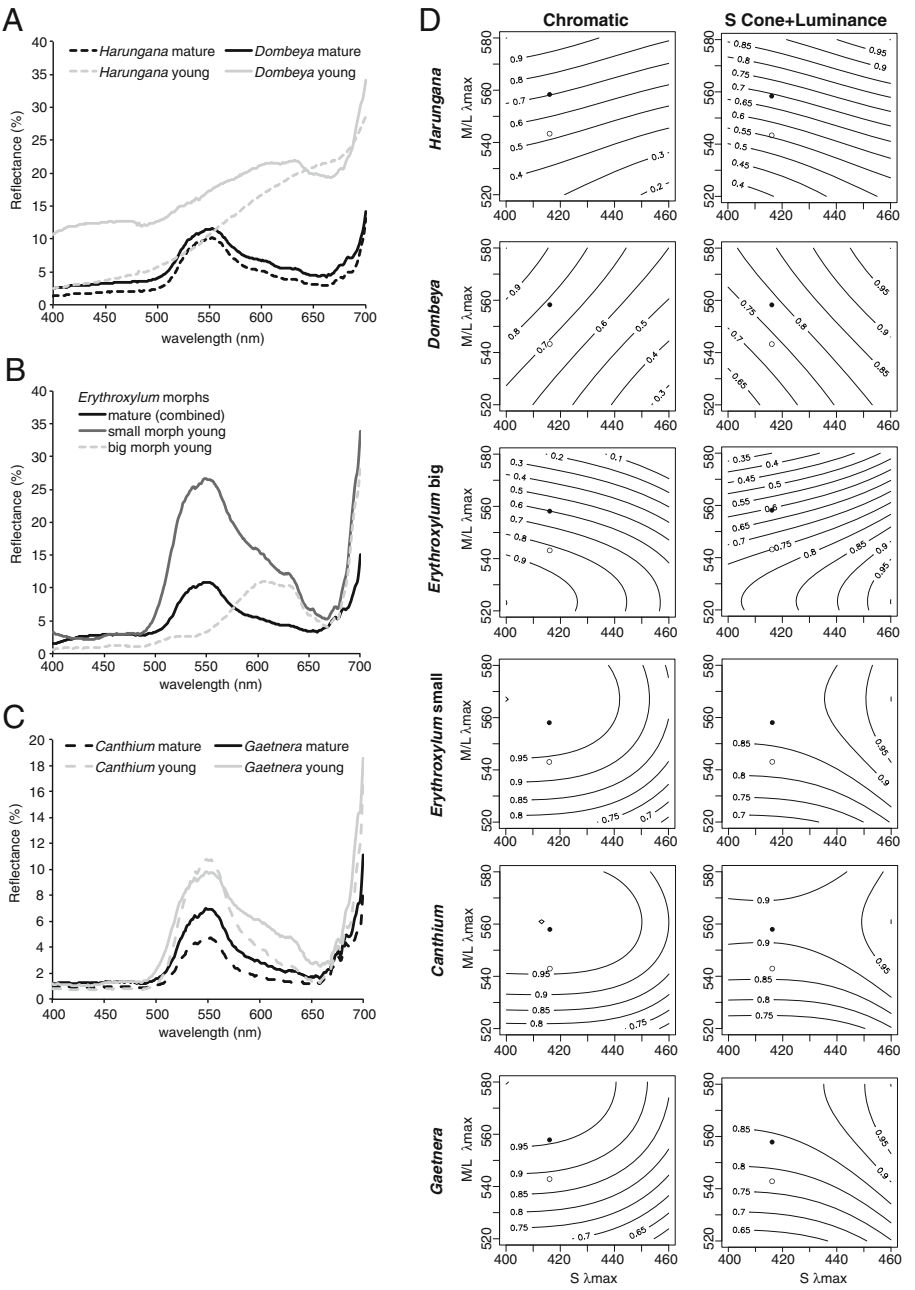


Fig. 4 Spectral reflectance **(a–c)** and optimal visual pigment models **(d)** for *Avahi*-consumed foods. Reflectances measured at RNP for **(a)** "red" young leaves (*Harungana madagascariensis, Dombeya pubescens*); **(b)** small and big leaf morph types of *Erythroxylum* sp.; **(c)** "green" young leaves (*Canthium* sp., *Gaetnera* sp.). **(d)** Contour plots of all pigment pair performances in chromatic and S cone+ luminance detection for each young leaf target against its mature leaf background under full moonlight open canopy irradiance (twilight results in Online Resource 4). Solid circle: observed *Avahi* pigments (416 nm, 558 nm), open circle: 543-nm allele alternative.

contribution is excluded, the significant effects/interactions of target detection type disappear in all models, although the significant leaf color ($P = 0.0002$) and color*light interactions ($P = 0.0198$) remain. These results suggest that while performance of the observed pair is substantially lower for red targets, particularly in twilight, the pigments of *Avahi* perform equally well in chromatic and luminance detection if S cones do not contribute to luminance.

Performance Comparisons with Alternate Visual Pigments When we compared the target detection performance for the observed pigment pair of *Avahi* with other potential pigments (M/L λ_{max} = 543 nm, S λ_{max} = 430 nm), we found that for all but one plant species, the 558-nm allele performs better at target detection than the 543-nm allele (Figs. 4d and 5a, b). The 543-nm allele is better at detecting the big leaf morph of *Erythroxylum* in chromatic and luminance detection tasks. Results from LMMs support these generalizations. Models including allele type (543 vs. 558 nm) in analyses of the effects of light environment (twilight, full moonlight) and detection type (chromatic, S cone+ luminance) provide a better fit to the data relative to models excluding them for "green" targets (LMM: $\chi^2 = 48.41$, df = 4, $P < 0.00001$), but not for "red" targets (LMM: $\chi^2 = 0.17$, df = 4, $P = 0.997$), even when the big leaf morph of *Erythroxylum* is excluded (LMM: $\chi^2 = 8.162$, df = 4, $P = 0.086$). The same results are achieved when the analyses are repeated for S cone– luminance: allele type is important for model fit for "green" targets (LMM: $\chi^2 = 63.42$, df = 4, $P < 0.00001$) but not "red" targets (LMM: $\chi^2 = 0.15$, df = 4, $P = 0.997$).

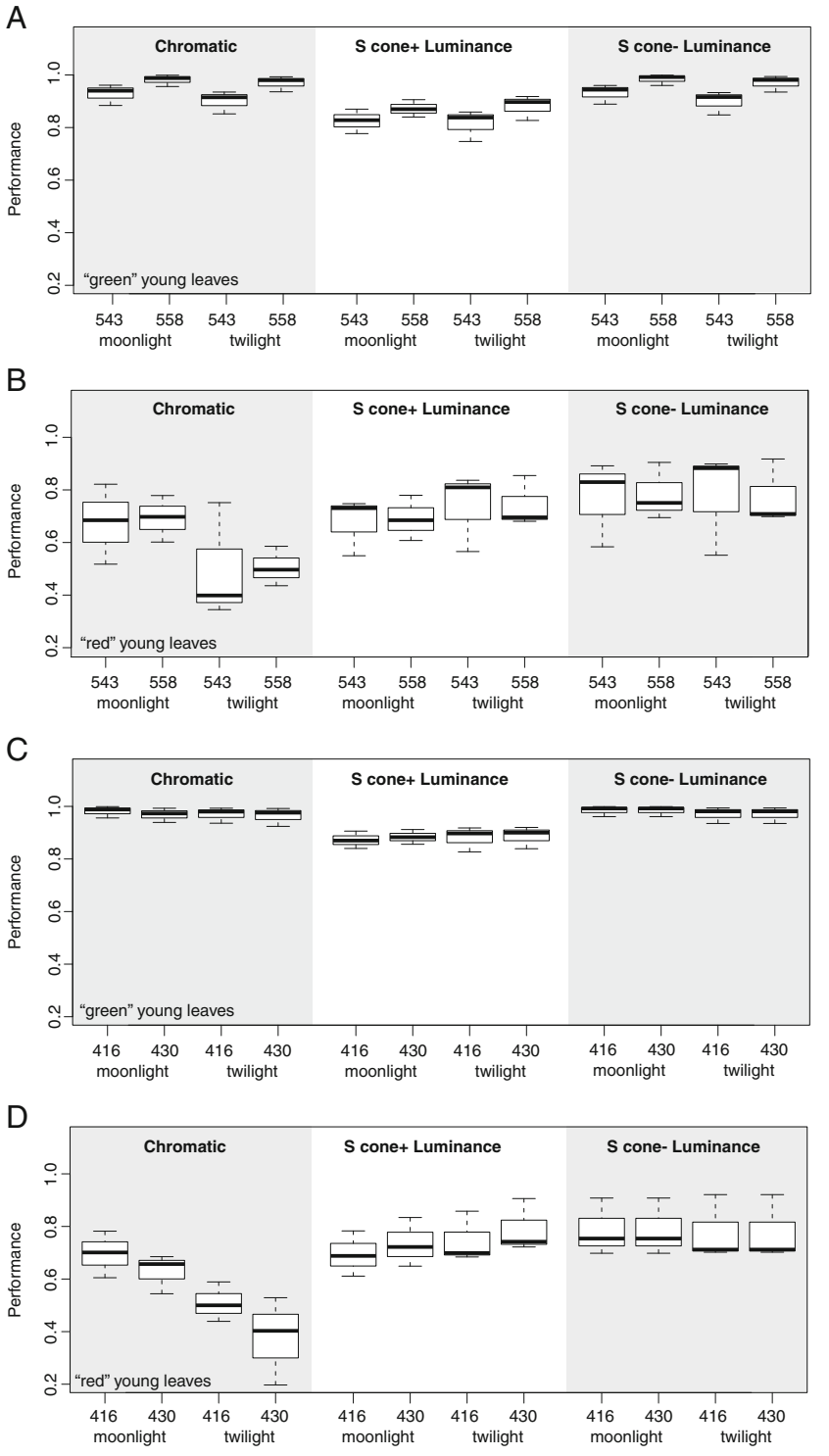
The *Avahi* S λ_{max} at 416 nm performs similarly or substantially better than the 430-nm alternative in chromatic detection, particularly for "red" targets (Fig. 5c, d). Moreover, chromatic performance is generally higher when $\lambda_{max} < 416$ nm (Fig. 4d), which is likely for actual S cones of *Avahi* (Veilleux *et al.* 2013). In contrast, the 430-nm pigment performs similarly or slightly better than 416 nm in S cone+ luminance detection (Fig. 5c, d). However, these differences are not statistically significant: including pigment type in models of the effects of light environment and detection type (chromatic, S cone+ luminance) does not fit the data significantly better for "green" (LMM: $\chi^2 = 7.344$, df = 4, $P = 0.118$) or "red" young leaves (LMM: $\chi^2 = 5.53$, df = 4, $P = 0.235$). Performance is identical for the 416-nm and 430-nm pigments for S cone– luminance (Fig. 5c, d).

Discussion

Color Vision in *Avahi*

In this study, we used molecular and ecological techniques to explore the significance of color vision for the nocturnal folivorous *Avahi*. Consistent with our hypothesis, the strength of purifying selection on the *SWS1* opsin gene did not significantly differ between the LCA of *Avahi* and day-active primates. This result suggests that *Avahi*

Fig. 5 Median target detection performance of observed and alternate *Avahi* visual pigments for "green" and "red" targets under full moonlight and twilight conditions. Boxes represent median (bar) and interquartile range, and whiskers represent minimum and maximum values. **(a–b)** M/L cone λ_{max}: 543 nm and 558 nm (observed). **(c–d)** S cone λ_{max}: 416 nm (observed) and 430 nm.


A
Chromatic
S cone+ Luminance
S cone- Luminance
Performance
0.2
0.4
0.6
0.8
1.0
"green" young leaves
543 558 543 558
moonlight twilight
B
"red" young leaves
C
416 430 416 430
D

has experienced consistent selection to retain dichromatic color vision throughout its evolutionary history, including during a hypothesized shift to nocturnality (Ganzhorn *et al*. 1985; Roos *et al*. 2004).

If color vision is important for leaf discrimination, we further predicted that the λ_{max} of observed *Avahi peyrierasi* cones should outperform alternatives in chromatic detection of *Avahi*-consumed young leaves at RNP. *M/LWS* opsin genotyping established the 558-nm allele in all *Avahi peyrierasi* and all other *Avahi*. Supporting our prediction, the 558-nm allele was substantially better at detecting almost all young leaf targets examined compared to the 543-nm allele for chromatic contrasts. In fact, the 558-nm allele only performed poorly for the big morph red young leaves of *Erythroxylum*. Because previous foraging studies at RNP did not differentiate *Erythroxylum* by morph type (Faulkner and Lehman 2006; Harcourt 1991), it is not clear at present which morph type *Avahi peyrierasi* prefers. Our results also suggest that more short wavelength-shifted S cones are better at chromatically detecting red young leaves (but exhibit no effect for young green leaves). However, we did not identify a significant effect of including S λ_{max} in models of target detection performance. It is possible that the power of the S pigment analysis was too low to detect an effect for red targets. Repeating this analysis after *Avahi* S λ_{max} has been experimentally determined could clarify these findings. We also currently cannot rule out the possibility that the particular tuning of the S cone has no adaptive significance for the foraging efficiency of *Avahi*.

Although the results of both the selection tests and detection analyses are consistent with a selective benefit of dichromatic color vision for discriminate folivory in *Avahi*, it is still possible that *Avahi* might be using luminance cues only for leaf detection. The observed 558-nm allele of *Avahi* did perform better in luminance detection of young green leaves (both when S cone contribution was included and excluded). However, we believe our results offer greater support for a role of chromatic detection. For example, although the 558-nm allele performed almost uniformly better in chromatic detection, the alternative 543-nm allele actually performed better in detecting young red leaves in both types of luminance analyses. Similarly, the alternative 430-nm pigment performed better than the observed 416-nm pigment for young red leaves in S cone+ luminance detection. Thus, the observed visual pigments of *Avahi* appear better suited for chromatically detecting consumed young leaves than for luminance detection.

Because the 558-nm allele was almost always better at detecting both young red and young green leaves, the uniformity of M/L spectral tuning across *Avahi* is not necessarily surprising. While variation in M/L λ_{max} among tarsiers has been linked to ecological differences (Melin *et al*. 2013; Moritz and Dominy 2010), it is possible that the young leaves and light environments used by all *Avahi* are relatively similar. In fact, our detection analyses found no difference in performance under nocturnal irradiance spectra from an open canopy dry deciduous forest or a closed canopy rain forest because the shapes of the spectra from these habitats do not differ substantially (Veilleux and Cummings 2012). Thus, our results may be applicable to *Avahi* across habitat types, although the spectral reflectance of *Avahi*-consumed leaves should be explored at other sites. Interestingly, dichromatic *Lepilemur*, which exhibits the 543-nm allele (Tan and Li 1999), is folivorous and sympatric with *Avahi* in many forests (Ganzhorn 1988). It would be informative to explore whether the 543-nm allele outperforms the 558-nm allele in detecting foods eaten by *Lepilemur*. Spectral

reflectance and color vision may thus offer an additional way for *Avahi* and *Lepilemur* to divide the nocturnal folivorous niche space.

Implications for Foraging Ecology of *Avahi*

One of the more surprising findings of our study was that visual pigments of *Avahi* appear optimally tuned for detecting young green rather than young red leaves. This was interesting, and contrary to our predictions, considering the most commonly eaten species (*Harungana*) has red young leaves. In both studies at RNP (Faulkner and Lehman 2006; Harcourt 1991), *Avahi* spent the greatest percent of time feeding on *Harungana* young leaves (42–48%). It was also the most commonly eaten species (27.5%) for *Avahi laniger* at Mantadia (Ganzhorn *et al*. 1985). Yet *Harungana* was one of the worst species for pigment detection performance of *Avahi*, second only to the big morph of *Erythroxylum*. Although our analyses were limited to the five plant species flushing during our study period, we were still able to sample foods that made up the majority (*ca*. 59.5–72%) of the feeding time budget of *Avahi* at RNP (Faulkner and Lehman 2006; Harcourt 1991). In general, the known young red species represent 49–54.4% of the total feeding time, while the known young green species represent 4.4–10.7% (Online Resource 1, excluding *Erythroxylum* because it is not clear which morph *Avahi* consumes).

There are several possible explanations for the discrepancy between pigment performance and feeding time budget of *Avahi*. One possibility is that the optimization of visual pigments of *Avahi* represents an adaptation for exploiting young leaves during critical fallback periods, similar to hypotheses for the evolution of routine trichromacy in catarrhines (Dominy and Lucas 2004). Both RNP feeding studies on *Avahi* were conducted May/June to August (dry season). Perhaps young green leaves make up a greater proportion of the diet during critical periods at other times of the year, such as during the rainier season (November–March) or developmentally critical times, e.g. female lactation. A second possibility could be that some species may exhibit intraspecific variation (individual or seasonal) in young leaf color and we happened to sample only individuals with young red leaves. It is also important to note that we restricted our target detection analyses to the tops of the leaves. *Avahi* may forage differently, e.g., detecting from above vs. below leaf, for different plant species. Finally, we cannot discount the possibility that the λ_{max} values of cones of *Avahi* do not reflect spectral tuning to specific aspects of the diet, but rather result from nonadaptive evolutionary mechanisms, such as genetic drift. Long-term foraging studies exploring spatial and temporal variation of leaf flush and variation in food preferences are needed to understand better the selective pressures influencing the visual ecology of *Avahi*.

Conclusions

In this study, we explored the ecology and evolution of color vision in the nocturnal lemur *Avahi*. If *Avahi* is secondarily nocturnal as some hypothesize (Ganzhorn *et al*. 1985; Roos *et al*. 2004), it offers an interesting comparison to secondarily nocturnal haplorhines. For example, similar to tarsiers and in contrast to *Aotus*, the strength of

purifying selection to maintain dichromacy did not change with *Avahi*'s hypothesized adaptation to nocturnality. For tarsiers, a preliminary analysis of cone spectral tuning suggests that dichromacy may be advantageous for prey detection in different environments (Moritz and Dominy 2010). Our target detection performance results suggest that foraging tasks may similarly be influencing selection for dichromacy and cone spectral tuning in *Avahi*. In particular, visual pigments of *Avahi* appear optimized for detecting young green leaves using color cues. Thus, these results offer tentative support for recent studies hypothesizing that nocturnal color vision is selectively important for some nocturnal primates (Melin *et al*. 2012; Perry *et al*. 2007; Veilleux *et al*. 2013). Overall, our results suggest that foraging for young leaves has influenced the evolution of color vision in *Avahi*.

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