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The evolution of color vision in red-bellied lemurs (*Eulemur rubriventer*)

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Abstract of the Dissertation

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Color vision in primates is a subject that has long been of interest to evolutionary biologists and anthropologists, largely owing to the unique capacity for trichromatic color vision in humans and many other primates (e.g., Jacobs 1981, 1993). Trichromatic color vision affords individuals the ability to make additional, more salient chromatic distinctions between red and green hues that most other placental mammals, which are primarily dichromatic (i.e., red-green colorblind), are unable to make (e.g., Jacobs 1981, 1993). This ability has been tied to two genetic mechanisms, making primate color vision an illustrative case study in molecular evolution.

Humans and other catarrhines, as well as New World howling monkeys (*Alouatta*), have one autosomal, short-wavelength (S) opsin gene, and two opsin genes on the X chromosome, one resulting in sensitivity to medium wavelengths of light (M) and the other to long wavelengths of light (L), allowing for routine trichromacy (Kainz et al. 1998; Dulai et al. 1999; Jacobs and

Deegan 1999; Nathans 1999). Many platyrrhines and lemurs have one autosomal, S opsin gene and one opsin gene on the X chromosome, for which there are two alleles (M and L), allowing for dichromatic color vision (e.g., Surridge et al. 2003). In some taxa, the X-linked opsin gene is polymorphic, providing heterozygous females the potential for trichromacy, while males and homozygous females are red-green color blind (Jacobs 1998; Tan and Li 1999; Jacobs and Deegan 2003).

Trichromatic color vision appears to characterize all diurnal catarrhines and platyrrhines through one mechanism or another (reviewed in Surridge et al. 2003; Jacobs 2007, 2008, 2009; Kawamura et al. 2012), but the same cannot be said of lemuriforms, in which polymorphic trichromacy has been identified in some diurnal and cathemeral species, while many others are strictly dichromatic (e.g., Tan and Li 1999; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. 2009).

Among diurnal haplorhines, trichromatic color vison has long been thought to result from positive selection favoring trichromacy over dichromacy (e.g., see Surridge et al. 2003 for review). Multiple hypotheses have been proposed suggesting various fitness-related tasks for which trichromatic color vision might be advantageous (e.g., Osorio and Vorobyev 1996; Lucas et al. 1998, 2003; Coss and Ramakrishnan 2000; Pessoa et al. 2014); the most long-standing hypothesis suggests advantages are conferred during foraging, primarily when foraging on red food items (e.g., fruit or young leaves; e.g., Allen 1879; Mollon 1989; Lucas et al. 1998, 2003). This hypothesis has received some support from studies that have modeled color perception of dietary items for different color vision phenotypes (e.g., Osorio and Vorobyev 1996; Lucas et al. 1998, 2003; Sumner and Mollon 2000a, b; Dominy and Lucas 2001; Regan et al. 2001; Osorio et al. 2004). In addition, trichromatic foraging advantages have been observed in captive settings

(e.g., Caine and Mundy 2000; Smith et al. 2003), but there is limited evidence for such advantages in wild populations (e.g., Vogel et al. 2007; Hiramatsu et al. 2008; Melin et al. 2008, 2009). Although the selective pressure(s) favoring trichromatic color vision is unknown, the apparent near ubiquity of trichromacy in haplorhines suggests there is positive selection (e.g., Surridge et al. 2003), and molecular studies have identified signatures of balancing selection to maintain color vision variation in platyrrhines (Hiwatashi et al. 2010; Kawamura et al. 2012).

Color vision in lemurs has been comparatively understudied, but similar adaptive explanations related to foraging behavior have been proposed to account for color vision polymorphisms in some species (Leonhardt et al. 2009). That said, there is a large amount of variation in color vision capacities across this lineage, and it is unclear why some lemur species have polymorphic trichromatic color vision, while other, closely related species are dichromatic (e.g., Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009). Thus, the overall goal of this dissertation was to identify potential evolutionary mechanisms that might account for observed color vision variation in lemurs. In so doing, this dissertation had three objectives: 1) to characterize the color vision capacity of a population of red-bellied lemurs (*Eulemur rubriventer*) in Ranomafana National Park (RNP) in southeastern Madagascar, 2) to examine color vision evolution in the genus *Eulemur* using phylogenetic methods to estimate ancestral color vision state, and 3) to explore potential nonadaptive and adaptive explanations for the type of color vision observed in this population of *E. rubriventer*.

To address the first objective, I sequenced exon 5 of the X-linked M/L opsin gene for 87 individual red-bellied lemurs ($N_{\text{X chromosomes}} = 134$). I found that this population is strictly dichromatic and has a single M/L opsin variant with peak spectral sensitivity at 558 nm (i.e., L opsin is fixed; chapter 2). When placed in a comparative context, this result identifies *E.*

rubriventer as unique among other species of *Eulemur*, for which data are available; some species/populations of *Eulemur* are dichromatic, but the peak spectral sensitivity of the M/L opsin is 543 nm (i.e., M opsin is fixed), while other species/populations are polymorphic, with both M and L opsins present within a population (Tan and Li 1999; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep).

To better understand the evolutionary history of color vision in this lineage, I compiled data on color vision phenotypes for strepsirrhines and mapped these data onto two time-calibrated phylogenetic trees (chapter 2). I then used a maximum likelihood approach to infer the ancestral state of *Eulemur*. Overall, results suggest that an M/L opsin polymorphism was likely the ancestral *Eulemur* condition. Therefore, this result suggests that the population of *E. rubriventer* in RNP likely lost polymorphic trichromatic color vision. Given that trichromatic color vision in other primates is thought to be adaptive, this begs the question of why a potentially advantageous trait would be lost from this population.

To address this question (objective 3), I explored the potential for a recent genetic bottleneck in the population of red-bellied lemurs in RNP (chapter 3). Madagascar has suffered from recent and large-scale forest loss (e.g., Harper et al. 2007), which, combined with other threats such as hunting (e.g., Schwitzer et al. 2014), has resulted in population declines, and genetic bottlenecks in a number of lemur species (e.g., Fredsted et al. 2007; Olivieri et al. 2008; Craul et al. 2009; Brenneman et al. 2012; Parga et al. 2012; Holmes et al. 2013). Genetic bottlenecks provide a potential nonadaptive mechanism through which genetic variation can be lost, because the impact of genetic drift increases in small populations, such that the strength of drift can be greater than selection and even result in loss of advantageous alleles (Futuyma 1998).

Using genotypes for 7 variable microsatellite loci from 55 adult red-bellied lemurs, I found that the *E. rubriventer* population in RNP exhibited significant heterozygosity excess compared to mutation-drift equilibrium, which suggests this population may have experienced a recent population bottleneck. However, I also found that this population did not exhibit significantly low *M* ratios (i.e., ratio of number of alleles to range in allele size) compared to mutation-drift equilibrium, which is not indicative of a genetic bottleneck. Taken together, these results provide mixed evidence that there was a recent genetic bottleneck in this population, and, therefore, this hypothesis cannot be rejected. Thus, polymorphic trichromatic color vision may have been lost through nonadaptive mechanisms in the population of *E. rubriventer* in RNP, and the L opsin may be fixed in this population as a result of genetic drift.

Although nonadaptive mechanisms might play a role in the evolution of color vision in this population, alternatively/additionally, there may be adaptive explanations (objective 3). In chapter 4, I addressed foraging hypotheses that might result in relaxed or disruptive selection (i.e., selection against trichromacy) on polymorphic color vision. I also addressed the potential for directional selection favoring the L opsin in red-bellied lemurs.

Using color modeling methods, I compiled reflectance data from 40 species including 72 plant parts consumed by red-bellied lemurs in RNP and modeled how these food items would be perceived by a trichromatic and dichromatic *Eulemur*. I used this approach to first address the hypothesis that food items consumed by *E. rubriventer* are primarily "dull" in coloration (e.g., green and brown; Dew and Wright 1998; Birkinshaw 2001), and therefore largely inconspicuous to a trichromat, which could result in relaxed selection to maintain polymorphic trichromatic color vision. I found that red-green chromaticities (only available to a trichromat) of many food items, particularly ripe fruit, were greater than chromaticities of background foliage, suggesting

that trichromatic color vision, theoretically, offers a potential advantage in detecting many food items.

I then modeled the chromatic and luminance contrasts of each food item from its leaf background as perceived by the two *Eulemur* dichromatic phenotypes (L opsin vs. M opsin) to determine if chromatic contrasts were significantly greater for the L opsin, which could result in directional selection favoring the L opsin over the M opsin. I found that chromatic contrasts were significantly greater for dichromats with the M opsin, but luminance contrasts were significantly greater for dichromats with the L opsin. This result suggests that *E. rubriventer* may rely on luminance cues during foraging, which could lead to relaxed selection to maintain polymorphic trichromacy or even selection against trichromatic color vision, as chromatic information may interfere with luminance vision (Osorio et al. 1998). Either of the two mechanisms could result in loss of polymorphic color vision. At the same time, fixation of the L opsin may be adaptive for maximizing luminance contrast and may have been driven to fixation through directional or purifying selection, suggesting a potential adaptive explanation for color vision in the *E. rubriventer* population in RNP.

In sum, the results of this dissertation suggest that color vision evolution in lemur populations is likely complex and may be influenced by both adaptive and nonadaptive mechanisms. Therefore, caution is warranted in assuming that observed color vision variation results from adaptation alone. Ultimately, evolutionary processes likely vary across lineages, species, and populations, and identifying how they vary will be important for understanding how evolution shaped the diversity in lemur color vision that we see today.

Dedication

For the red-bellied lemurs in Ranomafana and throughout Madagascar –

Thank you for allowing me a brief glimpse into your beautiful world.

I hope it can persist indefinitely.

Let this be my reminder to do more, be better, and appreciate what is really important in life.

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Acknowledgments

This dissertation is the result of a near decade-long roller coaster ride filled with unexpected turns, seemingly endless, anxiety-ridden uphill climbs, and overwhelming feelings of nausea, but it was certainly never dull, and in the end, it's something I'm glad I did but also something I'm thrilled is over. In getting through it all…the ups and downs, the twists and the turns, I have so many people to thank. I'm certain I will forget some, but let me try to thank you all.

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Chapter 1

Evolutionary mechanisms underlying color vision variation in primates

The diversity of organisms we see today is the product of past and ongoing evolutionary processes. One fundamental requirement of evolution is variation, and understanding the presence of variation within and across populations has long been of interest to evolutionary biologists (e.g., Darwin 1859; reviewed in Futuyma 1998). There is a large amount of theory surrounding the maintenance of variation in populations, which has identified multiple selective processes that may be at play (e.g., reviewed in Futuyma 1998). At the same time, variation is frequently lost in populations and this can result from nonadaptive evolutionary mechanisms and/or through selection (e.g., reviewed in Futuyma 1998). Accordingly, the distribution of variation observed both within and across natural populations results from both adaptive and nonadaptive mechanisms, which can be difficult to tease apart. Among non-model, long-lived species, such as primates, the challenge becomes even more pronounced.

Part of this challenge stems from the necessity that observed variation in populations is at least in part heritable (e.g., Darwin 1859). In assessing the role of selection, it is one thing to evaluate that a particular phenotype exhibits a fitness advantage compared to another, which in primates is challenging on its own, but to also demonstrate heritability of the phenotype often requires greater temporal commitments with potentially difficult-to-attain sample sizes. Such

challenges can be reduced when genetic variation underlying phenotypic variation is well-understood.

One example of a fairly well-established link between genotype and phenotype is color vision capacity in primates (see "Color vision genotypes underlying phenotypes" below). Primate color vision is highly variable both across and within species/populations, and this variation is tied to small changes at the molecular level (see review in Surridge et al. 2003). As a result, changes in an individual's genotype have the potential to greatly impact that individual's phenotype. Accordingly, understanding the potential fitness consequences (or lack there-of) of differential color vision capacities can provide interesting insight into evolutionary mechanisms underlying color vision variation within and across primate populations.

Primate Color Vision

Color vision is the ability to discriminate between stimuli that differ only in their chromatic properties, and as such, it is a behavioral phenomenon (e.g., Jacobs 1981, 1993). However, color vision is also a multi-stage process that requires both the presence of at least two cone photoreceptors in the retina that absorb different wavelengths of light and respond differentially to stimuli, as well as the neural mechanisms to compare and process the different responses (e.g., Walls 1942; Jacobs 1993; Dacey 2000; Kelber et al. 2003; Kelber and Roth 2006).

There are now decades of behavioral, physiological, and genetic research on color vision in primates (see reviews in Jacobs 1981, 1993, 2007, 2008, 2009; Surridge et al. 2003; Kawamura et al. 2012), and while many questions remain, the underlying genetic mechanisms of color vision are fairly well-understood (e.g., Nathans et al. 1986; Jacobs and Neitz 1987; Neitz et

al. 1991). Furthermore, for many primates, a direct link between color vision behavior (phenotype) and color vision genotype has been identified (e.g., *Macaca*: Devalois et al. 1974; Bowmaker et al. 1980; *Saimiri*: Jacobs 1984; Jacobs and Blakeslee 1984; *Saguinus*: Jacobs et al. 1987; but see Blakeslee and Jacobs 1985 on *Lemur catta*), which has been used by many primate studies, including those in wild populations, to infer color vision capacity based on genetic information (e.g., Tan and Li 1999; Tan et al. 2005; Melin et al. 2007, 2008, 2009; Hiramatsu et al. 2008, 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009).

Color vision genotypes underlying phenotypes. As stated above, color vision is related to the presence of two or more cone photoreceptors in the retina that also have differential peak spectral sensitivities (Walls 1942; Jacobs 1981, 1993). The sensitivities of cone photoreceptors are determined by their photopigment opsin proteins, which are coded by opsin genes (e.g., Nathans et al. 1986; Jacobs and Neitz 1987; Neitz et al. 1991). All primates have at least one cone photoreceptor that is sensitive to medium-long wavelengths of light (e.g., green, yellow, and red): M (medium-wavelength) cones and/or L (long-wavelength) cones (see reviews of primate color vision in Jacobs 1981, 1993, 2007, 2008, 2009; Surridge et al. 2003; Kawamura et al. 2012). The amino acid sequences of M/L cone photopigments are coded by one or more M/L opsin genes on the X chromosome, and variation in the peak spectral sensitivities of M/L cone photoreceptors is primarily linked to changes in just one to three amino acid sites (site 180 coded in exon 3; sites 277 and 285 coded in exon 5; e.g., Nathans et al. 1986; Jacobs and Neitz 1987; Neitz et al. 1991; Jacobs et al. 1993a; Tan and Li 1999). On its own, a single functional M/L opsin renders individuals completely color blind, a condition that has been documented in some

nocturnal mammals, including some nocturnal primates (Jacobs et al. 1993b; Jacobs et al. 1996a; Jacobs 2013; Veilleux et al. 2013).

Most primate retinas, however, also exhibit short-wavelength sensitive (S) cones, and the amino acid sequence of S cone photopigment is coded by an autosomal S opsin gene (Nathans 1986; Jacobs 2013; Veilleux et al. 2013). S cones coupled with a single M/L cone photoreceptor, results in dichromatic color, which characterizes the majority of eutherian mammals, as well as some primates (e.g., Jacobs 1981, 1993, 2009; Tan et al. 2005; Veilleux et al. 2013). Individuals with this type of color vision can readily distinguish colors reflecting in the short-wavelength range of light (e.g., blue) and colors reflecting in longer wavelengths (e.g., yellow), but have difficulty distinguishing colors reflecting in the middle-wavelength range of light (e.g., green) and colors reflecting in longer wavelengths (e.g., red; Jacobs 1981, 1993). The ability to also readily distinguish these latter colors (i.e., greens and reds) is conferred to individuals with three functional cone photoreceptors: S cones, M cones, and L cones (i.e., trichromatic color vision; Walls 1942; Jacobs 1981, 1993; Nathans et al. 1986; Neitz et al. 1991). Among extant eutherians, this condition is uniquely found in primates (e.g., Jacobs 1981, 1993) but the genetic mechanisms underlying trichromatic color vision vary across lineages (e.g., Jacobs, 1998; Dulai et al. 1999).

Trichromatic color vision in primates. Catarrhines (Old World monkeys and apes) and New World howling monkeys (*Alouatta*) have been characterized as routinely trichromatic (Kainz et al. 1998; Dulai et al. 1999; Jacobs and Deegan 1999; Nathans 1999). That is, species have separate M and L opsin genes on the X chromosome resulting in separate M and L cone photoreceptors in the retina (Nathans et al. 1986; Dulai et al. 1999). The presence of M and L

cones along with S cones provides all individuals in a population the potential for trichromatic color vision (Nathans et al. 1986; Jacobs et al. 1996b; Jacobs and Deegan 1999). This condition differs from many other primates that have polymorphic trichromatic color vision (see reviews in Surridge et al. 2003; Jacobs 2007, 2008; Kawamura et al. 2012).

More specifically, all diurnal New World monkeys (with the exception of *Alouatta* discussed above), as well as some lemurs, have the potential for trichromatic color vision through allelic variation of a single M/L opsin gene (reviews in Surridge et al. 2003; Jacobs 2007, 2008; Kawamura et al. 2012). Such species exhibit two or more M/L opsin alleles that code for two or more opsins, which vary in their medium-long wavelength sensitivities (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993a; Jacobs 1998; Tan and Li 1999). Given that this variation occurs on the X chromosome, only females that are heterozygous for the M/L opsin gene have the potential for trichromatic color vision, while homozygous females and all males are dichromatic (Jacobs and Neitz 1987; Neitz et al. 1991; Williams et al. 1992; Jacobs et al. 1993a; Tan and Li 1999).

The presence of routine trichromatic color vision in catarrhines and *Alouatta* resulted from two independent gene duplication events (Nathans et al. 1986; Jacobs et al. 1996b; Kainz et al. 1998; Dulai et al. 1999; Jacobs and Deegan 1999; Nathans 1999). Polymorphic trichromacy in New World monkeys appears to have a single early origin and is maintained in populations (Boissinot et al. 1998; Surridge and Mundy 2002; Hiwatashi et al. 2010; Kawamura et al. 2012). These scenarios are unlikely to occur in the absence of selection favoring trichromatic color vision (e.g., Surridge et al. 2003), and, therefore, much research on color vision in primates has focused on the adaptive value of trichromacy.

Adaptive hypotheses for trichromatic color vision. There have been multiple hypotheses proposed for the evolution of trichromatic color vision in primates, and most support for these hypotheses is based on quantitative models using data on photoreceptor responses to natural reflectance of items encountered in primate environments (e.g., Osorio and Vorobyev 1996; Lucas et al. 1998, 2003; Coss and Ramakrishnan 2000; Sumner and Mollon 2000a, b; Dominy and Lucas 2001; Regan et al. 2001; Osorio et al. 2004; Riba-Hernández et al. 2004; Changizi et al. 2006; Melin et al. 2014; Pessoa et al. 2014). Results of such studies suggest that trichromacy would be theoretically adaptive for several fitness-related tasks, such as detecting 1) conspecifics (Changizi et al. 2006; but see Fernandez and Morris 2007; Kamilar et al. 2013), 2) predators (Coss and Ramakrishnan 2000; Pessoa et al. 2014), 3) and food items, including ripe fruit and/or young leaves (e.g., Osorio and Vorobyev 1996; Lucas et al. 1998, 2003; Sumner and Mollon 2000a, b; Dominy and Lucas 2001; Regan et al. 2001; Osorio et al. 2004; Riba-Hernández et al. 2004; Melin et al. 2014). While the original selective pressure(s) favoring the evolution of trichromatic color vision is unknown, hypotheses related to food detection have remained the most prominent hypotheses used to explain color vision evolution in primates.

Among routine trichromats, there is limited variability in photopigment sensitivity and other color vision phenotypes are rare in populations, which makes adaptive hypotheses difficult to test (Bowmaker et al. 1991; Jacobs and Williams 2001). Not surprisingly, then, much color vision research in primates has focused on polymorphic populations (see below). Such populations are ideal for examining questions concerning selection and adaptation of primate color vision because multiple phenotypes are present in a population, making it possible to directly compare variation in behavior and fitness across individuals with different color vision capacities (Surridge et al. 2003). Furthermore, studies on polymorphic New World monkeys

have identified signatures of balancing selection, indicating that polymorphic trichromatic color vision is being maintained in at least some populations through natural selection (Surridge and Mundy 2002; Hiwatashi et al. 2010; Kawamura et al. 2012).

As stated above, there is much population genetic theory surrounding the maintenance of genetic variation in populations (Futuyma 1998), and multiple mechanisms have been hypothesized to maintain color vision variation in polymorphic populations (e.g., Mollon et al. 1984). Of these, heterozygote advantage and inverse frequency-dependent selection have received the most attention.

Heterozygote advantage (i.e., overdominance selection) suggests there is an overall advantage to the heterozygous (in this case trichromatic) condition (Mollon et al. 1984; Surridge et al. 2003). Again, quantitative models of New World monkey color vision indicate that in many species, trichromatic individuals should be better able to detect many fruits against a foliage background than dichromats (e.g., Regan et al. 2001; Osorio et al. 2004; Riba-Hernández et al. 2004; Melin et al. 2014). Empirical support for this hypothesis comes primarily from captive studies, which have found some evidence that trichromats forage more efficiently than dichromats on red-dyed food items against a green background (e.g., Caine and Mundy 2000; Smith et al. 2003). However, most studies on wild populations have failed to find evidence in support of trichromatic foraging advantages (e.g., *Cebus*: Vogel et al. 2007; Melin et al. 2008; *Ateles*: Hiramatsu et al. 2008; *Callicebus*: Bunce et al. 2011), although *Cebus* trichromats appear to forage more accurately on some *Ficus* fruits than do dichromats (Melin et al. 2009), and trichromatic tamarins catch more insect prey than do dichromats (Smith et al. 2012). Despite this potential support for trichromatic foraging advantages, there is so far no support that such advantages translate into overall greater fitness (i.e., increased survival and reproductive success)

for trichromats (Fedigan et al. 2014). Accordingly, many studies have turned to alternative mechanisms to explain color vision variation in populations, such as inverse frequency-dependent selection.

Inverse frequency-dependent selection refers to conditions when minority phenotypes have greater fitness compared to more common phenotypes (Futuyma 1998). In the case of color vision variation, minority phenotypes may experience less competition for food resources if they are better able to exploit them than are other phenotypes (Mollon et al. 1984). This mechanism would predict that different color vision phenotypes (e.g., dichromats and trichromats) have different advantages (Mollon et al. 1984). That is, trichromats would have an advantage, such as foraging on red food items, but dichromats would have a different advantage, potentially being better able to forage on other food items or detect predators (e.g., Mollon et al. 1984), and, in fact, primate studies have found greater support for dichromatic foraging advantages. More specifically, dichromatic individuals appear to be better able to detect camouflaged patterns. This has been shown in humans (Morgan et al. 1992) and captive non-human primates (Saito et al. 2005). There is also evidence that this ability translates into greater foraging success on camouflaged food items (e.g., insects) for dichromats compared to trichromats, which has been demonstrated in captivity (Caine et al. 2003, 2010), as well as in wild primate populations (Melin et al. 2007; Smith et al. 2012). These results suggest that inverse frequency-dependent selection may be acting to maintain color vision polymorphisms in at least some populations of platyrrhines, but, somewhat surprisingly, support for advantages of trichromatic color vision in wild primates remains mixed. As a result, despite extensive research in New World monkeys, the evolutionary mechanisms underlying color vision variation in this lineage remain unknown.

Color vision in lemurs. Compared to platyrrhines, color vision in lemurs has been understudied, owing largely to the fact that color vision diversity in lemurs has only more recently been documented (e.g., Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Veilleux et al. 2013; Bradley et al. in prep). It is now known that there is extensive variation in lemur color vision both across and within species (e.g., Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Veilleux et al. 2013; Bradley et al. in prep). Given current data, dichromatic color vision appears to characterize most lemur species, but color vision polymorphisms have been identified in several day-active lemurs (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep), and some nocturnal lemurs have lost color vision altogether (e.g., Veilleux et al. 2013).

Polymorphic trichromacy observed in lemurs is similar to that found in most New World monkeys. However, while many platyrrhine species are highly polymorphic, having more than two M/L opsin alleles (e.g., see reviews in Surridge et al. 2003; Kawamura et al. 2012), most polymorphic lemur species have only two alleles: a M opsin with peak spectral sensitivity (λ_{max}) at 543 nm and a L opsin with λ_{max} at 558 nm (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Differences in λ_{max} between the two opsins are the result of a single amino acid change at site 285 in exon 5 of the M/L opsin gene (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Interestingly, among dichromatic lemurs, species vary in whether a M

opsin or L opsin is fixed (Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux et al. 2014; Bradley et al. in prep).

As in haplorhines, it is currently unknown how evolution has shaped color vision variation in lemurs. However, similar adaptive hypotheses related to food detection have been proposed (Leonhardt et al. 2009; Veilleux et al. 2009). For example, in the only captive study to examine the relationship between foraging behavior and color vision in lemurs, at least some trichromatic individuals appear to forage faster on red food items compared to green food items (Leonhardt et al. 2009). However, feeding rates were not faster than dichromatic conspecifics, thus providing limited support for trichromatic foraging advantages (Leonhardt et al. 2009).

To account for the diversity in color vision across dichromatic species, Veilleux et al. (2014) suggest that the L opsin may have become fixed in the highly folivorous and nocturnal *Avahi* as an adaptation to foraging on young leaves. This hypothesis was proposed given that the dichromatic phenotype with the L opsin was superior to the dichromatic phenotype with the M opsin at detecting young leaves consumed by *Avahi* based on chromatic information (Veilleux et al. 2014). Consequently, fixation of either the M or L opsin could represent adaptation (through directional selection) for foraging on particular foods (Veilleux et al. 2014). However, additional analyses on other taxa and across color vision phenotypes is necessary to further explore this hypothesis.

Another factor, not unrelated to foraging, that might help account for some of the color vision variation in lemurs is habitat type, which is highly diverse in Madagascar (Veilleux et al. 2013). Research on the relationship of habitat light environments and selection to maintain functional S opsin genes (and therefore color vision) in nocturnal lemurs, suggests light environments may play a role, with strong purifying selection characterizing many species

occupying more open habitats (Veilleux et al. 2013). It is possible that variation in habitat characteristics also plays a role in color vision evolution across other lemur species.

Interestingly, although adaptive explanations are common in discussions of primate color vision, it has also been suggested that nonadaptive evolutionary mechanisms (i.e., genetic drift) may play a role in lemur color vision (Tan et al. 2005). The role of genetic drift in evolution is important, as it is always acting, it influences all genes and their allelic variation, and it is random (Futuyma 1998). Consequently, much variation within and across extant organisms likely results from random processes, and many have cautioned against a perspective that revolves around natural selection as the primary mechanism of evolution (i.e., Gould and Lewontin 1979). Given the distribution of M and L opsins in lemurs and invoking the principle of parsimony, Tan et al. (2005) suggested that a color vision polymorphism likely arose early in primate evolution, and that fixation of either the M or L opsin in dichromatic and monochromatic species results from random allele loss due to drift. This would represent a markedly different pattern from that observed in New World monkeys, in which a single origin of polymorphic trichromacy appears to have been maintained in most species over a long evolutionary time frame (Boissinot et al. 1998; Surridge and Mundy 2002). Although the origin(s) of polymorphic trichromacy in lemurs remains equivocal, this hypothesis introduces more complexity to understanding the distribution of color vision capacities across this lineage, and suggests that either or both nonadaptive and adaptive mechanisms might have resulted in the diversity of color vision observed in lemurs today.

In sum, the evolutionary processes responsible for color vision in primates are likely complex and may vary across lineages, species, and populations. This dissertation aims to

contribute to this field of research by addressing questions related to color vision evolution in a population of red-bellied lemurs (*Eulemur rubriventer*).

Dissertation Objective

The primary goal of this dissertation is to examine the evolutionary mechanisms potentially underlying color vision variation observed in lemurs using red-bellied lemurs (*Eulemur rubriventer*) as a case study in understanding molecular evolution. This dissertation first characterizes the color vision capacity of a population of *E. rubriventer* in southeastern Madagascar based on the frequency of M/L opsin alleles in this population and incorporates this result into a broader phylogenetic context to better understand the evolution of color vision across the genus *Eulemur* (chapter 2). This dissertation then explores potential nonadaptive explanations (i.e., genetic drift) for color vision in this population (chapters 3 and 4), as well as potential adaptive explanations based on foraging ecology (chapter 4). The results of this dissertation are synthesized in the final chapter (chapter 5), which includes a discussion of potential avenues for future research.

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Chapter 2

X-linked opsin gene allele frequencies in red-bellied lemurs (*Eulemur rubriventer*)

ABSTRACT

Polymorphic trichromatic color vision has been documented in some diurnal lemurs (*Propithecus*, *Varecia*) and more recently in a cathemeral taxon, *Eulemur flavifrons*. Its presence in *Eulemur* suggests cathemerality, rather than strict diurnality, is compatible with the evolution of trichromatic color vision. However, some species of *Eulemur* appear to be strictly dichromatic, making it unclear if polymorphic trichromacy arose early in this genus or independently in different species.

To further examine the evolutionary history of color vision in *Eulemur*, this study characterized M/L opsin allele frequencies in a population of red-bellied lemurs (*Eulemur rubriventer*) in Ranomafana National Park (RNP), Madagascar. Color vision genotypes were assigned based on amino acid site 285 in exon 5 for 87 individual red-bellied lemurs ($N_{X\ chromosomes} = 134$), and results indicate this population is strictly dichromatic with a long-wavelength (558 nm) opsin.

This result was included in a data set of strepsirrhine color vision phenotypes, and data were mapped onto two time-calibrated phylogenetic trees that hypothesize different relationships among *Eulemur* species. Four models were examined using a maximum likelihood approach to infer the ancestral state of *Eulemur*. Results of the two best-fit models based on Akaike weights suggest that in most analyses, an M/L opsin polymorphism was likely the ancestral *Eulemur*

condition. Therefore, polymorphic trichromacy was likely lost in the population of *E. rubriventer* in RNP and such losses may have occurred in other *Eulemur* as well. Potential nonadaptive and adaptive mechanisms are proposed to account for loss of color vision variation.

INTRODUCTION

One feature that distinguishes primates from all other mammals is an enhanced visual system (e.g., Martin 1990). Primates have extreme orbital convergence, resulting in considerable binocular visual field overlap (Heesy 2008). They also exhibit a number of derived features of the brain associated with vision, such as an expanded visual cortex (Kaas 2005). It is important to note, however, that visual systems vary across primate lineages. For example, all diurnal haplorhines have a cone-dominated and cone-dense fovea in the central retina, as well as low retinal summation (summation refers to a condition where single ganglion cells receive input from more than one photoreceptor; when retinal summation is relatively low, there are fewer photoreceptor cells interacting with single ganglion cells), resulting in high visual acuity (Kay and Kirk 2000; Kirk and Kay 2004). Visual acuity in strepsirrhines, though higher than in other mammals, is much lower compared to diurnal haplorhines (Kay and Kirk 2000; Kirk and Kay 2004; Veilleux and Kirk 2009). Strepsirrhines appear to have lower overall cone densities (Wikler and Rakic 1990; Peichl et al. 2001). They also lack a true fovea, with some species having instead an *area centralis* in the central retina with higher retinal summation (i.e., lower visual acuity; Rohen and Castenholz 1967; Wikler and Rakic 1990; Kay and Kirk 2000).

Trichromatic color vision, the ability to readily distinguish red and green hues, is another derived feature that sets primates apart from other eutherian mammals (with the possible exception of some megachirpoterans: Wang et al. 2004), which are primarily dichromatic and

red-green colorblind (Jacobs 1981, 1993). Until relatively recently, it was thought that this feature was only found in diurnal haplorhines (Jacobs 1981, 1993; Tan and Li 1999) and resulted from two genetic mechanisms (routine trichromacy in catarrhines and howling monkeys: Kainz et al. 1998; Dulai et al. 1999; Jacobs and Deegan 1999; Nathans 1999; polymorphic trichromacy in many platyrrhines: reviews in Surridge et al. 2003; Kawamura et al. 2012). It is now known that some lemur species have the potential for trichromatic color vision and exhibit a pattern similar to that of most New World monkeys (i.e., polymorphic trichromacy; Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003c; Veilleux and Bolnick 2009). This condition results in individuals within the same population having different color vision capacities. Specifically, some females have trichromatic color vision, while all males and other females are red-green colorblind (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993b).

The genetic basis for polymorphic trichromacy is fairly well-understood. Most lemurs and New World monkeys have a single autosomal short-wavelength (S) opsin gene that produces short-wavelength sensitive cone photopigments, as well as one medium-long-wavelength (M/L) opsin gene on the X chromosome producing cone pigments sensitive to longer wavelengths (e.g., Jacobs and Neitz 1987; Williams et al. 1992; Jacobs et al. 1993b; Tan and Li 1999). Having two cone photopigments spectrally tuned to different wavelengths results in dichromatic color vision (e.g., Jacobs 1993). However, in species with polymorphic trichromacy, there are two or more alleles of the M/L opsin gene that code for spectrally distinct photopigments (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993b; Jacobs 1998; Tan and Li 1999). As a result, females that are heterozygous for the M/L opsin gene are trichromatic, while homozygous females and all males are dichromats (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993b; Jacobs 1998; Tan and Li 1999).

Polymorphic trichromacy is present in all platyrrhine genera (for which data are available) (e.g., see Jacobs 2007 and Kawamura et al. 2012 for reviews; de Lima et al. 2015), with the exceptions of owl monkeys (*Aotus*), which are monochromatic, having lost a functional S opsin (Jacobs et al. 1993a), and howling monkeys (*Aloutta*), which appear to be routinely trichromatic, having separate functional M (medium-wavelength) and L (long-wavelength) opsin genes on the X chromosome (Jacobs et al. 1996; Dulai et al. 1999). Many New World monkey species are also highly polymorphic, possessing more than two M/L opsin alleles (e.g., Jacobs and Deegan 2003a, 2005). Within this lineage, it appears that color vision variation is maintained via balancing selection and has persisted for over 20 million years (Shyue et al. 1995; Boissinot et al. 1998; Hiwatashi et al. 2010; Kawamura et al. 2012). The selective pressure(s) maintaining color vision polymorphisms in platyrrhines remains unknown, but one of the most long-standing hypotheses suggests that trichromatic color vision (the heterozygous condition) confers an advantage when foraging on red food items that would ultimately translate into higher fitness (i.e., overdominance selection; e.g., Mollon et al. 1984).

There has been comparatively less research on color vision in strepsirrhines. Most lemur species with polymorphic trichromacy appear to have only two alleles, resulting from a single amino acid difference at site 285 in exon 5 of the M/L opsin gene (Tan and Li 1999; Tan et al. 2005; Jacobs and Deegan 2003c; Leonhardt et al. 2009; Veilleux and Bolnick 2009). Differences at only this site produce either a M opsin with peak spectral sensitivity (λ_{max}) at 543 nm or a L opsin with λ_{max} at 558 nm (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003c). Polymorphisms have been identified in some species of Lemuridae and Indriidae, while remaining species for which data are available appear to be dichromatic or monochromatic with either the M or L opsin (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003c; Tan et

al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep).

The evolutionary mechanisms underlying the distribution of color vision capacities across lemurs remain unknown, but adaptive explanations related to diet, activity pattern, predation, and habitat have been proposed (e.g., Yamashita et al. 2005; Veilleux and Cummings 2012; Veilleux et al. 2013, 2014). Relevant to this discussion as well is the origin(s) of color vision polymorphisms in lemurs, which is currently unknown. However, the presence of color vision polymorphisms in this lineage has raised the hypothesis that trichromatic color vision may have been important earlier in primate evolution than has been suggested previously (Tan and Li 1999; Tan et al. 2005).

Tan and Li (1999) were the first to identify polymorphic trichromacy in lemurs, finding both M and L opsins present in *Propithecus coquereli* and *Varecia rubra* (the identification of an M/L polymorphism in *Cheirogaleus major* was an error: see Heesy and Ross 2001; Jacobs and Deegan 2003c). This study also found that some species are monomorphic for either the M or L opsin (Tan and Li 1999). Based on the distribution of M/L opsins in the strepsirrhine lineage, the authors suggested that the ancestral strepsirrhine was likely polymorphic (Tan and Li 1999). They went further to suggest this may have been the ancestral primate condition, based on the principle of parsimony and the presence of both M and L opsins in tarsiers (Tan and Li 1999; Tan et al. 2005). This scenario is interesting, because it would suggest multiple independent losses of a color vision polymorphism in strepsirrhines, which is a very different pattern than that observed in New World monkeys (see Jacobs 2007 and Kawamura et al. 2012 for reviews). Relaxed selection might account for loss of trichromatic vision in nocturnal lineages, where light levels are often considered less conducive to color vision (e.g. Tan et al. 2005; but see Melin et

al. 2013), but loss among species described as diurnal or cathemeral (i.e., activity bouts occur throughout a 24-hour day: Tattersall 1987), which are active when light levels are more conducive to color vision, might require different adaptive or nonadaptive explanations.

A solution was provided by Heesy and Ross (2001) who responded to the hypothesis for an early origin of a color vision polymorphism in strepsirrhines by evaluating opsin evolution in primates using parsimony analyses and character reconstruction. Results from their study suggest that polymorphic trichromacy likely arose independently in *P. coquereli* and *V. rubra*, with the ancestral primate and ancestral strepsirrhine likely exhibiting dichromatic color vision with the M opsin only (Heesy and Ross 2001). Because *P. coquereli* and *V. rubra* have been generally considered diurnal (but see Rea et al. 2014 on *Varecia* cathemerality), it was suggested that diurnality is likely a necessary (but perhaps not sufficient) condition for the evolution of polymorphic trichromatic color vision in lemurs (Jacobs and Deegan 2003b). Loss of trichromacy in this scenario need not be explained.

Since the study of Heesy and Ross (2001), additional analyses of M/L opsins in lemurs have been conducted, making species-specific independent origins of color vision polymorphisms less parsimonious (e.g., Jacobs and Deegan 2003c; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Most notably, Veilleux and Bolnick (2009) identified both M and L opsins in *Eulemur flavifrons*, which was the first documentation of polymorphic trichromacy in this genus; other analyses of *Eulemur* species only recovered the M opsin (Tan and Li 1999; Leonhardt et al. 2009). The *Eulemur* clade is of particular interest primarily because many (if not all) species are considered cathemeral (Donati and Borgoginini-Tarli 2006), indicating that this activity pattern may also be compatible with the evolution of trichromatic color vision (Veilleux and Bolnick 2009).

Unfortunately, the presence of both M and L opsins in *E. flavifrons* does not necessarily imply that there is strong positive selection to maintain polymorphic trichromacy in this lineage, and, in fact, it appears that some *Eulemur* species may be strictly dichromatic with the M opsin only (Leonhardt et al. 2009; Bradley et al. 2009). Consequently, polymorphic trichromacy may not characterize the genus as a whole, which has implications for understanding color vision evolution in this lineage.

More specifically, the question remains if the ancestral *Eulemur* condition is polymorphic trichromacy, as would be likely if color vision polymorphisms arose earlier in lemur lineages (e.g., independently within the lemurids and indriids or prior to a lemurid and indriid split; Veilleux and Bolnick 2009). Observations of a single M/L opsin in *Eulemur* species would therefore represent loss of a color vision polymorphism and question the adaptive significance of polymorphic trichromacy in this lineage. Alternatively, if a color vision polymorphism arose independently in some *Eulemur* species, this may provide insight into important selective pressures underlying the evolution of trichromatic color vision in primates, particularly in relation to cathemeral activity patterns.

To better understand the evolutionary mechanisms underlying color vision in lemurs, and *Eulemur* in particular, the goals of this study are two-fold: 1) to determine the color vision capacity of a wild population of red-bellied lemurs (*Eulemur rubriventer*), and 2) to infer the evolutionary history of color vision and the ancestral state of *Eulemur* using an expanded data set of M/L opsin distributions in lemurs compared to previous studies, including additional *Eulemur* species (Bradley et al. 2009; Bradley et al. in prep). *Eulemur rubriventer* is of particular interest because, in some recent phylogenetic analyses, it has been hypothesized with strong support to

be sister to all other *Eulemur* taxa (Horvath et al. 2008; Springer et al. 2012), and as such, may be particularly informative for estimating the ancestral color vision state in this genus.

METHODS

All procedures in this study were in compliance with institutional (Stony Brook University IACUC: 2010/1803, 2011/1895) and national (Madagascar National Parks) guidelines.

Study site and animals

This study was conducted in and around Ranomafana National Park (RNP) between January 2012 and May 2013. RNP is an area of 41,000 ha of montane rainforest in southeastern Madagascar (E47°18' - 47°37', S21°02' - 21°25'; Wright et al. 2012). Data for this study were collected at five site localities within the park (Talatakely, Vatoharanana, Valohoaka, Sakaroa, and Sahamalaotra), and one site located just outside the park (Ambatolahy dimy) (Figure 2.1). The population of red-bellied lemurs in RNP has been the subject of previous research projects (1988-1990: Overdorff 1991; 2001: Durham 2003; 2003-2005: Tecot 2008) and is exposed to ecotourism activities to varying degrees, making many individuals habituated to observer presence.

E. rubriventer live in small, cohesive groups, ranging in size from 2-6 individuals (Wright 1992; Overdorff 1993; this study). Adults are usually pair-living, with group compositions generally composed of one adult male, one adult female, and immature individuals (Overdorff and Tecot 2006). Red-bellied lemurs defend small home ranges (11-19 hectares; Overdorff 1991), are sexually dichromatic, and exhibit individual variation in pelage

coloration/patterns. The latter allows observers to identify individuals based on unique variation in facial pelage patterns (Figure 2.2).

Fecal sample collection

Fresh fecal samples for genetic analyses were collected from individual red-bellied lemurs opportunistically as part of both behavioral data collection and survey on the red-bellied lemur population in RNP occurring throughout the study period. The goal was to collect three independent fecal samples from each individual, but the final sample size ranged from 1 to >10 fecal samples for each individual. Samples were collected from the ground immediately following defecation. Each sample (~ 5 grams of wet weight) was placed directly into a 50 mL plastic centrifuge tube pre-filled with 30 mL silica gel beads (for desiccation) using latex gloves and the untouched end of a freshly broken twig (Nsubuga et al. 2004). Tubes were labeled and sealed with parafilm and stored at ambient temperature in the field and later at +4°C in the lab (Yale Molecular Anthropology Laboratory; YMAL).

DNA extraction

Genetic analyses were conducted at the YMAL. Genomic DNA was extracted from dry fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen). Extraction procedures followed a protocol optimized by the YMAL, which deviated from the manufacturer's protocol in the following steps: samples were initially lysed at room temperature for 48 hours in ASL buffer, and all procedures following inhibitor adsorption were automated using a QIAcube® (Qiagen). All DNA extractions included at least one negative control.

DNA quantitation

DNA concentrations were quantified in duplicate for all samples using a Qubit® 2.0 Fluorometer (Invitrogen™) and the Qubit® dsDNA HS Assay Kit. This method quantifies total genomic DNA based on the signal from fluorescent dye that binds specifically to DNA. Therefore, this method does not distinguish lemur DNA from other DNA that may be present in the sample (e.g., microbial DNA). For a subset of samples (see chapter 3), DNA was also quantified using a real-time quantitative polymerase chain reaction assay (qPCR; Morin et al. 2001). This method amplifies an 81 base pair portion of the single copy *c-myc* gene (following Morin et al. 2001), and thus only primate DNA was targeted and quantified. The assay was performed in a Rotor-Gene Q real-time PCR cycler (Qiagen) with a total PCR volume of 25 μl: 12.5 μl of Rotor-Gene SYBR Green RT-PCR Master Mix (Qiagen), 1.25 μl each of forward and reverse primers (10 μM/μl), and 2.5 μl of DNA. Two-step thermal cycling conditions (denaturing and annealing only, with florescence measured at the end of each annealing step) were used with initial incubation at 95°C for 50 seconds followed by 50 cycles of 95°C for 5 seconds and 60°C for 10 seconds. Amplification was followed by a high resolution melt analysis to confirm specificity and lack of substantial secondary products.

A dilution series of human placental DNA of known quantities (25 ng, 20 ng, 6.25 ng, 3.125 ng, 1.5625 ng, 800 pg, 400 pg, 200 pg, 100 pg, and 25 pg) was included in triplicate in each qPCR run and used as standards to then quantify red-bellied lemur DNA in each fecal sample, which were included in duplicate. Each assay also included two negative controls (H_2O as template). Quantification was performed using the Rotor-Gene Q software package. The final quantity of DNA for fecal samples was determined by averaging the results from the two replicates, which were generally consistent across replicates.

Sex-typing

Each sample was genetically sex-typed by amplifying segments of the tetratricopeptide repeat protein gene on the Y chromosome (UTY), and the X-chromosomal homolog (UTX) using a multiplex (triple primer) PCR (Villesen and Fredsted 2006). This method was chosen, because it has been successfully tested in a number of lemur species, including one species of *Eulemur* (*E. fulvus*; Villesen and Fredsted 2006). It also yields short fragment sizes (Y = 86 bp, X = 127 bp), making it suitable for use with degraded DNA such as that extracted from non-invasive samples (e.g., feces; Villesen and Fredsted 2006).

PCR was performed in a total volume of 25 μl using an ABI 480 thermal cycler (Perkin-Elmer). PCR volume consisted of 12.5 μl of Qiagen HotStarTaq Master Mix, 2 μl of bovine serum albumin (BSA, 20 μM/μl), 4, 1, and 0.25 μl of UTY, UTXY, and UTX primers (10 μM/μl), respectively (see Villesen and Fredsted 2006 for primer sequences), and 4 μl of total template DNA. All PCR reactions included one negative control (H_2O as template). Cycling conditions were 95°C for 15 minutes and 36 cycles of 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 7 minutes. PCR fragments were separated on 2% agarose gels (120 volts, 1 hour) and visualized using GelRed (Biotium). If a sample yielded both X and Y fragments, the individual was typed male, and if a sample yielded a single X fragment, the individual was typed female. All samples were sex-typed in at least two independent reactions.

M/L opsin genotyping

The opsin gene complement of individual red-bellied lemurs was determined by amplifying and sequencing a 240 bp fragment of exon 5 of the X-linked M/L opsin gene. In

platyrrhines, functional variation in M/L opsins also results from amino acid site changes in exon 3 (e.g., Neitz et al. 1991), which does not vary in most lemur species for which data are available (e.g., Tand and Li 1999). Therefore, this study focuses on functional variation at exon 5. Fragments were amplified using PCR with a forward primer designed by B. J. Bradley (5'-GTAGCAAAGCAGCAGAAAGA – 3') and a previously published reverse primer (5' – CTGCCGGTTCATAAAGACGTAGATAAT – 3'; Jacobs et al. 2002). PCR reactions were performed in 25 μl total volume, comprising 12.5 μl of Qiagen HotStarTaq Master Mix, 2 μl of bovine serum albumin (BSA, 20 μM/μl), 1 μl each of forward and reverse primers (10 μM/μl), and 3-5 μl of total template DNA. All PCR reactions included one negative control (H_2O as template). Cycling conditions were 95°C for 15 minutes and 36 cycles of 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 7 minutes. PCR fragments were visualized on 2% agarose gels (120 volts, 1 hour) using GelRed. PCR products were sequenced (Sanger) in both directions using an Applied Biosystems 3730*xl* DNA Genetic Analyzer at the Yale DNA Analysis Facility. Sequence traces were visualized using FinchTV (Geospiza, Inc.), and genotypes were determined based on amino acid site 285 (codon translations: GCC = alanine; ACC = threonine). All individuals were replicated 1-3 times using independent PCR reactions and 1-2 independent fecal extractions. Different fecal samples were used for those replicated using two fecal extractions.

Ancestral state estimation

Data on color vision phenotypes for strepsirrhines (including lorisiforms as the outgroup of lemuriforms) based on DNA sequence translations and known residues of spectral tuning were compiled from the literature and expanded using unpublished data on multiple wild lemur

populations (Bradley et al. in prep; Table 2.1)[1]. These data were mapped onto two time-calibrated phylogenetic trees: 1) the phylogeny of Springer et al. (2012), which includes all species for which data are available and has robust bootstrap support (>95%) for all nodes of interest within the genus *Eulemur* (referred to as SEA throughout the remaining text); and 2) a modified Springer et al. (2012) phylogeny in which the *Eulemur* clade was extracted and replaced with the more recent *Eulemur* phylogeny of Markolf and Kappeler (2013) (referred to as SMK throughout the remaining text). This latter phylogeny was included because it hypothesizes different relationships among many *Eulemur* species, in particular the relationship of *E. rubriventer* to other *Eulemur* species.

For all analyses, color vision was treated as a single, multi-state, discrete character. Given that functional variation in lemur M/L opsins appears to be linked primarily to exon 5 of the M/L opsin gene (as opposed to exons 3 and 5, as in platyrrhines; Neitz et al. 1991; Tan and Li 1999), M/L opsins were classified based on amino acid differences at the critical site 285 in exon 5 of the M/L opsin gene only (i.e., alanine = M opsin, threonine = L opsin). Therefore, all M opsins, as well as all L opsins were considered to be potentially homologous. This approach is used here, as opposed to using longer M/L opsin sequences including noncritical sites to infer evolutionary history, because 1) for many species, only short fragments of critical tuning sites are available, and 2) there is strong evidence for gene conversion across M/L opsin alleles in New World monkeys, which can lead to misinterpretations of evolutionary history (Boissinot et al. 1998). Boissinot et al. (1998) found that analyzing sequence variation at noncritical sites suggested an independent origin of M/L opsin allele variation in multiple New World monkey species,

[1] NOTE: Opsin sequences and color vision status for many lemur species (Bradley et al. in prep) are unpublished data from a separate project, used here with permission. These data should not be cited without specific permission from Rachel Jacobs and/or Brenda Bradley.

whereas including only critical site differences resulted in the more parsimonious single origin for polymorphic trichromacy in platyrrhines.

Based on the above M/L opsin classification scheme, taxa that are presently documented as only exhibiting the M opsin were coded as 1 (routine dichromatic-M or monochromatic-M). Taxa that are presently documented as only exhibiting the L opsin were coded as 3 (routine dichromatic-L), and taxa in which both opsins have been documented were coded as 2 (polymorphic). In one species (*Propithecus verreauxi*), there is clear population-level variation in the presence of both M and L opsins. In one set of analyses, *P. verreauxi* was coded as 2, while an additional analysis incorporated population-level variation by adding a separate branch with a very short, non-zero (0.00001) branch length for the separate population of *P. verreauxi*. Finally, the presence of the L opsin in at least one population of *Lemur catta* remains equivocal. Therefore, separate analyses were run in which *L. catta* was classified as either 1) completely dichromatic with the M opsin, or 2) completely polymorphic. In an additional analysis, as for *P. verreauxi*, *L. catta* was split into two populations: one polymorphic and one dichromatic with the M opsin, with one population assigned a short, non-zero branch length. See Table 2.1 for details on the comparative sample.

Ancestral color vision states for each node were inferred using a maximum likelihood approach (Pagel 1994, 1999; Schluter et al. 1997). All analyses were implemented in R (version 3.1.2; R Core Team 2014) using the 'ace' function in the package 'ape', and maintaining the default 'marginal = FALSE' to compute marginal ancestral states (version 3.2; Paradis et al. 2004). This function infers the most likely set of ancestral states at internal nodes using all information (i.e., all states and nodes proportional to their probabilities) from the tree according to a joint estimation procedure (Pupko et al. 2000).

Four models were examined in this study. First, given the biology of this system, transitioning between states 1 and 3 would likely require even a brief polymorphic period. Therefore, in the first model, this character was treated as ordered and reversible, such that states could transition as follows: 1→2, 2→3, 3→2, and 2→1, but could not directly transition between states 1 and 3, and 3 and 1. The transition rates for this model were also constrained to be equal (i.e., equal rates or ER model), which is suggested for analyses with small sample sizes (Schluter et al. 1997). The other three models examined were as follows: 1) an unordered (all transitions could occur) ER model (as might occur if the polymorphic period is brief and fixation occurs rapidly); 2) a complex, ordered model in which all transition rates (for direct transitions only) were allowed to vary (i.e., all-rates-different or ARD model); and 3) a complex unordered ARD model. Akaike's Information Criterion (AIC) was calculated for each model and the relative fit of each model was assessed using the Akaike weight (AICw), which is the probability that one model is better than all other models (Burnham and Anderson 2002). It should be noted that this approach is not considered hypothesis testing *per se*, but rather assesses the relative fit of each model given the data (Burnham and Anderson 2002).

RESULTS

Sex and M/L opsin genotyping

Sequences for exon 5 of the M/L opsin gene were obtained for 87 adult and immature red-bellied lemurs (N_{female} = 47, N_{male} = 40, N_{adult} = 58, $N_{immature}$ = 29; n, $N_{X\ chromosomes}$ = 134; see Table 2.2). All individuals yielded codon ACC (amino acid = threonine) at site 285, and sex genotypes were consistent with color vision genotypes (i.e., males were not heterozygous).

Based on $N_{X\ chromosomes} = 134$, the frequency of the M allele is 0% and the L allele is 100%, indicating that all individuals are dichromats with the L opsin. This sample is more than sufficient to detect a color vision polymorphism present at a low (0.05 or less) frequency. That is, using binomial probability calculations, the probability of *not* detecting a rare (5% or less) allele given the sample size is < 0.001, which suggests the L opsin is likely fixed in the population of red-bellied lemurs in RNP.

Ancestral state estimation

Log-likelihood, AIC, and AICw values for 1) the ordered ER, 2) unordered ER, and 3) ordered ARD models are presented in Table 2.3. The fourth model (unordered ARD) could not be fit to the data. That is, the model could not return standard errors, likely as a result of the complexity of the model in relation to sample size, and so the results presented here are restricted to the former 3 models. In all cases, the ordered ARD model (AICw range = 0.653-0.840) was a better fit than the ordered ER model (AICw range = 0.133-0.225), and the unordered ER model AICw range = 0.041-0.234). The fit of the unordered ER model compared to the ordered ER model was only better under two analyses. Specifically, the fit of the former model was marginally better in analyses when *L. catta* was coded as 1 (SEA: AICw for unordered ER = 0.179 compared to AICw for ordered ER = 0.113; SMK: AICw for unordered ER = 0.234 compared to AICw for ordered ER = 0.126), but, again the ordered ARD was a better fit under all conditions. Although the ordered ARD was the best-fit model overall, AICw values indicate there is a large amount of uncertainty regarding the best fit model. This caveat in mind, ancestral estimations are presented for both the ordered ER (given only marginal differences with the unordered ER) and the ordered ARD models.

Ancestral state estimations for each node are presented for the ordered ER and ordered ARD models in Figures 2.3-2.5, which denote the ancestral state for each node with the highest probability, as well as the strength of the estimation based on relative probabilities. The proportional probabilities for each state and all estimations can be found in Appendices 1-4.

Results ordered ER: In all analyses under the ordered ER model, an M/L opsin polymorphism was returned as the color vision state with the highest probability in the ancestral *Eulemur* (0.62-0.94), although values vary depending on the phylogeny, the condition of *L. catta*, and the inclusion of population-level variation.

More specifically, a polymorphic state for the ancestral *Eulemur* was estimated with higher probability using the hypothesized relationships in SMK (0.81-0.94) compared to those of SEA (0.62-0.70). The lowest probabilities for a polymorphic ancestral *Eulemur* were obtained for each phylogeny when population-level variation was included in analyses (0.62 for SEA and 0.81 for SMK). The highest probabilities for both phylogenies were obtained when population variation was ignored and *L. catta* was classified as polymorphic (0.70 for SEA and 0.94 for SMK).

In all analyses, a polymorphic ancestral condition was recovered for the ancestral *Propithecus* (0.91-0.98) and the ancestral *Varecia* (>0.99). Furthermore, all analyses excluding those with population-level variation also suggest that an M/L opsin polymorphism may have an early single origin at the base of the [Lemuridae]/[Indriidae/Cheirogaleidae/Lepilemuridae] clade (0.46-610), albeit with low probabilities. However monomorphism for the M opsin had the lowest probability at this node in all scenarios (0.20-0.26). Again, probabilities vary depending on the character coding of *L. catta*, and support for a color vision polymorphism at this node was

highly equivocal when population-level variation was included in analyses (0.36), but monomorphism for the M opsin remained the condition with the lowest probability (0.24-0.25).

Results ordered ARD: Allowing the rates of the ordered transitions to vary improved the fit of the model. The major difference between this model and the other models is that the rate parameter for transition 1 to 2 was estimated to be effectively zero, whereas it was constrained to be equal in the ER models. The state with the highest probabilities at the root was either monomorphism for the L opsin (0.50-0.66) or polymorphic (0.52-0.56). The latter resulted in those analyses that coded *L. catta* as polymorphic.

Under the ordered ARD model, a polymorphic *Eulemur* ancestor had the highest probabilities under the SMK phylogeny and regardless of how *L. catta* was coded or whether population-level variation was included (0.90-0.92). Using the SEA phylogeny in analyses returned more equivocal results at the base of the *Eulemur* clade. That is, monomorphism for the L opsin had equal probability as an M/L opsin polymorphism when *L. catta* was coded as 1 (0.50), and was returned as the state with the highest probability when population-level variation was included in analyses (0.65). A polymorphic *Eulemur* ancestor had the highest (albeit only marginally) probability (0.54) when *L. catta* was coded as 2.

Monomorphism for the L opsin was returned as the state with the highest probabilities at nearly all internal lemuriform nodes for all analyses (see Figures 2.3-2.5 C and D). An M/L opsin polymorphism was returned as the state with the highest probability at the base of *Propithecus* (0.90-0.93), and *Varecia* (>0.99) under all analyses, as well as the base of *L. catta* (1.00) when population-level variation was included in analyses.

DISCUSSION

Results of this study indicate that color vision in the population of *E. rubriventer* in Ranomafana National Park (RNP) is, at the current state of knowledge, unique compared to other members of the genus *Eulemur* in being dichromatic with the L opsin only. Specifically, all sequences for exon 5 of the M/L opsin gene in this population have the amino acid threonine at site 285 (λ_{max} ~ 558 nm). All other species of *Eulemur* for which data are available appear to be either dichromatic with the M opsin (λ_{max} ~ 543 nm) or polymorphic (Tan and Li 1999; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). This raises the question as to why *E. rubriventer* exhibits a different pattern of color vision than other *Eulemur* species. However, it is important to emphasize that variation in color vision capacities has been documented at the population level in lemurs (i.e., *P. verreauxi*; Bradley et al. 2009; Bradley et al. in prep), which is notable given that the data analyzed here are from a single site (i.e., RNP) for a taxon with a relatively wide distribution in Madagascar (Irwin et al. 2005). Consequently, dichromacy for the L opsin may be a characteristic of this population, but may not characterize the entire species. Future research on color vision in *E. rubriventer* (as well as other lemurs) should include samples from multiple populations to determine if color vision capacities vary across populations.

With the above result, this study evaluated ancestral states for color vision phenotype in lemurs using different models of M/L opsin evolution and found that under most scenarios, the ancestral state with the highest probability for the *Eulemur* node was polymorphic, which suggests that *E. rubriventer* likely lost polymorphic trichromacy. Under a scenario where color vision state was treated as ordered and transition rates between states were constrained to be equal, an M/L opsin polymorphism was the most likely condition in both the hypothesized

relationships of the SEA (i.e., Springer et al. 2012) and SMK (Springer et al. 2012 with the *Eulemur* relationships hypothesized in Markolf and Kappeler 2013) phylogenies. The probability of a polymorphic *Eulemur* ancestor was higher in the SMK phylogeny, which is unsurprising given that *E. flavifrons* (sister to all other *Eulemur* in SMK) exhibits polymorphic trichromacy, while *E. rubriventer* (sister to all other *Eulemur* in SEA) is dichromatic with the L opsin only.

When ordered transition rates were allowed to vary, the model was a better fit to the data and also returned an M/L opsin polymorphism as the ancestral *Eulemur* state under many conditions. Specifically, analyses using the SMK phylogeny always returned a polymorphic *Eulemur* ancestor with the highest probability. Results were more equivocal using the SEA phylogeny. That is, in all analyses, monomorphism for the L opsin was returned as the ancestral condition in near equal probabilities as an M/L opsin polymorphism. Importantly, while this model was a relatively better fit to the data compared to other models, the probabilities for this model being better than all others only ranged between 0.65 and 0.84, indicating there is some uncertainty in the best model. Furthermore, an all-rates-different model may not be appropriate with small samples sizes, because it can lead to estimations of high transition rates away from unique states found on shorter branches, such that if change is rare, unique states might appear at the root (Schluter et al. 1997). This factor may have contributed to the high probabilities of either a monomorphic L opsin or M/L opsin polymorphism at the root. This result is counter to other parsimony-based analyses that suggest the M opsin is the ancestral primate condition (Heesy and Ross 2001; but see Tan and Li 1999; Tan et al. 2005), and also counter to estimations under equal rates models that identify monomorphism for the M opsin at the root (albeit equivocally). To tease apart the ancestral conditions at these deeper nodes and improve model fit, future

research should aim to increase samples size and include additional taxa, such as haplorhines and primate outgroups.

With these caveats in mind, results of these analyses are also equivocal regarding the origin(s) of polymorphic color vision in lemurs. Equal rates models suggest that the color vision polymorphism in lemurs may have an ancient single origin, potentially at the base of the [Lemuridae]/[Indriidae/Cheirogaleidae/Lepilemuridae] clade. That is, an M/L opsin polymorphism was returned as the state with the highest probability (albeit ≤ 0.61) under all scenarios, with the exceptions of those including population-level variation, where all states were returned at near equal probabilities. All-rates-different models, however, suggest multiple independent origins for an M/L opsin polymorphism. That is, monomorphism for the L opsin had the highest probability at nearly all internal lemur nodes with a polymorphic state returned in *Propithecus*, *Varecia*, and *Eulemur* lineages, as well as *L. catta* in analyses including population-level variation. This model also suggests rapid transitions to monomorphism with the M opsin in some lineages (e.g., lorisiforms, *Cheirogaleus*), all of which may be related to potential problems with fitting complex models when samples sizes are small (see above).

One additional and important note regarding these analyses is that family-level relationships in lemurs remain highly contentious, particularly in regard to the placement of the Indriidae. Alternative hypotheses suggest a lemurid/indriid clade to the exclusion of the Lepilemuridae and Cherogaleidae, although support for this relationship remains limited (e.g., DelPero et al. 2001; Roos et al. 2004; Masters et al. 2013). Additional clarification of relationships across lemurs will be necessary to further evaluate the evolution of color vision polymorphisms throughout the lemur lineage.

Despite limitations to broader interpretations of color vision evolution in lemurs, results of this study suggest that the ancestral *Eulemur* condition is most likely an M/L opsin polymorphism and, based on current M/L opsin allele frequency data, identifies multiple independent losses of polymorphic trichromacy in this lineage. More specifically, polymorphic trichromacy has likely been lost in *E. rubriventer* as well as other *Eulemur* taxa (i.e., *E. mongoz* and potentially *E. collaris*, although data on the latter are limited to 5 samples from a captive population; Leonhardt et al. 2009). *E. flavifrons* and *E. rufifrons*, on the other hand, likely maintain the ancestral polymorphic condition. Multiple losses of polymorphic trichromacy in this lineage stands in contrast to other polymorphic lineages (i.e., *Propithecus* and *Varecia*) in which such losses appear to be more limited.

Multiple losses of color vision variation in *Eulemur* is a pattern unlike that observed in New World monkeys, where color vision polymorphisms are highly consistent and likely maintained in populations through positive selection (Shyue et al. 1995; Boissinot et al. 1998; Hiwatashi et al. 2010; Kawamura et al. 2012). This raises the question as to why the capacity for trichromatic color vision, which is a feature long considered to be adaptive in primates (e.g., Allen 1879; Mollon et al. 1984; Mollon 1989), would be lost in some *Eulemur* species/populations.

Indeed, trichromatic color vision is thought to be advantageous for foraging on red food items (particularly red fruit or leaves) under bright and/or mesopic (i.e., dim) light conditions (e.g., Mollon 1989; Osorio and Vorobyev 1996; Lucas et al. 1998; Dominy and Lucas 2001; Osorio et al. 2004). While there is limited evidence for trichromatic foraging advantages in wild primate populations (e.g., Vogel et al. 2007; Melin et al. 2008, 2009; Smith et al. 2012; Fedigan et al. 2014), such advantages have been observed in captive settings (e.g., Caine and Mundy

2000; Smith et al. 2003), and this remains the most long-standing hypothesis for the evolution of trichromatic color vision in primates (e.g., Allen 1879).

Under this adaptive scenario, the ecology of *Eulemur* species, including *E. rubriventer,* is a classic example of species in which the evolution of trichromatic color vision would likely be maintained through positive selection. *Eulemur* species are cathemeral, so while they have some activity at night (light levels less conducive to color vision), they are also readily active during the day (light levels more conducive to color vision), and are also highly frugivorous (reviewed in Mittermeier et al. 2010). Accordingly, loss of polymorphic trichromacy in some *Eulemur* species demands explanation, and there are multiple, not necessarily mutually exclusive, evolutionary mechanisms that may account for such losses.

First, loss of polymorphic trichromacy may result from relaxed selection to maintain color vision variation (Jacobs and Deegan 2003b; Kawamura et al. 2012). Under this scenario, fixation of the L or M opsin would be the consequence of random allele loss due to genetic drift (Futuyma 1998). Again, trichromatic color vision is generally considered to be adaptive in some primates, but signatures of positive selection for polymorphic trichromacy have only been documented in haplorhines (Hiwatashi et al. 2010; Kawamura et al. 2012), and it remains unknown whether or not color vision polymorphisms are maintained through positive selection (i.e., balanced selection) in lemurs.

It has been suggested that color vision may be less acute in lemurs, which might lead to loss of color vision variation through relaxed selection (e.g., Jacobs and Deegan 2003b; Kawamura et al. 2012). This hypothesis notes that, as mentioned above, strepsirrhine visual systems differ in multiple features compared to the visual systems of haplorhines (e.g., lower cone densities, lack of a fovea; e.g., Rohen and Castenholz 1967; Wikler and Rakic 1990; Kay

and Kirk 2000; Kirk and Kay 2004) that may result in relatively lower color vision acuity (e.g., Jacobs and Deegan 2003b; Kawamura et al. 2012).

The visual systems of *Eulemur* also differ from those of diurnal strepsirrhines and appear to represent a compromise between the competing demands of high light and low light level conditions (Kirk 2006). For example, cone densities in *Eulemur* are considerably lower than those in diurnal *Propithecus*, but higher than nocturnal primates (Peichl et al. 2001; Kirk 2006). Retinal summation is similarly intermediate in *Eulemur* (Kay and Kirk 2000; Kirk and Kay 2004), and the single study of visual acuity in this genus (*E. flavifrons*) indicates that *Eulemur* acuity actually resembles that of nocturnal strepsirrhines (Veilleux and Kirk 2009). Consequently, color vision acuity may be lower in species of *Eulemur* (and potentially other cathemeral taxa), and account for the seemingly higher frequency of allele loss in *Eulemur* compared to that of other polymorphic lineages, in particular *Propithecus*.

This hypothesis, however, does not accord well with the evolutionary history of polymorphic trichromacy and its relationship to cathemerality in *Eulemur*. It has been proposed that cathemeral activity in some lemurs (e.g., *Eulemur*) represents a recent transition from nocturnality to more diurnal behavior as the result of recent (~2,000 years) extinctions of large diurnal lemurs and raptors that, once extirpated, opened up new niches for previously nocturnal species to exploit (van Shaik and Kappeler 1996). This hypothesis suggests that the visual systems of cathemeral lemurs may not be well-adapted to diurnal conditions (van Schaik and Kappeler 1996), which could potentially account for multiple losses of polymorphic trichromacy in this lineage. Interestingly, this scenario would imply, given that polymorphic trichromacy likely occurs at the base of the *Eulemur* clade, that this trait may have evolved in species with a nocturnal activity pattern. If color vision polymorphisms arose early in the lemur lineage, this

would also suggest they evolved under a nocturnal scenario, given ancestral estimations of lemur activity patterns (e.g., Heesy and Ross 2001; Santini et al. 2015). Intriguingly, a recent color vision study on tarsiers suggests that nocturnality may be compatible with the evolution of polymorphic trichromatic color vision, as crown tarsiers were likely polymorphic, and identifies moonlight and twilight conditions, rather than strict diurnality, as potentially favoring the evolution of this trait (Melin et al. 2013).

All that said, more recent analyses of *Eulemur* eye morphology (Kirk 2006), as well as recent ancestral estimations of *Eulemur* activity patterns (Santini et al. 2015), indicate that cathemerality is likely the ancestral *Eulemur* condition. Given that polymorphic trichromacy occurs at the base of the *Eulemur* lineage, and it is presently documented in some *Eulemur* species, this would suggest that polymorphic trichromacy has a long evolutionary history despite likely lower visual acuity, making it less likely that relaxed selection as a result of *Eulemur* visual systems accounts for loss of color vision variation. However, given that visual systems of many lemurs have not been well-studied, it is possible that more subtle variation in visual systems across species plays a role in the maintenance of color vision polymorphisms.

Given that polymorphic trichromacy appears to be broadly compatible with *Eulemur* visual systems and cathemerality, other explanations for loss of this trait may be warranted, and it may be better to take a more species/population-specific approach. If polymorphic trichromacy is theoretically adaptive in *Eulemur* species, loss of variation can occur in populations with low effective population sizes, such that genetic drift plays a larger role than selection (Futuyma 1998). *E. rubriventer* is largely pair-living and potentially monogamous (Merenlender 1993; note that polygamous groups may occur; R. Jacobs unpublished data), which suggests that, theoretically, effective population size should be greater for this taxon compared to other

polygamous taxa (Frankham 2007), such as *Eulemur rufifrons* (Ostner and Kappeler 1999), which has polymorphic trichromacy. However, a number of factors can influence effective population size and the strength of genetic drift, another being random reductions in population size (i.e., genetic bottlenecks). When population sizes are reduced, genetic drift can increase and result in loss of genetic variation, even when it is under positive selection (Futuyma 1998). Such processes may be particularly relevant to species living in Madagascar, given the historical and present-day large-scale forest destruction occurring throughout the island (Dewar 2003; Harper et al. 2007). Forest loss and fragmentation combined with other anthropogenic disturbances, such as hunting, as well as natural climatic events have led to the extirpation of multiple lemur species and extreme reductions in the current distributions of some extant taxa (Godfrey et al. 1999; Perez et al. 2005; Crowley 2010; Quéméré et al. 2012). Therefore, current genetic variation observed in lemur species/populations may be the result of severe population declines and genetic bottlenecks (see chapter 3) rather than selective pressures.

The above scenarios highlight the potential role of nonadaptive evolutionary mechanisms shaping color vision in some species of *Eulemur*. However, variation in color vision across species may have adaptive explanations. For example, in *E. rubriventer* there may have been directional selection favoring the L opsin (Futuyma 1998). It has been hypothesized that dichromacy with the L opsin in *Avahi* may be an adaptation to foraging on young leaves, given that this color vision phenotype had superior performance compared to dichromacy with the M opsin for detecting foods readily consumed by this genus (Veilleux et al. 2014). It is possible, therefore, that different selective processes are acting on different species, and this may be related to variation in species' ecologies. Similar techniques modeling the detectability of lemur

food items to different color vision phenotypes may help elucidate potential adaptive explanations for the variable distribution of color vision observed in lemurs (see chapter 4).

Related to this hypothesis is the potential that fixation of one opsin or another represents disruptive selection (selection against trichromacy) in some species (Futuyma 1998). Although it may seem difficult to envision a scenario in which trichromatic color vision would be disadvantageous, there are proposed costs to trichromacy. In particular, chromatic information may actually corrupt luminance vision (Osorio et al. 1998), which is used for visual tasks such as motion and shape detection, and may be important for detecting camouflaged objects (e.g., some predators and food items) (Livingstone and Hubel 1988; Morgan et al. 1992). If dichromatic *Eulemur* species (e.g., *E. rubriventer*) rely heavily on luminance vision, this scenario could result in dichromacy being favored over trichromacy (Osorio et al. 1998; Osorio et al. 2004), in which case the opsin with the highest frequency would become fixed in a population (Futuyma 1998).

Importantly, any one of these potential hypotheses is unlikely to explain all or even most of the color vision variation observed in *Eulemur* and other lemur species/populations. Multiple adaptive and nonadaptive mechanisms are likely at play, and they may be related to variation in a number of factors, such as species' ecologies and past demographic histories. Distinguishing among alternative (not mutually exclusive) hypotheses will be a challenge, but it will be important for understanding how both adaptive and nonadaptive mechanisms might shape the large variation in color vision observed in primates today.

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Figure 2.1 Study sites within and around Ranomafana National Park. This figure was modified with permission from Baden (2011).

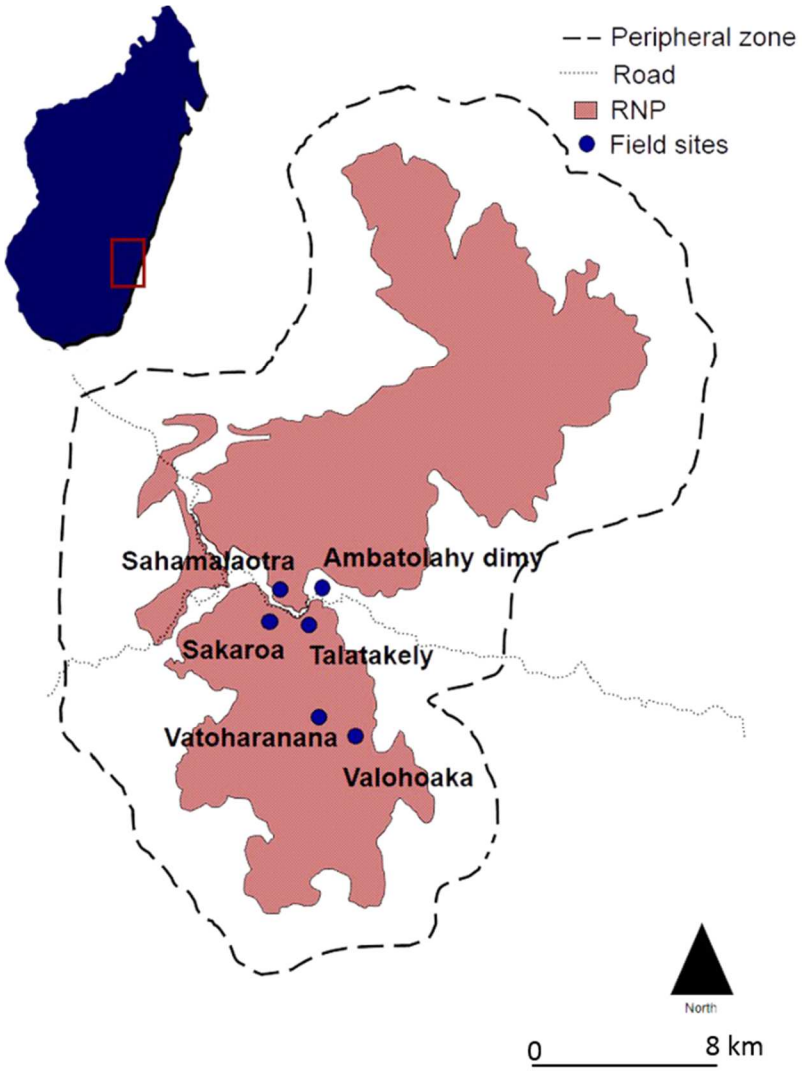


Figure 2.2 Frontal facial photographs of 12 adult female (top) and 12 adult male (bottom) red-bellied lemurs in Ranomafana National Park. Photographs illustrate individual variation in facial pelage patterns used to identify individuals during data collection.

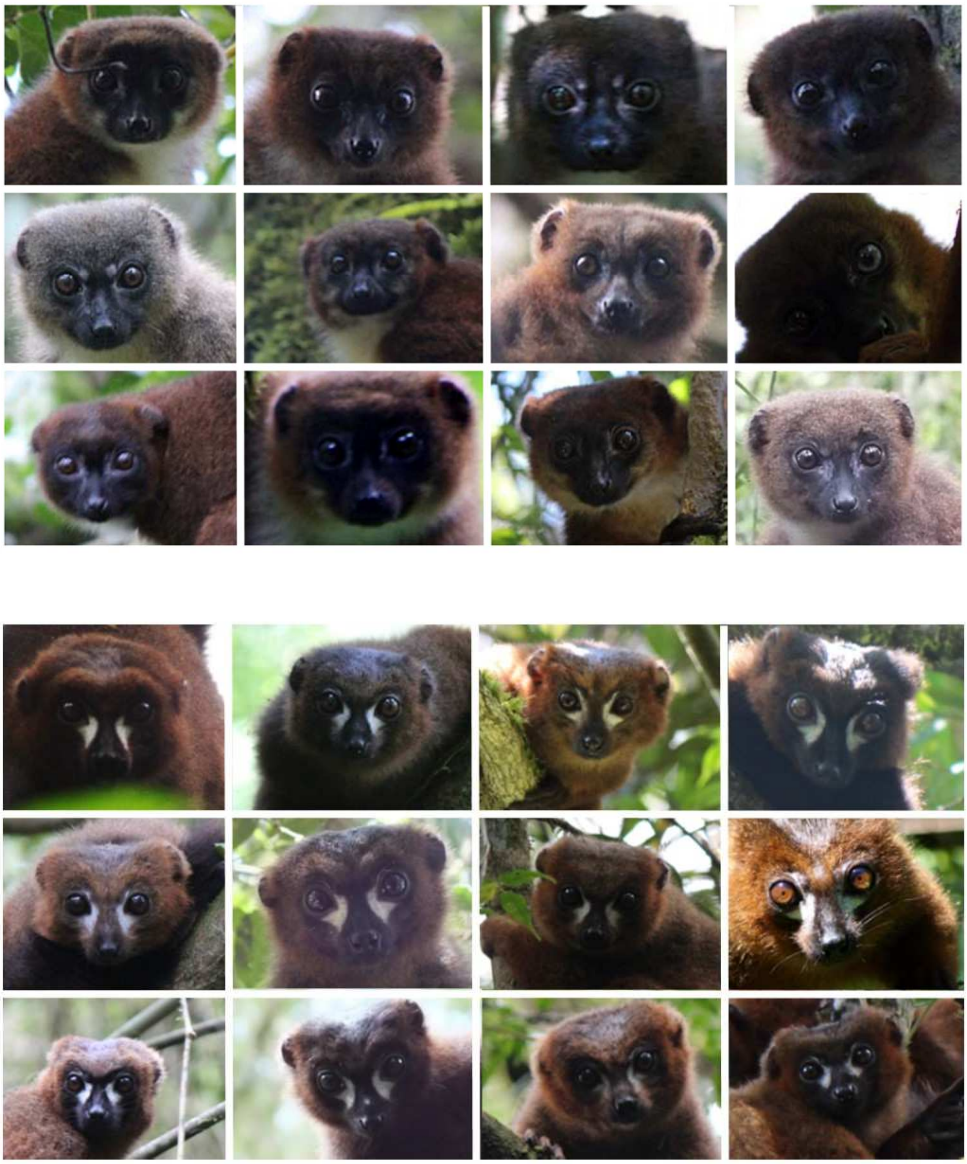
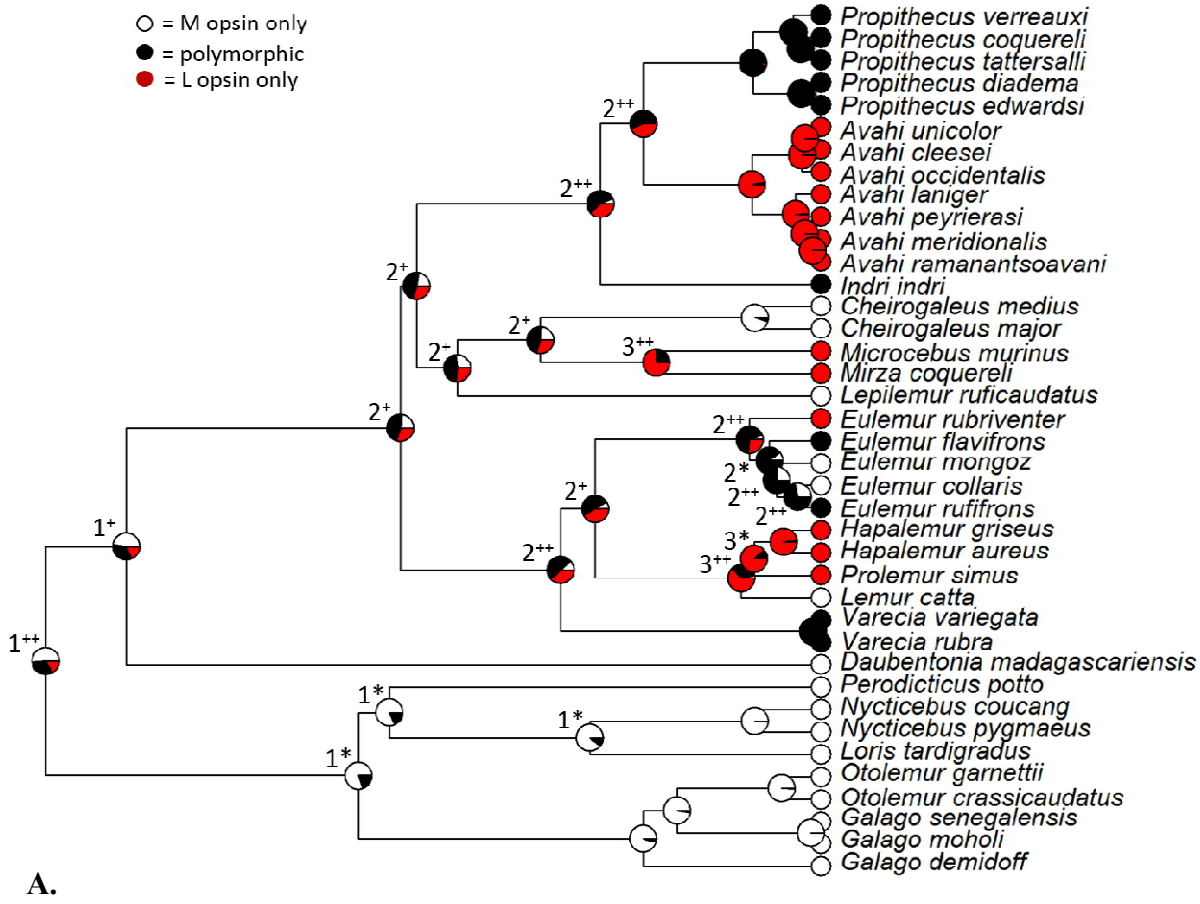
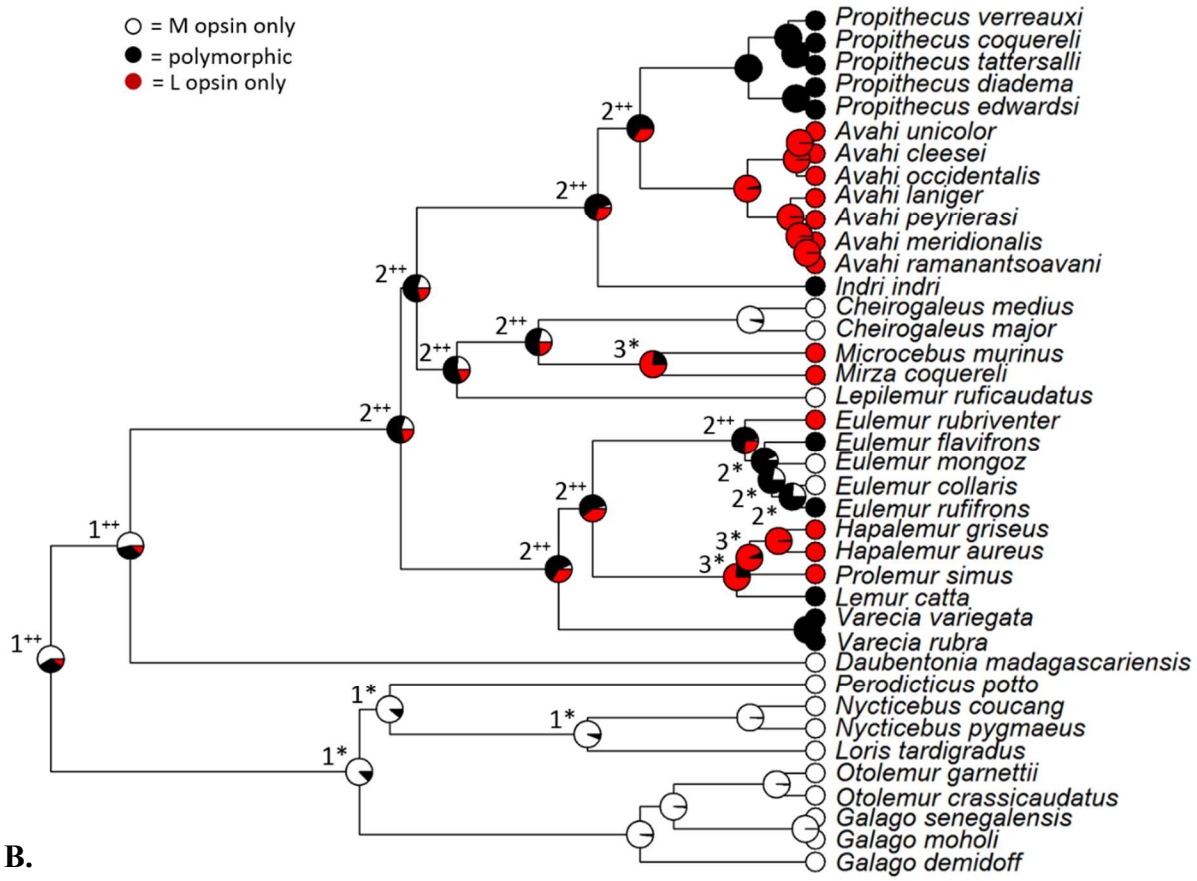


Figure 2.3 Ancestral state estimations using maximum likelihood for color vision phenotype across strepsirrhines based on the SEA phylogenetic tree. Figures depict ancestral estimations for the ordered ER model with *L. catta* coded as **A)** dichromat with the medium allele and **B)** polymorphic, and ancestral estimations for the better-fit ordered ARD with *L. catta* coded as **C)** dichromat with the medium allele and **D)** polymorphic. Pie charts at each node depict relative probabilities for each color vision phenotype. Numbers at each node represent the color vision phenotype with the highest relative probability (1 = monomorphic M opsin, 2 = polymorphic, 3 = monomorphic L opsin). Symbols represent the strength of the estimation for the state with the highest relative probability (+ = < 50% of relative probability, ++ = 50-74.99%, * = 75-94.99%, no symbol = ≥ 95%). For exact values at each node, see Appendix 1.

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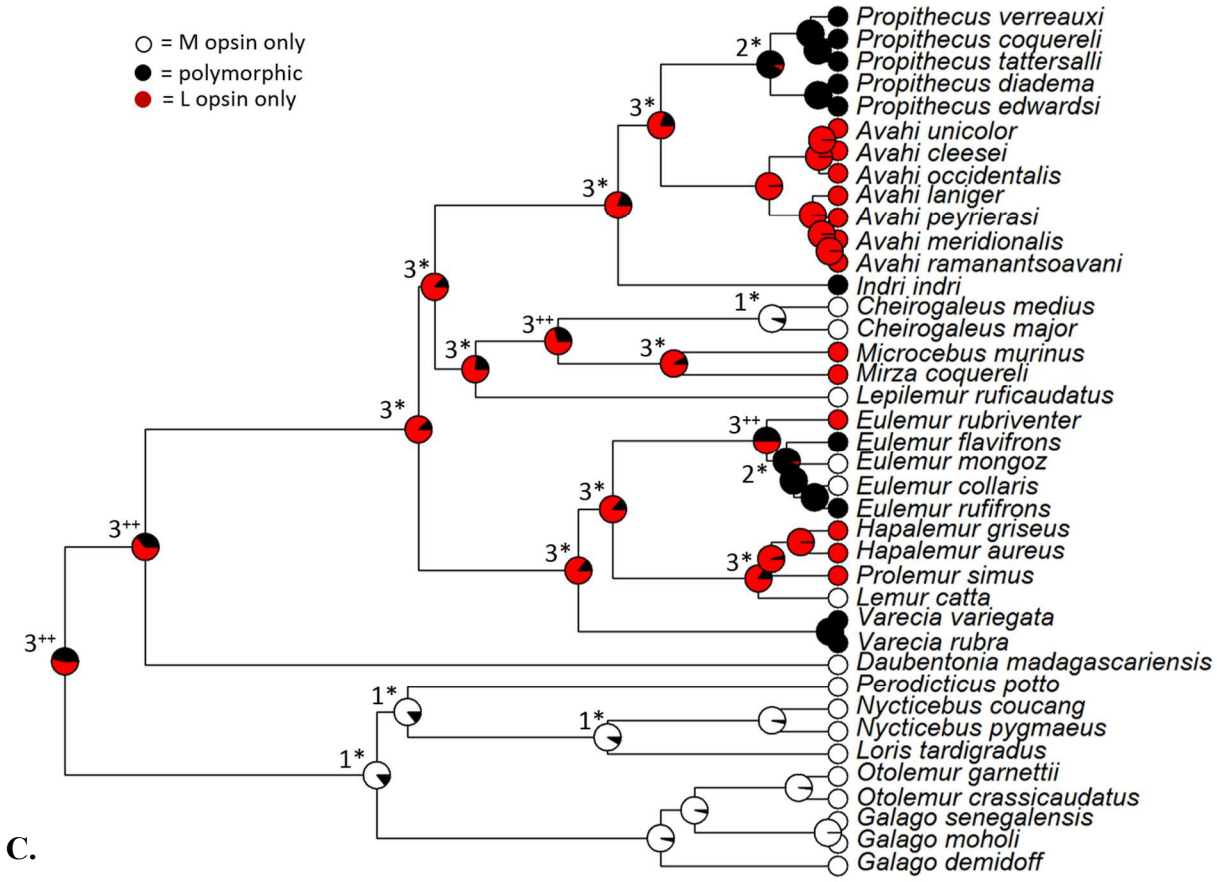
Propithecus tattersalli
Propithecus diadema
Propithecus edwardsi
Avahi unicolor
Avahi cleesei
Avahi occidentalis
Avahi laniger
Avahi peyrierasi
Avahi meridionalis
Avahi ramanantsoavani
Indri indri
Cheirogaleus medius
Cheirogaleus major
Microcebus murinus
Mirza coquereli
Lepilemur ruficaudatus
Eulemur rubriventer
Eulemur flavifrons
Eulemur mongoz
Eulemur collaris
Eulemur rufifrons
Hapalemur griseus
Hapalemur aureus
Prolemur simus
Lemur catta
Varecia variegata
Varecia rubra
Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
Loris tardigradus
Otolemur garnettii
Otolemur crassicaudatus
Galago senegalensis
Galago moholi
Galago demidoff
1++
2++
3*
2*
1*

B.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Avahi occidentalis
Avahi laniger
Avahi peyrierasi
Avahi meridionalis
Avahi ramanantsoavani
Indri indri
Cheirogaleus medius
Cheirogaleus major
Microcebus murinus
Mirza coquereli
Lepilemur ruficaudatus
Eulemur rubriventer
Eulemur flavifrons
Eulemur mongoz
Eulemur collaris
Eulemur rufifrons
Hapalemur griseus
Hapalemur aureus
Prolemur simus
Lemur catta
Varecia variegata
Varecia rubra
Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
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Otolemur crassicaudatus
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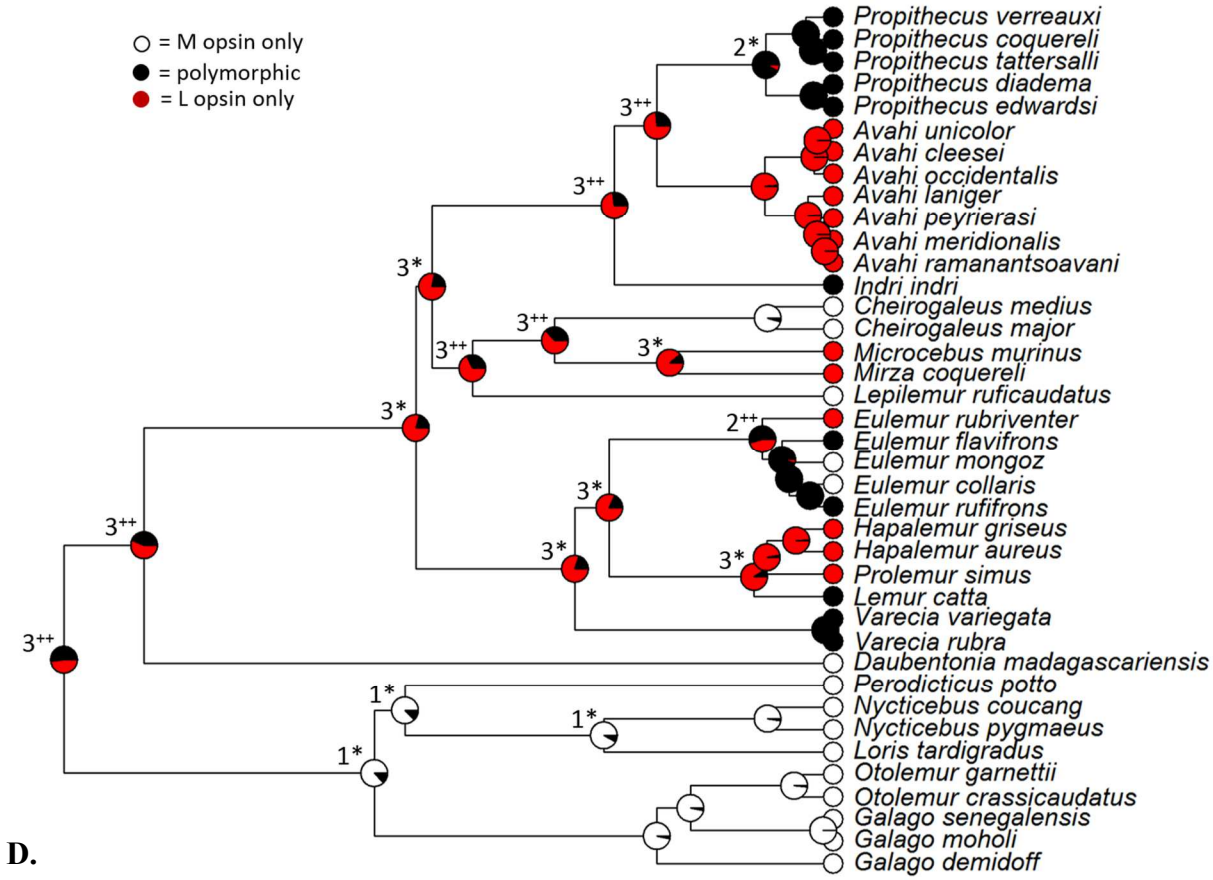
Galago moholi
Galago demidoff

C.

D.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Avahi meridionalis
Avahi ramanantsoavani
Indri indri
Cheirogaleus medius
Cheirogaleus major
Microcebus murinus
Mirza coquereli
Lepilemur ruficaudatus
Eulemur rubriventer
Eulemur flavifrons
Eulemur mongoz
Eulemur collaris
Eulemur rufifrons
Hapalemur griseus
Hapalemur aureus
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Lemur catta
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Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
Loris tardigradus
Otolemur garnettii
Otolemur crassicaudatus
Galago senegalensis
Galago moholi
Galago demidoff
2*
3++
3++
3*
3++
3++
3*
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2++
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3++
3++
1*
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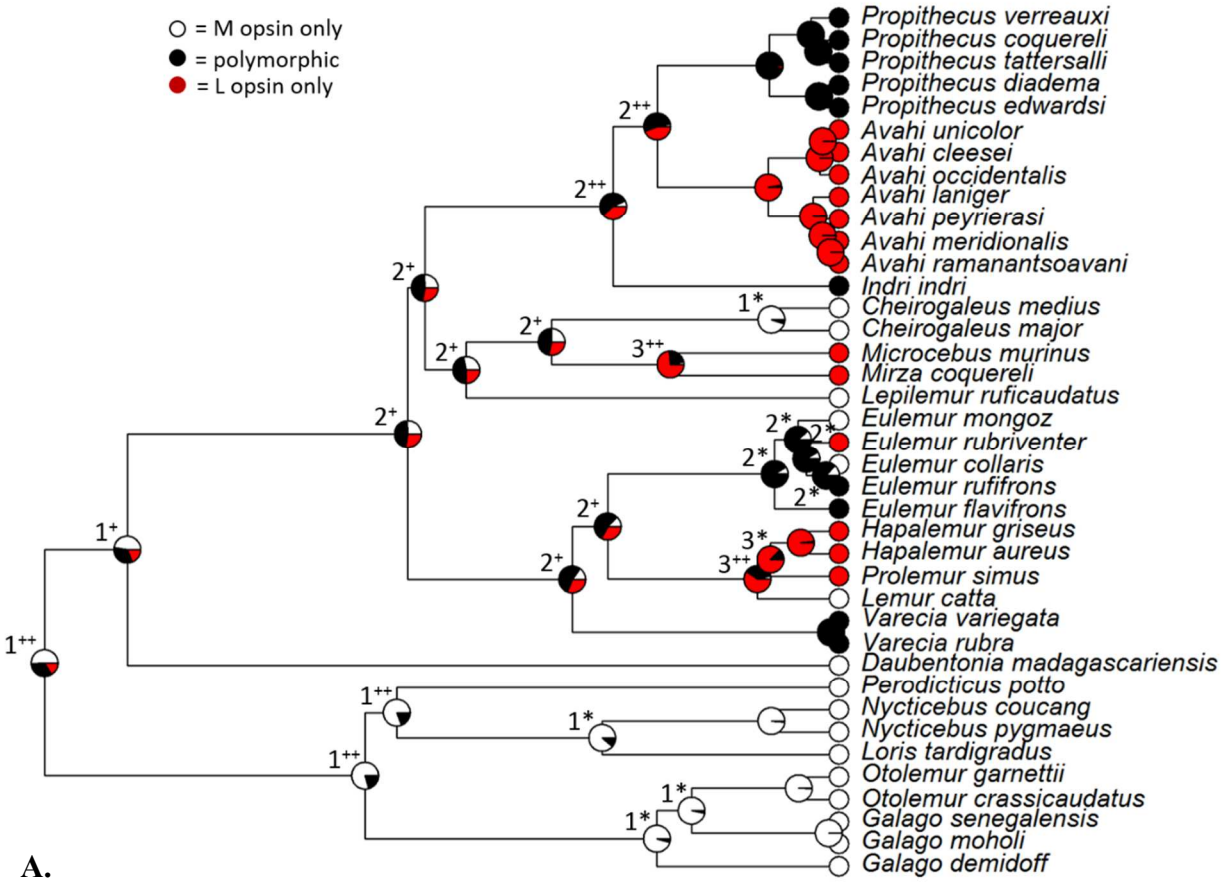
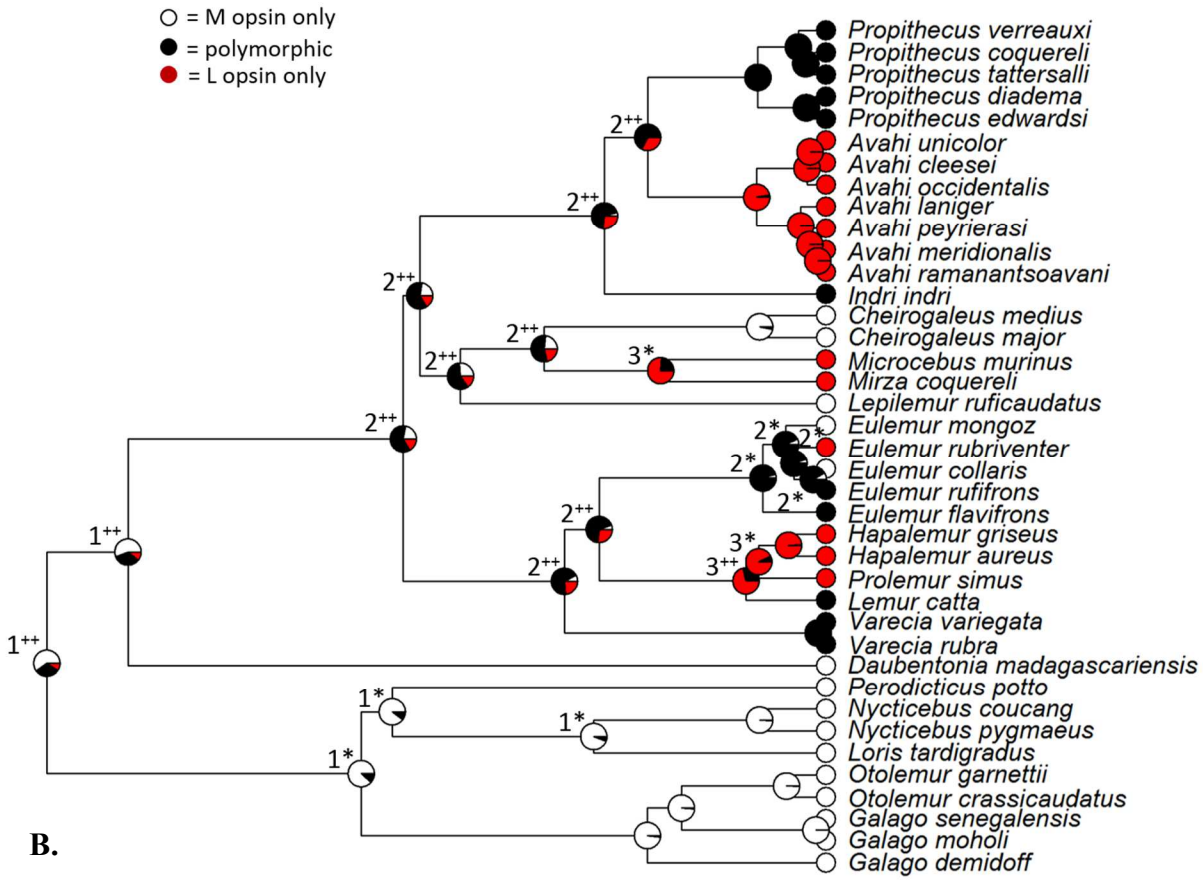
1*

Figure 2.4 Ancestral state estimations using maximum likelihood for color vision phenotype across strepsirrhines based on the SMK phylogenetic tree. Figures depict ancestral estimations for the ordered ER model with *L. catta* coded as **A)** dichromat with the medium allele and **B)** polymorphic, and ancestral estimations for the better-fit ordered ARD with *L. catta* coded as **C)** dichromat with the medium allele and **D)** polymorphic. Pie charts at each node depict relative probabilities for each color vision phenotype. Numbers at each node represent the color vision phenotype with the highest relative probability (1 = monomorphic M opsin, 2 = polymorphic, 3 = monomorphic L opsin). Symbols represent the strength of the estimation for the state with the highest relative probability (+ = < 50% of relative probability, ++ = 50-74.99%, * = 75-94.99%, no symbol = ≥ 95%). For exact values at each node, see Appendix 2.

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Avahi occidentalis
Avahi laniger
Avahi peyrierasi
Avahi meridionalis
Avahi ramanantsoavani
Indri indri
Cheirogaleus medius
Cheirogaleus major
Microcebus murinus
Mirza coquereli
Lepilemur ruficaudatus
Eulemur mongoz
Eulemur rubriventer
Eulemur collaris
Eulemur rufifrons
Eulemur flavifrons
Hapalemur griseus
Hapalemur aureus
Prolemur simus
Lemur catta
Varecia variegata
Varecia rubra
Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
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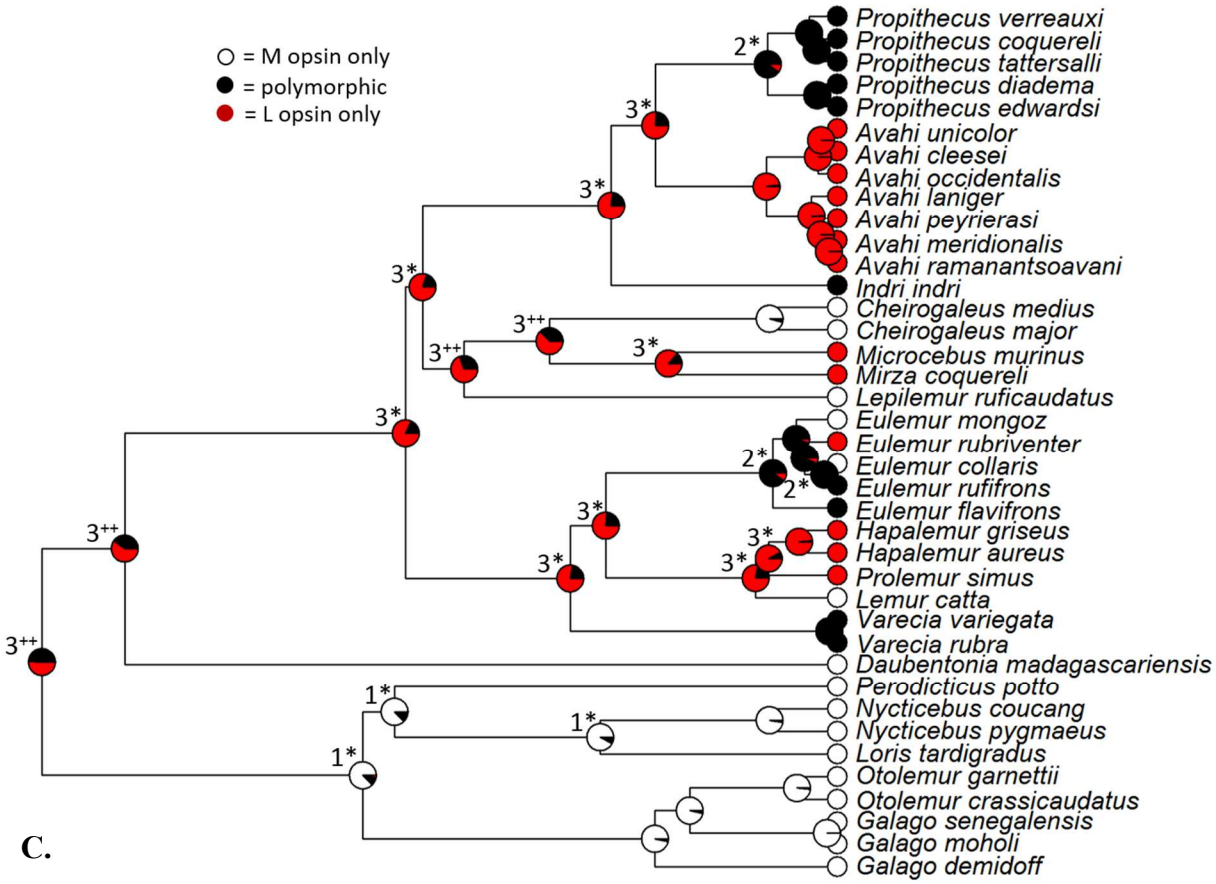
Galago moholi
Galago demidoff
B.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Mirza coquereli
Lepilemur ruficaudatus
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Eulemur collaris
Eulemur rufifrons
Eulemur flavifrons
Hapalemur griseus
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Prolemur simus
Lemur catta
Varecia variegata
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Nycticebus coucang
Nycticebus pygmaeus
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Otolemur crassicaudatus
Galago senegalensis
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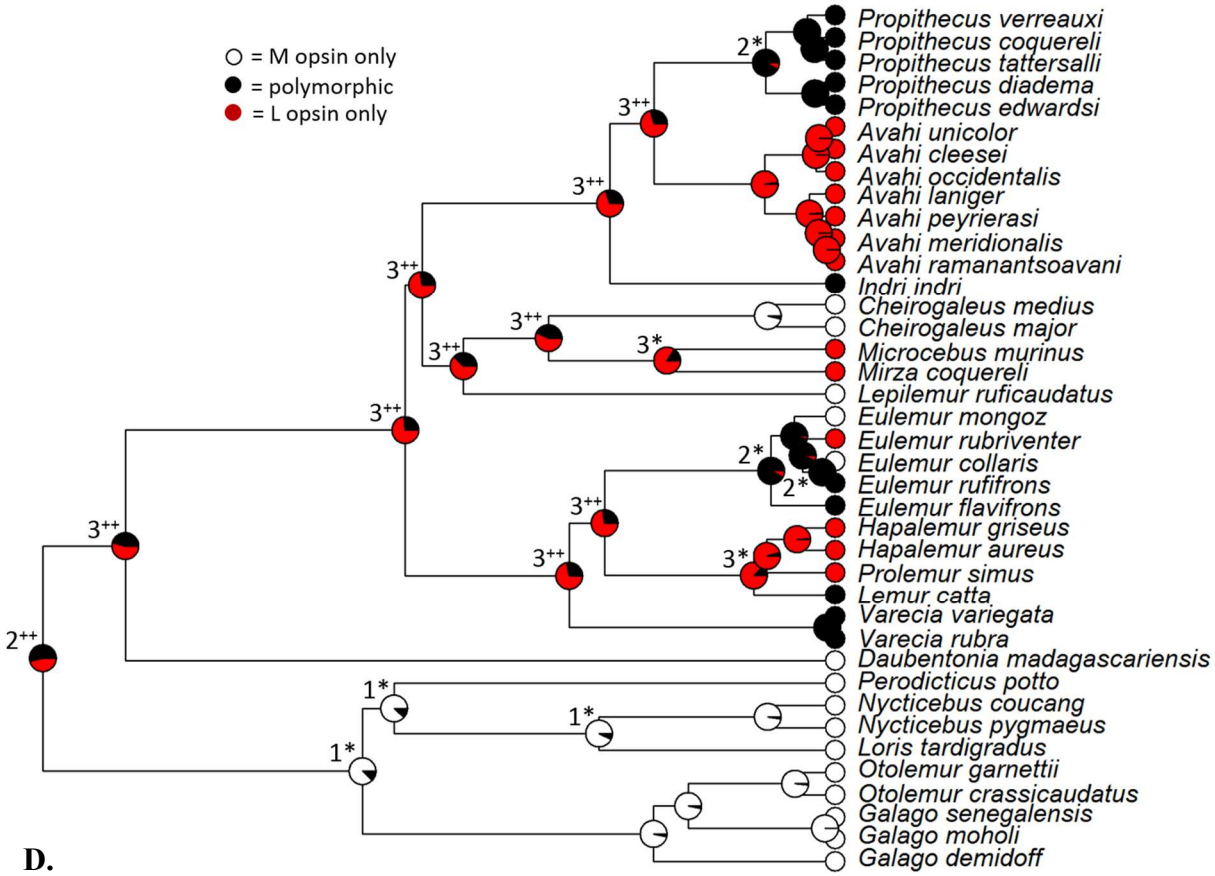
Galago demidoff
C.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Hapalemur griseus
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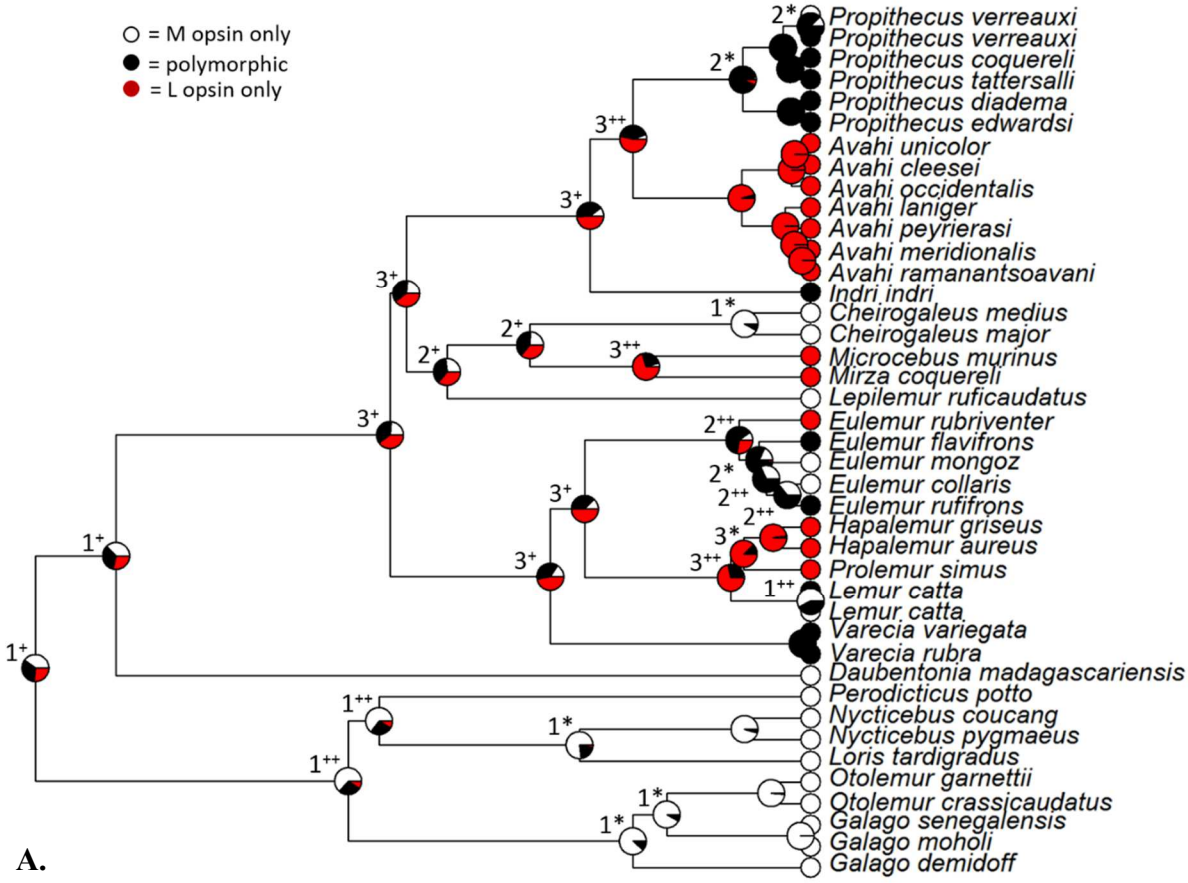
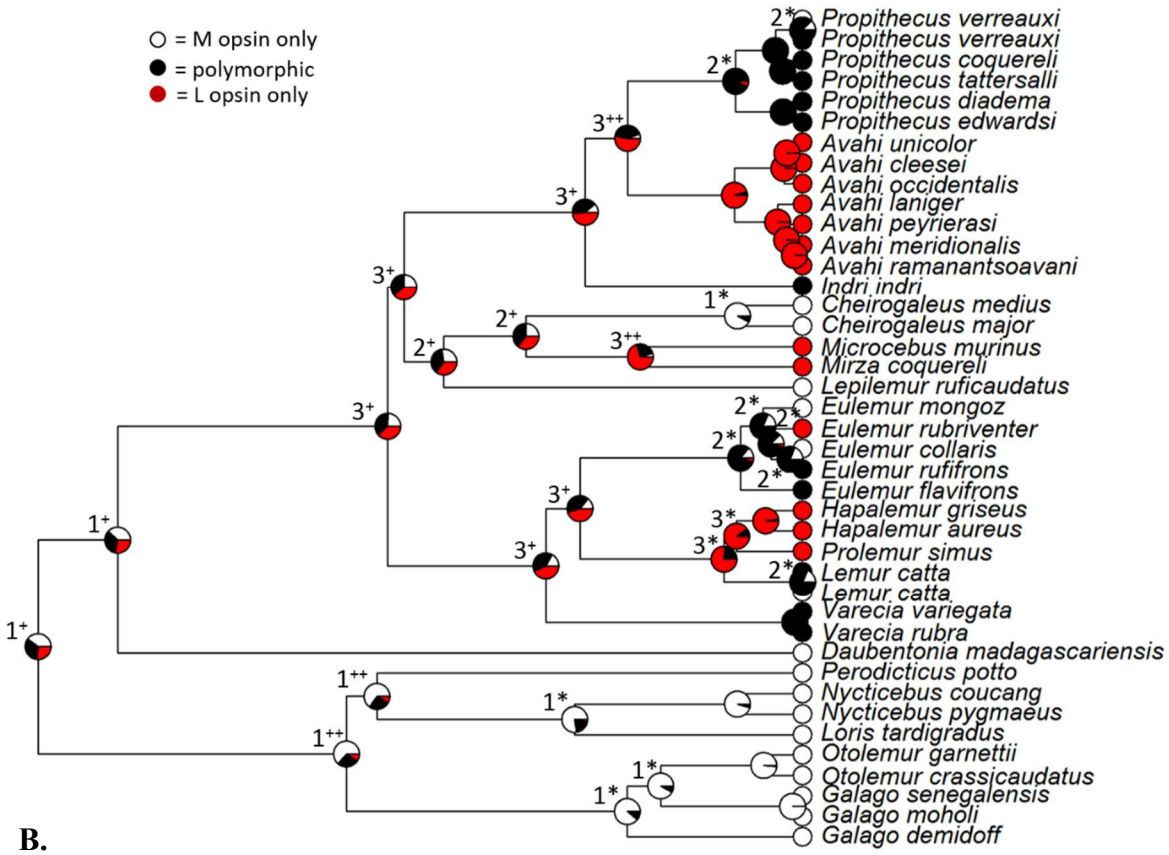
Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
Loris tardigradus
Otolemur garnettii
Otolemur crassicaudatus
Galago senegalensis
Galago moholi
Galago demidoff
2++
3++
1*
2*
3*

D.

Figure 2.5 Ancestral state estimations using maximum likelihood for color vision phenotype that include population-level variation for *P. verreauxi* and *L. catta* for two phylogenetic trees. Figures depict ancestral estimations for the ordered ER model using the phylogenetic relationships of **A)** SEA and **B)** SMK, as well as ancestral estimations for the better-fit ordered ARD model using the phylogenetic relationships of **C)** SEA and **D)** SMK. Pie charts at each node depict relative probabilities for each color vision phenotype. Numbers at each node represent the color vision phenotype with the highest relative probability (1 = monomorphic M opsin, 2 = polymorphic, 3 = monomorphic L opsin). Symbols represent the strength of the estimation for the state with the highest relative probability (+ = < 50% of relative probability, ++ = 50-74.99%, * = 75-94.99%, no symbol = ≥ 95%). For exact values at each node, see Appendices 3-4.

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Prolemur simus
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Lemur catta
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Perodicticus potto
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Nycticebus pygmaeus
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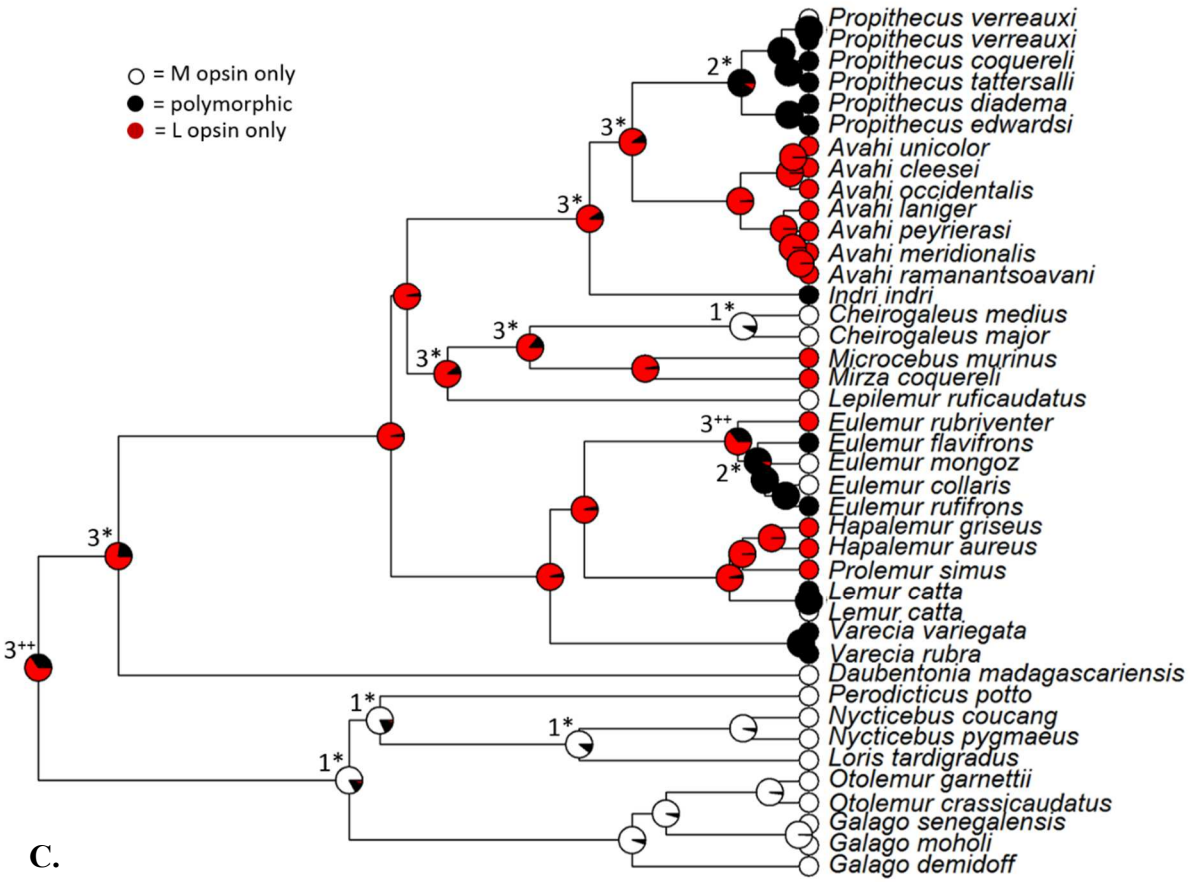
Galago demidoff

B.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Hapalemur aureus
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Lemur catta
Lemur catta
Varecia variegata
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Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
Loris tardigradus
Otolemur garnettii
Otolemur crassicaudatus
Galago senegalensis
Galago moholi
Galago demidoff
2*
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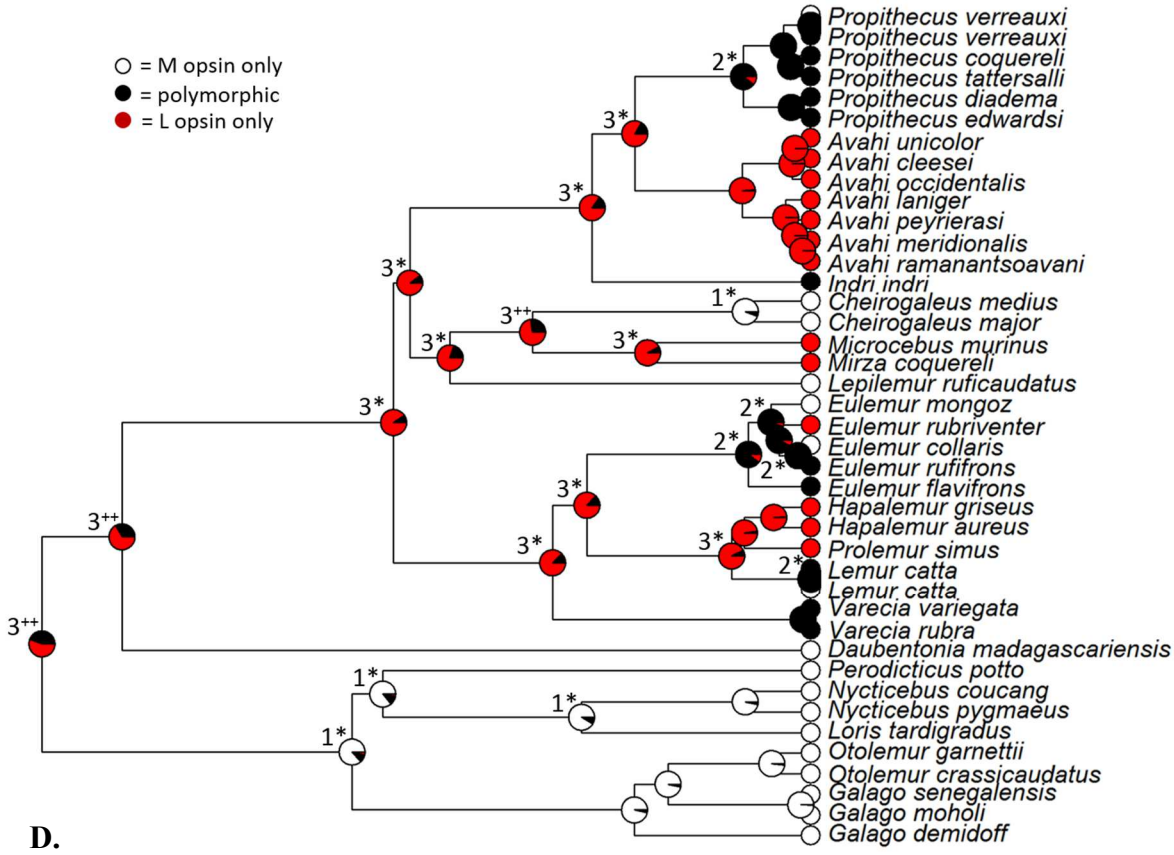
C.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Daubentonia madagascariensis
Perodicticus potto
Nycticebus coucang
Nycticebus pygmaeus
Loris tardigradus
Otolemur garnettii
Otolemur crassicaudatus
Galago senegalensis
Galago moholi
Galago demidoff

D.

Table 2.1. Comparative sample of strepsirrhine M/L opsin peak sensitivities based on exon 5, site 285 only. "X" = presence of the allele.

Species	Exon 5 Site 285		References[a]	Code
	Alanine (~543 nm)	Threonine (~558 nm)		
Galagonidae				
Galago moholi	X		1, 2	1
Galago senegalensis	X		1, 2	1
Galago demidoff	X		2	1
Otolemur crassicaudatus	X		1, 2	1
Otolemur garnetti	X		1, 2	1
Loridae				
Loris tardigradus	X		1, 2	1
Nycticebus pygmaeus	X		2	1
Nycticebus coucang	X		1, 2	1
Perodicticus potto	X		1, 2	1
Cheirogaleidae				
Cheirogaleus major	X		1, 2	1
Cheirogaleus medius	X		1, 2	1
Microcebus murinus		X	1, 2	3
Mirza coquereli		X	1, 2	3
Daubentoniidae				
Daubentonia madagascariensis	X		1, 2	1
Lemuridae				
Eulemur collaris	X		3	1
Eulemur mongoz	X		1, 2, 4	1
Eulemur flavifrons	X	X	5	2
Eulemur rufifrons	X	X	4	2
Eulemur rubriventer		X	This study.	3
Hapalemur griseus		X	1, 2, 4	3
Hapalemur aureus		X	4	3
Lemur catta	X	X?	1, 2, 3, 4	1, 2[b]
Prolemur simus		X	4	3
Varecia rubra	X	X	1, 2, 3, 4	2
Varecia variegata	X	X	4, 6	2
Lepilemuridae				
Lepilemur ruficaudatus	X		4	1
Indriidae				
Avahi cleesei		X	7	3
Avahi laniger		X	7	3
Avahi meridionalis		X	7	3
Avahi occidentalis		X	7	3
Avahi peyrierasi		X	7	3
Avahi ramanantsoavani		X	7	3
Avahi unicolor		X	7	3
Indri indri	X	X	4	2
Propithecus coquereli	X	X	1, 2, 3, 4	2
Propithecus tattersalli	X	X	4	2
Propithecus edwardsi	X	X	4	2

Propithecus verreauxi[c]	X	X	4	2, 1
Propithecus diadema	X	X	4	2

[a] References for M/L opsin gene data. 1 = Tan and Li (1999), 2 = Tan et al. (2005), 3 = Leonhardt et al. (2009), 4 = Bradley et al. (2009); Bradley et al. (in prep), 5 = Veilleux and Bolnick (2009), 6 = Jacobs and Deegan (2003c), 7 = Veilleux et al. (2014)

[b] Because the presence of the L opsin remains equivocal in at least one population of *L. catta*, this taxon was separately coded as "1" and then "2" representing the taxon as a whole, as well as "1" and "2" representing different populations in three different analyses.

[c] This species exhibits population-level variation in the presence of the color vision polymorphism (Bradley et al. 2009; Bradley et al. in prep). Data for *P. v. deckenii* and *P. v. coronatus* have been collapsed into *P. verreauxi*, as their species/subspecies status remains questionable (Pastorini et al. 2001).

Table 2.2 *E. rubriventer* samples from Ranomafana National Park that were genotyped at exon 5 of the M/L opsin gene. Immatures were considered any individuals in the group that appeared to observers not to have attained full adult body size.

Site	Groups	Adult males	Adult females	Immature males	Immature females	X chromosomes
Ambatolahy dimy	1	1	0	0	0	1
Sahamalaotra	4	2	4	0	1	12
Sakaroa	6	2	4	1	2	15
Talatakely	9	9	8	2	4	35
Valohoaka	9	7	7	5	7	40
Vatoharanana	7	7	7	4	3	31
Total	**36**	**28**	**30**	**12**	**17**	**134**

Table 2.3 Number of rate parameters (k), log-likelihood (LL) values, Akaike's Information Criterion (AIC), and Akaike weights (AICw) for the 1) ordered equal transition rates (ER) model, 2) unordered ER model, and 3) ordered all-rates-different (ARD) model. Results are presented for analyses using the SEA and SMK phylogenies.

	Ordered ER				**Unordered ER**				**Ordered ARD**			
					SEA							
Analysis	**k**	**LL**	**AIC**	**AICw**	**k**	**LL**	**AIC**	**AICw**	**k**	**LL**	**AIC**	**AICw**
L. catta code = 1	1	-29.42735	60.8547	0.126	1	-29.07694	60.1539	0.179	4	-24.7172	57.4343	0.696
L. catta code = 2	1	-27.39207	56.7841	0.225	1	-28.99912	59.9982	0.045	4	-23.2167	54.4334	0.730
Population variation	1	-51.30487	104.6097	0.189	1	-52.84134	107.6827	0.041	4	-46.9005	101.8010	0.770
					SMK							
L. catta code = 1	1	-31.19441	64.3888	0.113	1	-30.47156	62.9431	0.234	4	-26.4930	60.8867	0.653
L. catta code = 2	1	-29.21222	60.4244	0.202	1	-30.38525	62.7705	0.062	4	-24.9182	57.8364	0.736
Population variation	1	-62.84543	127.6909	0.134	1	-64.49804	130.9961	0.026	4	-58.0132	124.0264	0.840

Chapter 3

The potential role of nonadaptive evolutionary mechanisms shaping color vision in red-bellied lemurs (*Eulemur rubriventer*)

ABSTRACT

Color vision in primates has long been suggested to be adaptive for multiple fitness-related tasks (e.g., food foraging), but nonadaptive explanations have also been proposed to account for color vision variation across the lineage. Lemuriforms, in particular, are highly variable in their color vision capacities, but it is currently unclear why some species are polymorphic, while many lemurs are strictly dichromatic.

Red-bellied lemurs (*Eulemur rubriventer*) in Ranomafana National Park (RNP) are dichromatic, which likely represents loss of a color vision polymorphism. This result may be due to nonadaptive processes, such as relaxed selection and/or recent reductions in population size (i.e., genetic drift). To address the latter, 55 adult red-bellied lemurs from four localities within RNP were genotyped at seven microsatellite loci to test for a signature of a genetic bottleneck.

Under a two-phase mutation model, this study found significant heterozygosity excess (indicative of a population bottleneck) compared to heterozygosity at mutation-drift equilibrium in the *E. rubriventer* population. The result held under a combined female and male data set, as well as a female-only data set, but not a male-only data set. Results of *M*-ratio tests reveal that this population does not exhibit a significantly low *M*-ratio compared to equilibrium (not indicative of a population bottleneck). Despite the mixed results, it is suggested that a genetic

bottleneck may have occurred in this populations and genetic drift may play a role in the evolution of color vision in this population.

INTRODUCTION

Color vision is highly variable across the Order Primates, and this variation occurs both within and across species (e.g., see Surridge et al. 2003 and Jacobs 2009 for reviews). Many primates have trichromatic color vision, allowing individuals the ability to discriminate between a wider range of colors (e.g., greens and reds) than most other eutherian mammals, which are primarily red-green colorblind (i.e., dichromatic) (e.g., Jacobs 1981, 1993). Trichromatic color vision occurs across multiple primate lineages, but the underlying genetic mechanisms resulting in trichromacy differ (e.g., Nathans et al. 1986; Jacobs 1998; Kainz et al. 1998; Dulai et al. 1999; Jacobs and Deegan 1999; Tan and Li 1999).

All catarrhines (for which data are available) are routine trichromats, which is achieved through having three functional opsin genes: one autosomal short-wavelength (S) opsin gene and two separate medium- (M) and long-wavelength (L) opsin genes on the X chromosome (Nathans et al. 1986; Ibbotson et al. 1992). Each gene codes for a distinct opsin protein resulting in three spectrally distinct retinal cone photopigments, thus affording all individuals the potential for trichromatic color vision (Nathans et al. 1986; Bowmaker et al. 1991; Neitz et al. 1991; Ibbotson et al. 1992). The presence of two separate M/L opsin genes is the result of a gene duplication and subsequent fixation that occurred at the base of the catarrhine lineage (Hunt et al. 1998).

Trichromacy is also present in most New World monkeys but is achieved through two mechanisms. Howling monkeys (*Aloutta*) are routinely trichromatic and have two separate M and L opsin genes as in catarrhines (Jacobs et al. 1996; Dulai et al. 1999). Other species exhibit

trichromatic color vision through M/L opsin gene allelic polymorphisms, which results in intra-species/population color vision variation (i.e., polymorphic trichromacy; Jacobs and Neitz 1987; Williams et al. 1992; Jacobs et al. 1993b; Jacobs 1998). Specifically, polymorphic trichromacy occurs when species with only two functional opsin genes (one autosomal S opsin and one M/L opsin on the X chromosome) also possess two or more M/L opsin alleles (Jacobs and Neitz 1987; Williams et al. 1992; Jacobs et al. 1993b; Jacobs 1998). Alleles must be functionally variable, which results from changes in just a few key amino acid sites (site 180 coded in exon 3; sites 277 and 285 coded in exon 5) that lead to differences in the spectral tuning of cone photopigments (Jacobs and Neitz 1987; Neitz et al. 1991; Williams et al. 1992; Jacobs et al. 1993b). Given that allelic variation is X-linked, this provides heterozygous females with the potential for trichromatic color vision, while homozygous females and all males are dichromatic (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993b).

Among New World monkeys, color vision polymorphisms appear to characterize all genera (that have been studied) (e.g., see Jacobs 2007 and Kawamura et al. 2012 for reviews; de Lima et al. 2015), with the exceptions of nocturnal owl monkeys (*Aotus*), which are monochromatic (Jacobs et al. 1993a), and howling monkeys (*Aloutta*), which, as mentioned above, are routinely trichromatic (Jacobs et al. 1996), although it should be noted that the presence of hybrid opsin sequences in *Alouatta* species suggests there is individual variation in the spectral sensitivities of photopigments (Matsushita et al. 2014). Among polymorphic taxa, the distributions in number and peak sensitivities of M/L opsins (2-5 alleles) are highly variable (e.g., see Surridge et al. 2003 and Jacobs 2007 for reviews), but polymorphic trichromacy in this lineage likely has a single origin occurring early in platyrrhine evolution (>20 million years ago; Boissinot et al. 1998).

Lemuriforms may exhibit the most variation in color vision capacities across species (Figure 3.1; e.g., Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Veilleux and Bolnick 2009; Veilleux et al. 2013; Bradley et al. in prep). Some nocturnal cheirogaleids, like all lorisiforms, have non-functional S opsin genes and are monochromatic (e.g., Kawamura and Kubotera 2004; Tan et al. 2005; Veilleux et al. 2013). Dichromatic color vision characterizes multiple species from all five lemur families, and polymorphic trichromacy has been documented in some species of Lemuridae and Indriidae (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003b; Tan et al. 2005; Bradley et al. 2009; Veilleux and Bolnick 2009; Veilleux et al. 2013, 2014; Bradley et al. in prep). Polymorphic trichromacy in lemurs results from a similar genetic mechanism as that in New World monkeys (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003b). However, most polymorphic lemurs exhibit only two M/L opsin gene variants caused by a single nucleotide polymorphism at amino acid site 285 coded in exon 5 (e.g., Tan and Li 1999; Bradley et al. 2009; Bradley et al. in prep). This results in two photopigments with peak spectral sensitivities at 543 nm (M opsin) and 558 nm (L opsin; e.g., Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003b).

Polymorphic trichromacy in lemurs, unlike in New World monkeys, is more sparsely distributed across species (Tan and Li 1999; Bradley et al. 2009; Bradley et al. in prep), and this trait may have been lost in multiple taxa (chapter 2). Among the *Eulemur* clade, for example, some species are polymorphic, while others may be monomorphic for either the M or the L opsin (Tan and Li 1999; Bradley et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep; chapter 2). Dichromatic color vision in this lineage appears to represent independent loss of color vision variation from a polymorphic *Eulemur* ancestor (chapter 2). Polymorphic trichromacy may also have a single early origin in lemur evolution, which would indicate additional losses of variation

in some species (Tan and Li 1999; Tan et al. 2005; Veilleux and Bolnick 2009; chapter 2). Loss of a color vision polymorphism has also been documented at the population level in at least one species (*Propithecus verreauxi*; Bradley et al. 2009; Bradley et al. in prep).

The seemingly near ubiquity of polymorphic trichromacy in New World monkeys contrasts with its sporadic distribution in lemurs and suggests that the evolutionary mechanisms underlying color vision variation might differ across these two lineages. In platyrrhines, it has been shown that M/L opsin polymorphisms are likely maintained in some populations through positive selection (Hiwatashi et al. 2010; Karamura et al. 2012). Indeed, trichromatic color vision has been suggested to provide an adaptive advantage over dichromacy, with the most long-standing hypothesis proposing an advantage to trichromats when foraging on red food items (e.g., fruit, young leaves; e.g., Allen 1879; Mollon et al. 1984; Mollon 1989; Osorio and Vorobyev 1996; Lucas et al. 1998; Dominy and Lucas 2001; Osorio et al. 2004).

An overall trichromatic advantage would explain fixation of the M/L opsin duplications in catarrhines and howling monkeys, as well as long-term maintenance of color vision polymorphisms in most platyrrhines through overdominance selection (i.e., heterozygote advantage; Mollon et al. 1984; Mollon 1989; Jacobs et al. 1996; Hunt et al. 1998). This hypothesis has received some theoretical support (e.g., Osorio and Vorobyev 1996; Osorio et al. 2004), as well as some empirical support in captive settings (e.g., Caine and Mundy 2000; Smith et al. 2003), but there is limited support for trichromatic advantages (foraging or otherwise) in wild primate populations (e.g., Vogel et al. 2007; Hiramatsu et al. 2008; Melin et al. 2008, 2009; Smith et al. 2012; Fedigan et al. 2014). Given these mixed results, alternative processes whereby differential advantages are conferred to trichromats and dichromats (e.g., inverse frequency-dependent selection, niche divergence, and mutual benefit of association) might underlie color

vision variation in platyrrhines (Mollon et al. 1984; Melin et al. 2007, 2008; Kawamura et al. 2012), and in fact dichromatic foraging advantages (i.e., greater foraging efficiency on insects) have been documented in wild platyrrhine populations (Melin et al. 2007; Smith et al. 2012). Regardless of the exact mode(s) of selection in polymorphic platyrrhines, however, there is some evidence for the view that color vision variation is maintained in populations via natural selection in this lineage.

Color vision in lemurs remains largely understudied compared to that in New World monkeys, but similar adaptive scenarios related to trichromatic advantages have been proposed for the presence of polymorphic trichromacy in some species (Leonhardt et al. 2009). Loss of polymorphic trichromacy in some species/populations, on the other hand, suggests there may be more complex evolutionary scenarios underlying color vision in this lineage. Adaptive processes (e.g., directional selection or disruptive selection) might theoretically account for loss of polymorphisms and subsequent fixation of either the M or L opsin in some species/populations (Futuyma 1998; chapter 2). Indeed, adaptation has been proposed for the high frequency of the L opsin in *Avahi* (Veilleux et al. 2014), as well as observed variation in the frequency/presence of color vision polymorphisms in some lemur populations (Bradley et al. 2009). However, there are reasons to suspect that nonadaptive processes, specifically genetic drift, might also play a role.

First, there is some indication that color vision may be less acute in lemurs compared to haplorhine primates. Although lemurs have the physiological and neuronal capacity to detect, transmit, and process chromatic information (Yamada et al. 1998), they lack many of the visual specializations that characterize haplorhines and are associated with high visual acuity and color vision. For example, lemurs have lower overall cone densities compared to haplorhines (Wikler and Rakic 1990; Peichl et al. 2001). Many lemurs have a tapetum lucidum, which increseases

sensitivity of the retina but may reduce visual acuity (Martin 1990). Lemurs also lack a true fovea in the central retina, and have higher retinal summation, such that single ganglion cells receive and transmit information from multiple cone photoreceptors for neural processing (Rohen and Castenholz 1967; Wikler and Rakic 1990; Kay and Kirk 2000). Overall, these differences result in lower visual acuity in lemurs compared to haplorhines (Kay and Kirk 2000; Kirk and Kay 2004; Veilleux and Kirk 2009), suggesting that the capacity for color vision in lemurs may also be less acute (Jacobs and Deegan 2003a; Kawamura et al. 2012). Less acute color vision could ultimately lead to relaxed selection for maintaining polymorphic trichromacy (Kawamura et al. 2012), and, consequently, the observed distribution of M and L opsins in many dichromatic lemurs may be the result of random allele loss due to genetic drift rather than selective processes. However, generally lower color vision acuity in strepsirrhines would not explain why allelic trichromacy originally evolved in this lineage and why it appears to be maintained in some lemurs and not others, especially when such variation occurs within genera (although more subtle species- or population-level variation in lemur visual systems may play a role; e.g., Jacobs and Deegan 2003).

Second, lemur populations may be particularly vulnerable to random reductions in population size (i.e., population bottlenecks) that might result in rapid loss of polymorphisms through genetic drift even in the presence of positive selection (Futuyma 1998). Madagascar has experienced recent island-wide, large-scale forest destruction and fragmentation (Dewar 2003; Harper et al. 2007), largely owing to human subsistence strategies, such as slash-and-burn agriculture and charcoal production (e.g., Green and Sussman 1990; Dewar 2003). During a brief fifty-year time-span (1950-2000), ≥40% of forest was cleared in the eastern and western regions of Madagascar, and as of the year 2000, 45% of all forest occurred in fragments <500 km^2

(Harper et al. 2007). Evidence also suggests that human predation likely played a large role in extirpating at least 17 lemur species during the last 2000 years (Perez et al. 2005; Crowley 2010). Habitat destruction and hunting continue to threaten Madagascar's fauna (Schwitzer et al. 2014), and large-scale range contractions have been documented in extant lemurs (Godfrey et al. 1999).

The present patchy forest landscape in Madagascar has produced similarly patchy distributions of many lemur species, resulting in genetic signatures of severe population decline and collapse (i.e., bottleneck) across multiple taxa (e.g., Fredsted et al. 2007; Olivieri et al. 2008; Craul et al. 2009; Brenneman et al. 2012; Parga et al. 2012; Holmes et al. 2013). Genetic bottlenecks have been documented in lemur populations occurring in a variety of habitats and ranging from small unprotected fragments to larger protected tracts of forest (Craul et al. 2009; Parga et al. 2012; Holmes et al. 2013). In some taxa, population sizes have been reduced by orders of magnitude in the last 500 years (Olivieri et al. 2008; Craul et al. 2009). Given that population declines can reduce genetic diversity, and smaller populations are more susceptible to genetic drift and inbreeding (Nei et al. 1975), demographic histories of populations are important for interpreting present genetic diversity. In the case of color vision polymorphisms, allele loss may occur in bottlenecked populations at varying rates and under either relaxed or positive selection (Futuyma 1998).

Although adaptive explanations for observed color vision variation in lemurs are attractive, in order to avoid falsely inferring adaptive evolution, it is important to also explore nonadaptive explanations. Toward this end, the goal of this study is to examine the potential role of nonadaptive processes in the evolution of color vision in a population of red-bellied lemurs (*Eulemur rubriventer*) in Ranomafana National Park (RNP). Previous research has shown that the *E. rubriventer* population in RNP is dichromatic with the L opsin only (chapter 2). This

condition is unique among other species of *Eulemur* (that have been studied), which are either dichromatic for the M opsin or polymorphic (Tan and Li 1999; Bradley et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep), and it likely represents loss of a color vision polymorphism (chapter 2). Loss of polymorphic trichromacy in this population is unexpected given that they are readily active during the day and highly frugivorous (Overdorff 1991; Tecot 2008; R. Jacobs, unpublished data). This represents a classic ecological description for a species that would benefit from maintaining trichromatic color vision (e.g., Mollon 1989; Melin et al. 2014). Given that lemur species vary in the presence and frequency of color vision polymorphism across populations (Bradley et al. 2009; Bradley et al. in prep), it is currently unknown when loss of polymorphic trichromacy occurred in this lineage and/or whether dichromatic color vision characterizes all *E. rubriventer* or represents population-level variation. If the latter, color vision in the red-bellied lemur population in RNP may represent local allele loss due to nonadaptive mechanisms or local adaptation. As a first step in addressing the former, this study aims to evaluate the past demographic history (i.e., determine if there is a genetic signature of a bottleneck) of the population of red-bellied lemurs in RNP.

MATERIALS AND METHODS

All methods were approved by and in compliance with institutional (Stony Brook University IACUC: 2010/1803, 2011/1895) and national guidelines (Madagascar National Parks).

Study subjects

Red-bellied lemurs (*Eulemur rubriventer*) are small to medium-sized, frugivorous primates that are distributed across the eastern rainforests of Madagascar (Mittermeier et al. 2010; Figure 3.2A). Despite their apparent wide range, red-bellied lemurs can be considered patchily distributed, as Madagascar's eastern rainforests are increasingly fragmented and are not continuous (Irwin et al. 2005; Harper et al. 2007). Where they do occur, red-bellied lemurs are usually found at low population densities (Irwin et al. 2005).

E. rubriventer live in small, cohesive groups (2-6 individuals) that usually consist of an adult male-adult female pair and immature individuals (Wright 1992; Overdorff 1993). Groups actively defend small home ranges (11-19 hectares; Overdorff 1991), and group compositions are relatively stable apart from births and juvenile emigrations (Overdorff and Tecot 2006), although turnovers in adult males and adult females have been documented (R. Jacobs, unpublished data).

Red-bellied lemurs are sexually dichromatic (described in Mittermeier et al. 2010). They have reddish-brown pelage coloration, which characterizes the dorsal and ventral coats of males. In females, the dorsal coat is reddish-brown, while the ventrum is white in coloration. This white ventral coat variably extends to the neck and face of females. Males have patches of white skin around the eyes that are reduced or absent in females. Individual pelage coloration and patterns are variable, and this variation allowed observers to readily identify individuals during this study.

Study site

Data were collected in Ranomafana National Park (RNP). RNP is an area of 41,000 ha of montane rainforest in southeastern Madagascar (E47°18' - 47°37', S21°02' - 21°25'; for a more

detailed description of the site, see Wright 1992). Although RNP is protected habitat, the areas to the east and west of the park have become almost entirely denuded, with extensive forest loss occurring over the last century (Harper et al. 2007). The forest has become disconnected just slightly north of RNP (around 21°S; Irwin et al. 2005), and while there may be narrow physical connectivity just south of the park (Irwin et al. 2005; Harper et al. 2007), its functional connectivity for lemur species is unknown. Therefore, lemur populations in RNP have become potentially isolated and given the scale of recent forest destruction throughout southeastern Madagascar, may have experienced recent and severe reductions in population size. Furthermore, prior to its park status in 1991, areas within RNP were subjected to intensive selective logging of large fruiting trees (Wright et al. 2012) with unknown demographic impact on some of the frugivorous lemur populations, including *E. rubriventer*.

During January 2012-May 2013, fecal samples were collected from individual red-bellied lemurs at four sites located within the park (Talatakely: $N = 37$ individuals, Vatoharanana: $N = 36$ individuals, Valohoaka: $N = 38$ individuals, and Sahamalaotra: $N = 11$ individuals; Figure 3.2B; Table 3.1). All sites are within 8 kilometers of one another and are assumed to be in migratory contact, representing the same population. It should be noted, however, that Ranomafana is bisected by the Namorona River and a parallel paved roadway (Wright et al. 2012), which separates the more northern site, Sahamalaotra, from the other three sites located in the southern parcel. While red-bellied lemurs readily cross the road (R. Jacobs, personal observation), it is currently unknown whether the Namorona River represents a significant barrier to gene flow for this species.

Fecal sample collection

Fresh fecal samples were collected opportunistically from individual red-bellied lemurs for genetic analyses. Samples (~5 grams wet weight) were collected from the ground immediately following defecation and placed directly into a 50 mL plastic centrifuge tube pre-filled with 30 mL silica gel beads for desiccation (Nsubuga et al. 2004). Samples were collected using latex gloves and the untouched end of a freshly broken twig. Tubes were labeled and sealed with parafilm and stored at ambient temperature in the field and later at +4°C in the lab (Yale Molecular Anthropology Laboratory; YMAL).

DNA extraction

All genetic analyses were conducted at the YMAL. Genomic DNA was extracted from dry fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen) following a protocol optimized by the YMAL. This protocol was modified from the manufacturer's protocol in the following manner: samples were initially lysed at room temperature for 48 hours in ASL buffer and all procedures following inhibitor adsorption were automated using a QIAcube® (Qiagen). A negative control was included in all extraction procedures to assess the potential for contamination.

DNA quantitation

DNA concentrations for each sample were determined using two methods. First, DNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen™) with the Qubit® dsDNA HS Assay Kit. This procedure measures total genomic DNA based on the signal from fluorescent dye that binds specifically to DNA and, therefore, does not distinguish lemur DNA from other DNA that

may be present in the sample (e.g., microbial DNA). Therefore, DNA concentrations were also determined using real-time quantitative PCR (qPCR) following methods described in Morin et al. (2001). This method targets an 81 base pair portion of the single copy *c-myc* gene (Morin et al. 2001), and, therefore, only primate DNA was quantified. A dilution series of human placental DNA of known quantities (25 ng, 20 ng, 6.25 ng, 3.125 ng, 1.5625 ng, 800 pg, 400 pg, 200 pg, 100 pg, and 25 pg) was included as a standard to then quantify red-bellied lemur DNA in each fecal sample.

C-myc assays were performed in a Rotor-Gene Q real-time PCR cycler (Qiagen). The total PCR volume was 25 μl comprising 12.5 μl of Rotor-Gene SYBR Green RT-PCR Master Mix (Qiagen), 1.25 μl each of forward and reverse primers (10 μM/μl), and 2.5 μl of DNA. The reaction conditions used a two-step thermal cycling method (denaturing and annealing only, with florescence measured at the end of each annealing step) and were as follows: initial incubation at 95°C for 50 seconds followed by 50 cycles of 95°C for 5 seconds and 60°C for 10 seconds. A high resolution melt analysis followed amplification to confirm specificity and lack of substantial secondary products. Each assay included standards (in triplicate), samples (in duplicate), and two negative controls (H_2O as template). The final quantity of DNA for each fecal sample was determined using the Rotor-Gene Q software package, and results from the two replicates, which were generally consistent, were averaged. DNA quantitation was used to assess an appropriate number of PCR replications for obtaining confident microsatellite genotypes (Morin et al. 2001).

Sex-typing

Each sample was sex-typed using a multiplex (triple primer) PCR to amplify segments of the tetratricopeptide repeat protein gene on the Y chromosome (UTY), and the X-chromosomal

homolog (UTX) (Villesen and Fredsted 2006). This method yields short fragment sizes (Y = 86 bp, X = 127 bp) and is suitable for use with degraded DNA such as that extracted from feces (Villesen and Fredsted 2006).

PCR was performed in a total volume of 25 μl using G-Storm GS1 thermal cyclers. The PCR volume consisted of 12.5 μl of Qiagen HotStarTaq Master Mix, 2 μl of bovine serum albumin (BSA, 20 μM/μl), 4, 1, and 0.25 μl of UTY, UTXY, and UTX primers (10 μM/μl), respectively (see Villesen and Fredsted 2006 for primer sequences), and 4 μl of total template DNA. All PCR reactions included one negative control (H_2O as template). Cycling conditions were 95°C for 15 minutes and 36 cycles of 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 7 minutes. PCR fragments were separated on 2% agarose gels (120 volts, 1 hour) and visualized using GelRed (Biotium). If a sample yielded both X and Y fragments, the individual was typed male, and if a sample yielded a single X fragment, the individual was typed female. All samples were sex-typed in at least two independent reactions.

Microsatellite genotyping

A total of 59 adult samples (N_{males} = 30, $N_{females}$ = 29; N_{groups} = 29; $N_{Talatakely}$ = 19 individuals, $N_{Sahamalaotra}$ = 5 individuals, $N_{Valohoaka}$ = 12 individuals, $N_{Vatoharanana}$ = 19 individuals) were included in microsatellite genotyping. Samples were amplified at 7 variable microsatellite loci using previously published primers (Andriantompohavana et al. 2007; Table 3.2).

Loci were amplified using PCR in G-Storm GS1 thermal cyclers. PCR conditions were carried out in 12.5 μl total volume comprising 6.25 μl of Qiagen HotStarTaq Master Mix, 2 μl of bovine serum albumin (BSA, 20 μM/μl), 1 μl each of forward (fluorescently labeled) and reverse

primers (10 μM/μl), and 2 μl of total template DNA. For some, lower quality samples, larger reaction volumes were used with higher quantities (3-5 μl) of DNA template to avoid errors associated with allelic dropout (Morin et al., 2001). All PCR reactions included one negative control (H_2O as template). Cycling conditions were 95°C for 15 minutes and 37 cycles of 95°C for 30 seconds, a locus-specific annealing temperature (see Table 3.1) for 30 seconds, and 72°C for 30 seconds, with a final extension step of 72°C for 10 minutes.

Fragment analyses (i.e., size estimates of microsatellite loci) were carried out in the DNA Analysis Facility at Yale University. PCR products were separated using capillary electrophoresis with an ABI 3730*xl* 96-Capillary Genetic Analyzer. Genescan Rox-500 was added as the facility size standard. GeneMapper® (Applied Biosystems) and GeneMarker® (SoftGenetics) software were used to identify allele fragment length. Binning was conducted manually based on visual assessment of allele peaks, and genotypes were determined based on multiple independent PCR reactions (Taberlet et al. 1996; Morin et al. 2001). Specifically, homozygous genotypes were confirmed with a minimum of 4 and up to 7 independent replications (Morin et al. 2001). Heterozygous individuals were confirmed when each allele was scored twice based on two or more independent PCR reactions (Taberlet et al. 1996; Morin et al. 2001).

Data analysis

Data screening. Microsatellite genotypes were screened for errors (i.e., scoring errors, allelic dropout, and null alleles) prior to data analysis using the software MICRO-CHECKER (van Oosterhout et al. 2004). In addition, genotype errors (e.g., allelic dropout) were estimated

by calculating the proportion of PCRs that successfully amplified DNA, as well as the proportion of successful PCRs for heterozygous consensus genotypes that yielded both consensus alleles. Genepop version 4.2 was used to test for linkage disequilibrium among all combinations of microsatellite loci using the log-likelihood ratio statistic and evaluated with 10,000 permutations (Raymond and Rousset 1995; Rousset 2008).

Summary statistics for each locus (e.g., the number of alleles - k, number of individuals typed, observed and expected heterozygosity, and polymorphic information criterion), as well as goodness-of-fit tests for Hardy-Weinberg equilibrium were calculated in CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). Allelic richness (the number of alleles per locus independent of sample size) for each locus was calculated using FSTAT version 2.9.3.2.

Sex-biased dispersal. New alleles may be introduced to populations through immigration, and this can potentially mask the genetic signature of a bottleneck (Busch et al. 2007; Lawler 2011). To overcome this potential issue, some studies incorporate additional bottleneck analyses using data from the philopatric sex (in species that exhibit sex-biased dispersal) only (Lawler 2008, 2011; Parga et al. 2012). At the same time, sex-biased dispersal can result in increased genetic substructure within the philopatric sex (e.g., Chesser 1991), which may have implications for interpreting results of bottleneck analyses (see "Population structure" below).

Behavioral data suggest that both sexes disperse in red-bellied lemurs (Merenlender 1993; Overdorff and Tecot 2006), a pattern that is documented in pair-living species (e.g., Dobson 1982; Fernandez-Duque 2009; see Koenig and Borries 2012 for review on hylobatids). However, among pair-living species, males and females may differ in their dispersal distances,

such that males and females may be considered "more" or "less" philopatric (e.g., hylobatids; reviewed in Koenig and Borries 2012). In order to determine if additional sex-specific bottleneck analyses were warranted for this red-bellied lemur population, the hypothesis that there is no sex-bias in dispersal within this population was evaluated using genetic data.

FSTAT 2.9.3.2 was used to implement five tests for sex-biased dispersal in which individuals were grouped by sampling locality. The first two tests use F-statistics (F_{IS} and F_{ST}) as measures of population structure. F_{IS} (Weir and Cockerham 1984) measures the fit of genotype frequencies within a population with that expected under Hardy-Weinberg Equilibrium (Hartl and Clark 1997). Under conditions of sex-biased dispersal, the dispersing sex should exhibit significantly higher F_{IS} (heterozygote deficit) compared to the philopatric sex, because the population should include a mixture of residents and immigrants from the dispersing sex. F_{ST} (Weir and Cockerham 1984), which is the proportion of total genetic variance that occurs among subpopulations (Hartl and Clark 1997), is expected to be significantly lower in the dispersing sex compared to the philopatric sex (Goudet et al. 2002). This is due to the expectation that allele frequencies will be more similar in the dispersing sex (resulting from increased gene flow) compared to the philopatric sex (Goudet et al. 2002). The third test examines differences between male and female relatedness (*r*), and, similar to F_{ST}, the expectation is that relatedness is significantly lower in the dispersing sex compared to the philopatric sex (Goudet et al. 2002).

The last two sex-biased dispersal tests are based on corrected individual assignment indices (*AI*: Paetkau et al. 1995; Favre et al. 1997; Goudet et al. 2002). An individual's *AI* is the probability that its genotype occurs by chance in the sampled population given the allele frequencies within that population (Paetkau et al. 1995; Favre et al. 1997; Goudet et al. 2002). The individual probabilities are then corrected (*AIc*) after log-transformation by subtracting the

mean *AI* in order to correct for potential differences arising from variation in gene diversity across populations (Favre et al. 1997; Goudet et al. 2002). Following correction, average *AIc* for populations is zero, so individuals with negative *AIc* values are likely immigrants, as their genotypes are less likely to occur in the sample, and positive *AIc* values are likely residents (Favre et al. 1997; Goudet et al. 2002). From the individual probabilities, mean *AIc* (*mAIc*) and variance of *AIc* (*vAIc*) are calculated for each sex, with the expectation that the dispersing sex exhibits significantly lower *mAIc* (Goudet et al. 2002). The dispersing sex is also expected to have significantly larger *vAIc* compared to the resident sex, as the dispersing sex includes both residents and immigrants (Goudet et al. 2002). Importantly, given that the distances over which individuals may disperse are unknown, F_{ST}-based tests may be sensitive to dispersal distances, being better able to detect sex-bias dispersal when it occurs over short distances, but *AIc* tests should be less influenced by dispersal distances (Goudet et al. 2002). That said, given that all sites occur within 8 km of each other, these tests may not capture sex biases in long range or long-term dispersal. All tests (F_{IS}, F_{ST}, *r*, *mAIc*, and *vAIc*) were two-tailed and significance was calculated over 10,000 permutations.

Population structure. An important assumption of bottleneck analyses is that there is no substructure within the samples (e.g., Cornuet and Luikart 1996; Garza and Williamson 2001). The presence of substructure has the potential to mimic a signature of a population bottleneck (Wakeley 1999; Chikhi et al. 2010). More specifically, Chikhi et al. (2010) found that spurious bottleneck signals increased as genetic differentiation ($F_{st} \geq 0.1$) increased. Therefore, while acknowledging small sample sizes within sites, pairwise F_{st} values (Weir and Cockerman 1984) were calculated across the four study sites using the program Genodive 2.0b27 (Meirmans and

van Tienderen 2004). Significance was evaluated using 10,000 permutations with significance set to $p < 0.05$ and adjusted using Bonferroni correction for multiple comparisons.

Genetic bottleneck analyses: heterozygosity excess. The program BOTTLENECK was used to test for a genetic signature of a population bottleneck (Cornuet and Luikart 1996; Luikart and Cornuet 1998; Piry et al. 1999). The program computes expected heterozygosity (H_{eq}) at mutation-drift equilibrium (based on allele number and sample size) for each locus under three mutation models: infinite allele model (IAM), stepwise mutation model (SMM), and the two-phase model (TPM) (Piry et al. 1999). The program compares H_{eq} to Hardy-Weinberg heterozygosity (H_e) with the expectation that in recently bottlenecked populations, there will be significant excess H_e compared to H_{eq}, because allele number should be reduced faster than heterozygosity (Cornuet and Luikart 1996; Piry et al. 1999).

BOTTLENECK performs multiple tests, but the Wilcoxon test is considered to be robust when using a small number of polymorphic loci (< 20) and most appropriate for microsatellite data, and therefore is used in this study. H_{eq} is calculated under TPM, as this mutation model is also considered the most appropriate model for microsatellite loci, with IAM and SMM representing more extreme mutation models (Di Rienzo et al. 1994; Piry et al. 1999).

Importantly, tests for heterozygosity excess have the potential to produce both type I and type II errors based in part on incorrect assumptions of mutation model parameters (Peery et al. 2012). Specifically, TPM assumes that mutations during microsatellite evolution can result in small changes in a single repeat motif (i.e., single-step mutations, which characterize most mutations), as well as larger changes in multiple repeat motifs (i.e., multi-step mutations, which characterize fewer mutations; Di Rienzo et al. 1994; Peery et al. 2012). Consequently, TPM

requires knowledge (or assumptions) about the proportion and size of multi-step mutations in the microsatellite data of interest (Peery et al. 2012). The program BOTTLENECK requires the proportion of multi-step mutations and the variance in the mean size of multi-step mutations to be specified (Piry et al. 1999), and it has been shown that type I and type II errors can result from errors in assumed values for these parameters (Williamson-Natesan 2005; Peery et al. 2012). One way to help avoid such errors is to use reasonable and appropriate values for the mutation model parameters (Peery et al. 2012). Based on their review of 18 studies of microsatellite evolution in vertebrates, Peery et al. (2012) suggest 0.22 and 12 to be appropriate values for the proportion of multi-step mutations and variance in mean size of multi-step mutations, respectively. The former value deviates from the more commonly used proportion of 0.10 (Peery et al. 2012). Because overestimating this value increases the likelihood of a type I error in heterozygosity excess tests, 0.10 may be considered a more conservative value (Williamson-Natesan 2005; Peery et al. 2012). Therefore, the Wilcoxon test was run twice under the TPM and setting the proportion of multi-step mutations to 0.22 and 0.10, respectively, with a variance of 12 for each analysis. Significance ($p < 0.05$) was assessed using 10,000 iterations.

Genetic bottleneck analyses: *M*-ratio. A signature of a population bottleneck was also assessed using the *M*-ratio test implemented in the program M_P_val (Garza and Williamson 2001). This test computes *M*, which is the ratio of *k* (total number of alleles) to *r* (range in allele size) averaged across all microsatellite loci, and compares this ratio to a simulated distribution of *M* values at mutation-drift equilibrium (Garza and Williamson 2001). In populations that have experienced a bottleneck, the expectation is that observed *M* should be lower than *M* values at equilibrium, because rare alleles are likely to be lost in bottlenecked populations but should not

be biased toward the smallest or largest allele sizes (Garza and Williamson 2001). Therefore, *k* is expected to reduce faster than *r* (Garza and Williamson 2001).

Calculating *M* requires three input parameters, and, similar to heterozygosity excess tests, incorrect assumptions about these parameters can produce both type I and type II errors (Peery et al. 2012). *M*-ratio tests require assumptions about p_s (the proportion of one-step mutations) and Δ_g (the average size of one-step mutations; Garza and Williamson 2001). Following the recommendation of Peery et al. (2012), p_s was set to 0.78, as well as the more commonly used 0.90, and Δ_g was set to 3.1. The *M*-ratio test also requires the input parameter pre-bottleneck θ ($=4N_e\mu$; N_e = effective population size; μ = mutation rate; Garza and Williamson 2001). Given that pre-bottleneck N_e is unknown as is μ, a range of values for θ (0.2-20) was tested (Busch et al. 2007; Parga et al. 2012). If one assumes $\mu = 5.0 \times 10^{-4}$, which is a commonly used microsatellite mutation rate (Weber and Wong 1993; Garza and Williamson 2001), these values correspond to pre-bottleneck N_e values: 100-10000 individuals. Observed *M* is considered significant and indicative of a population bottleneck if < 5% of simulated values fall below the observed *M* (Garza and Williamson 2001).

RESULTS

Data screening

Sex genotypes for samples were in accordance with sex assignments based on field observations. Of the 59 adult individuals used in microsatellite genotyping analyses, 55 yielded confident genotypes at a minimum of 4 microsatellite loci and comprise the final data set (Table 3.3). Genotypes were 91% complete for 7 microsatellite loci (range 60-100% complete) across

the 55 adult red-bellied lemurs (Table 3.4). MICRO-CHECKER found no evidence for scoring errors, allelic dropout, or the presence of null alleles across each of the 7 loci. Overall, the proportion of PCRs that successfully amplified DNA was 81% (range 70-92% across loci), and the proportion of successful PCRs for heterozygous consensus genotypes that yielded both consensus alleles was 74% (range 65-83% across loci). Of the 21 locus combinations, no combinations showed evidence for linkage after Bonferroni's correction ($p > 0.002$). Summary statistics for all loci are presented in Table 3.4. Across the 7 microsatellite loci, the mean number of alleles (*k*) was 5.857 (range 3 to 9). Mean allelic richness was 5.663 (range 3.000 to 8.309). Mean observed heterozygosity for the population was 0.687 (range 0.519 to 0.800) and mean expected heterozygosity was 0.697 (range 0.586 to 0.810). Goodness-of-fit tests showed no significant deviations from Hardy-Weinberg equilibrium for all loci.

Sex-biased dispersal

Results of the sex-biased dispersal tests suggest there is no evidence for sex-biased dispersal in this population of red-bellied lemurs (Table 3.5), corroborating behavioral observations. Specifically, all five sex-biased dispersal tests (F_{IS}, F_{ST}, *r*, *mAIc*, and *vAIc*) revealed no significant difference between males and females. However, all results were in the predicted direction of male-biased dispersal. That is, males exhibited higher F_{IS}, lower F_{ST}, and lower *r* values compared to females. Males also had lower *mAIc* and larger *vAIc* values compared to females. Therefore, all bottleneck analyses were run using a combined male and female data set, as well as female-only and male-only data sets.

Population structure

Results of pairwise F_{st} tests are presented in Table 3.6. Overall average F_{st} was low (0.051), and all pairwise values suggest limited, albeit some significant, genetic differentiation across sites. All pairwise F_{st} values fall below 0.1, with one near exception: between Sahamalaotra and Vatoharanana. The highest significant F_{st} values occur between Sahamalaotra from the northern parcel and two sites from the southern parcel. Although pairwise F_{st} values are low, because population substructure ($F_{st} \geq 0.1$; Chikhi et al. 2010) can result in spurious bottleneck effects, bottleneck analyses were run using both the full data set and a data set excluding the samples from Sahamalaotra.

Genetic bottleneck analyses

Results of the Wilcoxon test for heterozygosity excess revealed that under TPM, the population of red-bellied lemurs in RNP exhibits significant excess heterozygosity compared to mutation-drift equilibrium. This was true when the proportion of multi-step mutations was set to 0.22 ($p < 0.01$) and the more conservative 0.10 ($p < 0.05$). Results were similar using an adult-female-only data set: $p < 0.01$ and $p < 0.05$ for proportions of multi-step mutations set to 0.22 and 0.10, respectively. However, results were not significant using an adult-male-only data set: $p = 0.055$ when the proportion of multi-step mutations was set to 0.22 and 0.10.

Analyses using the full data set but excluding samples from Sahamalaotra ($N = 50$ individuals) yielded similar results. There was significant heterozygosity excess under both multi-step mutation assumptions: $p < 0.01$ (0.22 multi-step mutations) and $p < 0.05$ (0.10 multi-step mutations). However, when only females were analyzed in this sample ($N = 26$), heterozygosity excess was significant when the proportion of multi-step mutations was set to

0.22 only ($p < 0.05$; $p = 0.148$ with 0.10 multi-step mutations). Results were similar when only males were analyzed. Heterozygosity excess was significant when the proportion of multi-step mutations was set to 0.22 only ($p < 0.05$; $p = 0.344$ with 0.10 multi-step mutations).

Results of the *M*-ratio tests revealed that observed average *M* values in the population of red-bellied lemurs in RNP were high (0.935-0.974) and not significantly lower than expected under mutation-drift equilibrium (Table 3.7). This was the case for the combined male and female, female-only, and male-only data sets. Results also held under both scenarios for the proportion of one-step mutations (0.78 and 0.90), as well as the data sets excluding the Sahamalaotra samples.

DISCUSSION

The results of this study provide some, albeit mixed, evidence that the population of *E. rubriventer* in RNP may have experienced a population bottleneck. Specifically, this study found evidence for significant heterozygosity excess in the population of *E. rubriventer*, but did not find evidence for a significantly low *M*-ratio. Therefore, at this time, the present study cannot reject the hypothesis that a genetic bottleneck occurred in the red-bellied lemur population in RNP. As a result, present genetic diversity observed in this population may have been influenced by a recent reduction in population size. Thus, a population bottleneck could provide a potential nonadaptive mechanism through which polymorphic trichromacy may have been lost in this population.

Although a population bottleneck was only detected using heterozygosity excess tests, similar inconsistent patterns (significant heterozygosity excess coupled with high *M*-ratios) have been identified in other vertebrate species (e.g., ornate box turtles: Kuo and Janzen 2004;

northern spotted owls: Funk et al. 2010; Siberian tigers: Alasaad et al. 2011). The opposite pattern, in which *M*-ratio tests but not heterozygosity excess tests show signatures of bottlenecks, has also been observed in multiple populations (e.g., tiger salamanders: Spear et al. 2006; copperbelly water snakes: Marshall et al. 2009; elk: Hundertmark and Van Daele 2010; bottlenose dolphins: Galov et al. 2011). One proposed explanation for ambiguous results suggests that different tests are better able to detect bottlenecks that vary in timing/duration and/or severity (Williamson-Natesan 2005). Simulation studies have found that heterozygosity excess tests may be better at detecting recent or less severe bottlenecks, as well as bottlenecks occurring in populations with a small pre-bottleneck θ (Williamson-Natesan 2005). Conversely, the *M*-ratio test appears to be better able to detect bottlenecks in populations with large pre-bottleneck θ, in populations that have had some recovery time, or in populations that have experienced longer-term bottlenecks (i.e., multiple generations; Williamson-Natesan 2005). This has led many of the aforementioned studies to interpret inconsistent results as suggestive of "recent" (in the case of significant heterozygosity excess tests) or "historical" (in the case of significant *M*-ratio tests) population bottlenecks (Spear et al. 2006; Marshall et al. 2009; Hundertmark and Van Daele 2010; Alasaad et al. 2011). However, caution is warranted in this interpretation given that these analyses do not date or quantify population bottlenecks, and they may be capable of detecting bottlenecks over a wide temporal range (Cornuet and Luikart 1996).

For example, heterozygosity excess in populations is temporary and will be detected up to 0.2-4N_e generations, where N_e is the bottlenecked effective population size (Luikart and Cornuet 1998). Although N_e is unknown for this population, it may be possible to obtain rough estimates using census data (Frankham 2007). The most recent census data from RNP estimate the *E. rubriventer* population to be approximately 1,800 individuals, including adults and

juveniles (Wright et al. 2012). If adults account for approximately 60% of the population (R. Jacobs, unpublished data), the adult population is estimated to be 1,080 individuals. However, effective population size (N_e) is generally much lower than adult census size (N), with a recent estimate suggesting that N_e/N is approximately 0.1 (Frankham 2007), resulting in a value of 108 individuals for N_e. Given a generation time of approximately 8 years (Pacifici et al. 2013), this method would theoretically detect bottlenecks occurring before ~173-3,456 years ago. This lower end of the spectrum would accord well with the recent large-scale forest destruction that has occurred in the Ranomafana region (and across the eastern rainforests; e.g., Harper et al. 2007). But given the long temporal period over which this test might detect a bottleneck, as well as the very rough estimation obtained here, such a scenario is currently speculative at best. Additional analyses designed to evaluate the timing of population decline, such as the Bayesian method of Storz and Beaumont (2002), may help refine this temporal range.

It is important to acknowledge, however, that multiple factors can contribute to generating a false signature of a population bottleneck. First, both methods are sensitive to violations of assumed mutation model parameters (Peery et al. 2012). While this study used reasonable and appropriate mutation model parameters to reduce the potential for both type I and type II errors (Peery et al. 2012), results differ if the microsatellite loci actually exhibit one of the more extreme mutation models (i.e., IAM or SMM). Loci evolving under a stepwise mutation model (SMM) are less likely to exhibit heterozygosity excess if it is small (Cornuet and Luikart 1996). On the other hand, heterozygosity excess tests are more powerful at detecting bottlenecks when loci evolve under an infinite allele model (IAM), but also have the potential to identify heterozygosity excess in the absence of a population bottleneck (Cornuet and Luikart 1996).

As mentioned above, bottleneck analyses are also sensitive to substructure within samples (Chikhi et al. 2010). All samples used in this study were collected from multiple groups at sites located within 8 km of each other and appear to be in migratory contact, as exhibited by low pairwise F_{st} values across all sites, although sample sizes for each site are small. However, genetic differentiation was significant in some cases, with the greatest differentiation occurring between Sahamalaotra and two sites located south of the Namorona River. Although all pairwise F_{st} values fell below 0.1 (when more significant spurious bottleneck effects are obtained; Chikhi et al. 2010), when Sahamalaotra was removed from the data set, evidence for significant heterozygosity excess remained. As a result, substructure within the sample may not account for the bottleneck signature obtained in this study. That said, results of sex-biased dispersal tests, though not significant, were in the direction of male-biased dispersal. Such results could indicate variation between the sexes in dispersal distances, as has been shown for other taxa in which both sexes disperse (e.g., Harrison et al. 2014), and may introduce different levels of substructure that are sex-specific (e.g., Perrin and Mazalov 2000). Such differences might account for the variation in heterozygosity excess observed between female-only and male-only data sets. If females are the more philopatric sex, this might introduce a level of social substructure (e.g., Chesser 1991) resulting in a more pronounced bottleneck signature in females as observed in this study. Importantly, the tests used in this study are sensitive to sex-bias dispersal rates, requiring them to be high, and the power of these tests increases with the number of individuals sampled (Goudet et al. 2002), suggesting that larger sample size might help identify whether or not there is significant sex-bias dispersal, and consequent sex-specific substructure, in *E. rubriventer*.

Additional factors that might create spurious bottleneck effects include sampling scheme and immigration. Related to the discussion above, Chikhi et al. (2010) found that in highly

structured populations, false bottleneck signatures were more likely to be obtained when sampling from a single "deme". In order to counter this, they suggested sampling from multiple demes (Chikhi et al. 2010). Although this study used samples collected from multiple localities and groups, likely minimizing this potential effect, it is possible that the population of *E. rubriventer* in RNP exhibits larger-scale structure, with the samples used here representing a single deme.

Finally, one of the assumptions of the heterozygosity excess test is the absence of migration between populations (Cornuet and Luikart 1996). This assumption is often violated, and while low-level immigration likely masks a bottleneck effect (Busch et al. 2007), high levels of immigration can actually mimic a population bottleneck (Pope et al. 2000). Although RNP is disconnected from forest tracts to the north, a narrow corridor to larger tracts of forest to the south remained as of 2000 (Harper et al. 2007). Whether or not this physical connectivity actually facilitates migration is unknown, but if there is a high level of immigration from southern populations, this could potentially result in a bottleneck signal without population collapse. At the same time, a spurious bottleneck effect can be obtained when once-connected populations become completely disconnected, without actual population collapse (Broquet et al. 2010). Such a scenario may be applicable to RNP, which was historically connected to larger and continuous tracts of forest (Harper et al. 2007). Future studies incorporating simulations, as well as additional data from RNP and other populations of red-bellied lemurs, will help tease apart the potential effects of population collapse, population structure, and migration on the excess heterozygosity observed in this study.

Despite the above confounding factors, the potential for a recent genetic bottleneck in the *E. rubriventer* population in RNP cannot be rejected, and a severe population collapse could

provide a nonadaptive mechanism through which polymorphic trichromacy may be lost. The results of this study coupled with the large amount of color vision variation observed in lemurs introduce some important caveats when interpreting this variation. First, characterizing the color vision of a species should include data from multiple populations. Population-level variation has been documented in the presence and frequency of color vision polymorphisms in multiple taxa (Bradley et al. 2009; Bradley et al. in prep), and this study, among others (e.g., Fredsted et al. 2007; Olivieri et al. 2008; Craul et al. 2009; Brenneman et al. 2012; Parga et al. 2012; Holmes et al. 2013), has demonstrated that lemur populations may be differentially affected by nonadaptive evolutionary mechanisms that could potentially account for population-level variation in color vision capacity. Therefore, single populations may not accurately represent color vision variation at the species level. For red-bellied lemurs in particular, it will be necessary to sample from other populations to determine if dichromatic color vision with the L opsin characterizes the species or is specific to the RNP population.

Along similar lines, this study suggests that the unique color vision phenotype observed in red-bellied lemurs may not be the result of adaptation. To be clear, however, this study does not address adaptive hypotheses for fixation of the L opsin in *E. rubriventer*. Furthermore, fixation of the L opsin may have occurred much earlier in *E. rubriventer* evolution and may not be specific to the RNP population, although nonadaptive processes may still be responsible for fixation of the L opsin under such scenarios. In any case, given the absence of additional data on other red-bellied lemur populations, and in light of a potential bottleneck in the RNP population, adaptive explanations for the evolution of color vision in *E. rubriventer* should not be overstated. Research on color vision evolution in lemurs, especially those represented by a single population, should be similarly cautious in inferring adaptive evolution, as nonadaptive

mechanisms may play a role in the observed distribution of color vision capacities across this lineage.

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Figure 3.1 Distribution of strepsirrhine color vision mapped onto the phylogeny of Springer et al. (2012). "?" depicts that the presence of an opsin is uncertain in the case of *Cheirogaleus major* and *Lemur catta*, and that the spectral tuning of M/L opsins in *Allocebus* and *Phaner* is unknown. The "*" indicates that the presence of an L opsin in *Propithecus verreauxi* is variable at the population level. References: Tan and Li (1999), Jacobs et al. (2002), Jacobs and Deegan (2003), Kawamura and Kubotera (2004), Tan et al. (2005), Bradley et al. (2009), Veilleux and Bolnick (2009), Carvalho et al. (2012), Veilleux et al. (2013, 2014), Bradley et al. (in prep).

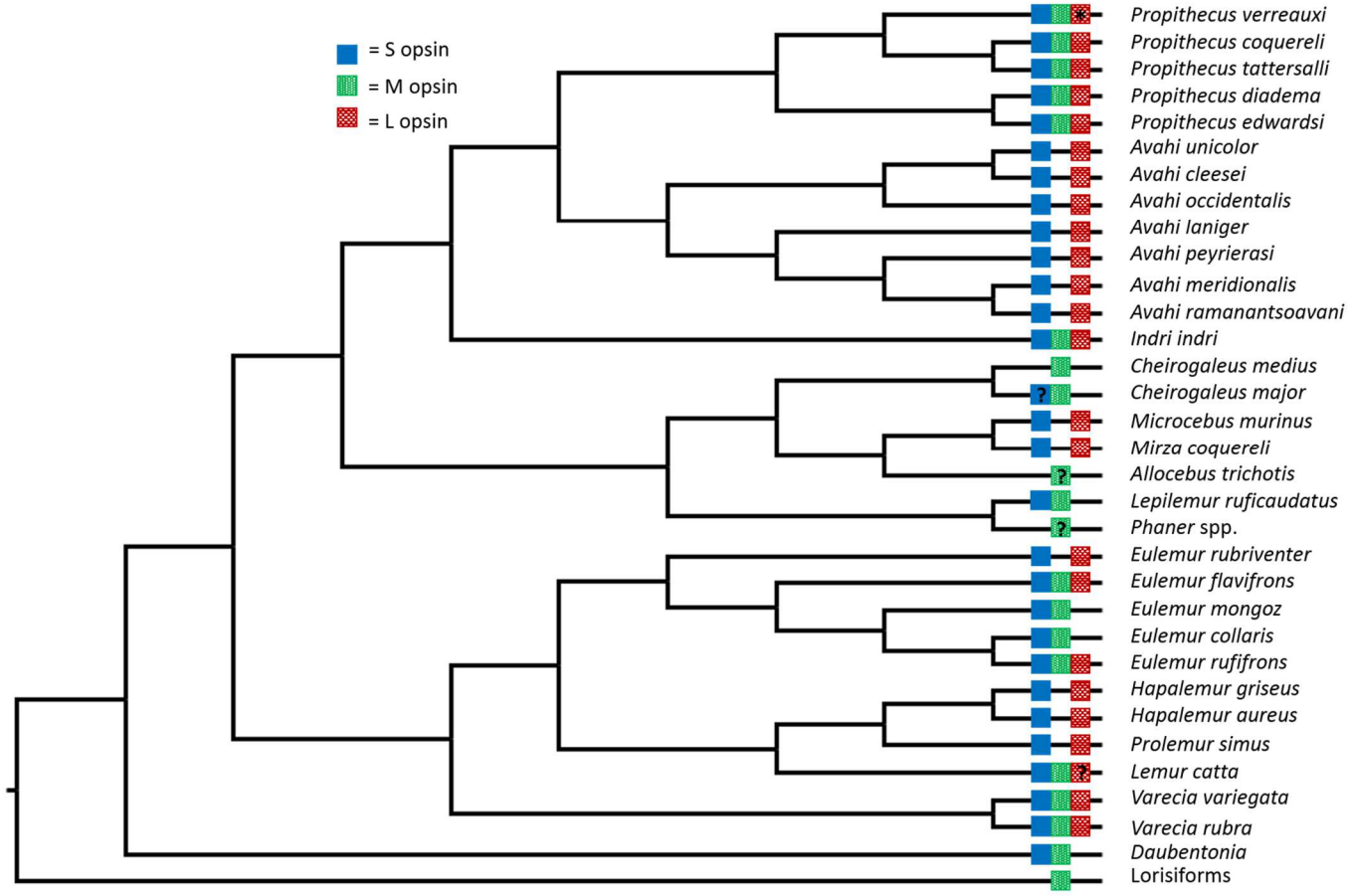

Figure 3.2 A. Range map for *Eulemur rubriventer* modified from the IUCN Red List (www.iucnredlist.org; Andriaholinirina et al. 2014). Data downloaded July 5, 2015. B. Map of Ranomafana National Park depicting fecal sample collection sites. This figure was modified with permission from Baden (2011).

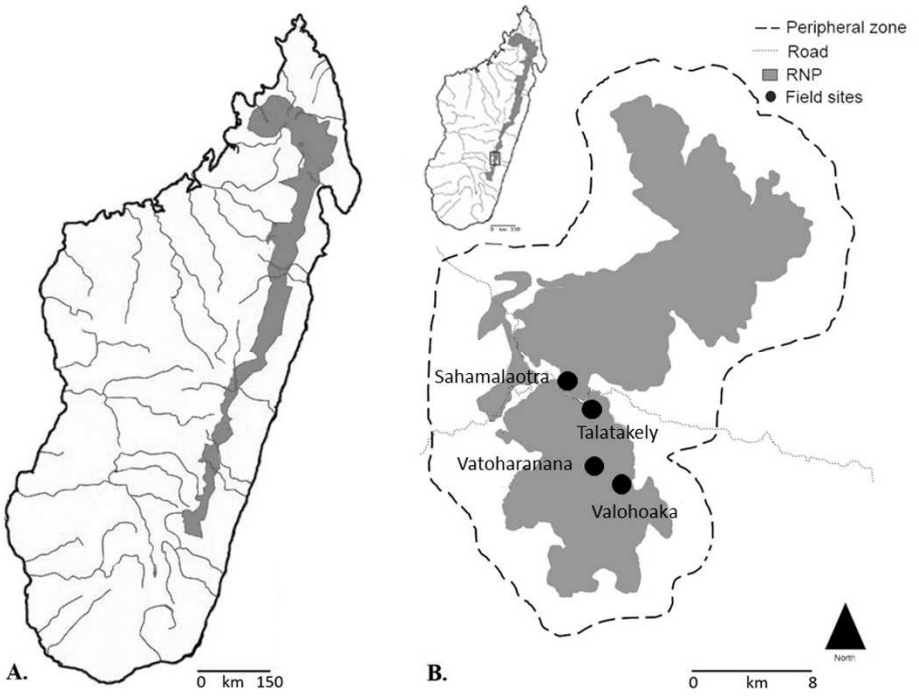

Table 3.1 Number of individual red-bellied lemurs for which fecal samples were collected in Ranomafana National Park. Samples were collected between January 2012 and May 2013.

Site	N_{groups}	N_{males}	$N_{females}$	$N_{immature\ females}$	$N_{immature\ males}$	$N_{individuals}$
Talatakely	11	11	13	9	4	37
Sahamalaotra	4	3	4	2	2	11
Valohoaka	10	10	10	9	9	38
Vatoharanana	12	12	12	5	7	36
Total	**37**	**36**	**39**	**25**	**22**	**122**

Table 3.2 Characteristics of 7 variable microsatellite loci for *E. rubriventer* that were used in this study. Locus names, primer sequences, and repeat motifs are from Andriantompohavana et al. (2007). Size ranges represent size ranges obtained in this study. Annealing temperatures (*T*) were modified when necessary from Andriantompohavana et al. (2007).

Locus	**Primer sequence**	**Repeat motif**	**Size range (bp)**	***T* (°C)**
44HDZ005	F: GAGCCCAGAGTGCCTTTG R: GAGATTAGAGAAGTATGTGTGTTTGG	$(GT)_{17}$	161-169	54
44HDZ011	F: TGTGGATTCAGCATTTGGC R: TCTGTCAGGGATTTGCGAG	$(CA)_{16}$	162-182	56
44HDZ035	F: ACCTCACCTCGCCTAGTCC R: TGCCTCTCGTGTTTGGTTC	$(AC)_{15}$	148-160	54
44HDZ119	F: TGGTTTTGCCACAAGTTATGTC R: TGAAGCCATCTAAGGAGGTTG	$(CA)_{12}$	158-162	60
44HDZ124	F: TACACCCCCTCCCCCAA R: GGCAAGTCTTTTGTCTAATGGAA	$(CA)_{16}$	132-140	54
44HDZ193	F: TCTGTGTAAGAAAAATGGGGAC R: AGCCAGGAACTGTGGACG	$(CA)_{14}$	171-185	54
44HDZ287	F: GTTTCCCCTACCAAGCTGC R: ATGGAAAAGGAGGTAGCAATG	$(CA)_{23}$	173-179	57

Table 3.3 Sample used in data analysis includes all individuals that yielded confident genotypes at a minimum of 4 microsatellite loci.

Site	N_{groups}	N_{males}	$N_{females}$	$N_{individuals}$
Talatakely	10	9	10	19
Sahamalaotra	4	2	3	5
Valohoaka	6	6	6	12
Vatoharanana	9	9	10	19
Total	**29**	**26**	**29**	**55**

Table 3.4 Summary statistics for 7 microsatellite loci (*N* = 55 individuals) for the red-bellied lemur population in Ranomafana National Park.

Locus	**K**	**AR**	**N**	H_{Obs}	H_{Exp}	PIC	NE_{1P}	NE_{2P}	NE_{PP}	NE_{ID}	NE_{SibID}	*p*	F (Null)
44HDZ005	5	4.938	55	0.764	0.689	0.626	0.737	0.573	0.397	0.157	0.448	0.178	-0.0626
44HDZ011	9	8.309	47	0.766	0.800	0.761	0.583	0.405	0.222	0.074	0.373	0.737	0.0186
44HDZ035	7	6.537	55	0.745	0.763	0.719	0.640	0.461	0.274	0.096	0.396	0.434	0.0080
44HDZ119	3	3.000	33	0.576	0.597	0.522	0.827	0.682	0.531	0.236	0.515	0.897	0.0026
44HDZ124	5	4.930	53	0.642	0.634	0.564	0.786	0.634	0.465	0.202	0.487	0.877	-0.0087
44HDZ193	8	7.938	55	0.800	0.810	0.782	0.545	0.367	0.177	0.059	0.363	0.878	0.0004
44HDZ287	4	3.989	52	0.519	0.586	0.496	0.826	0.702	0.556	0.261	0.525	0.487	0.0604
Statistics for combined loci													
Mean number of alleles												5.857	
Mean H_{Exp}												0.697	
Mean PIC												0.639	
Combined NE_{1P}												0.080	
Combined NE_{2P}												0.119	
Combined NE_{PP}												5.88×10^{-4}	
Combined NE_{ID}												8.30×10^{-7}	
Combined NE_{SibID}												3.16×10^{-3}	

k = number of alleles, AR = allelic richness, N = number of individuals genotyped, H_{Obs} = observed heterozygosity, H_{Exp} = expected heterozygosity under Hardy-Weinberg equilibrium, PIC = polymorphic information content, NE_{1P} = non-exclusion probability (first parent), NE_{2P} = non-exclusion probability (second parent), NE_{PP} = non-exclusion probability (parent-pair), NE_{ID} = non-exclusion probability (identity), NE_{SibID} = non-exclusion probability (sibling identity)

Table 3.5 Results of sex-biased dispersal tests. Results presented are mean values for each sex and significance based on 10,000 randomizations.

Test	Prediction for dispersing sex	Male	Female	*P*
F_{IS}	Significantly higher	0.0607	-0.0625	0.124
F_{ST}	Significantly lower	0.0240	0.0338	0.722
R	Significantly lower	0.0443	0.0694	0.636
mAIc	Significantly lower	-0.3713	0.3329	0.228
vAIC	Significantly larger	5.1304	3.2346	0.166

Table 3.6 Pairwise F_{st} values for each sample locality within RNP. F_{st} values are above the diagonal and *p* values are below. *p* values that were below the Bonferroni-corrected significance value of 0.05 ($p < 0.008$) are in bold.

	Sahamalaotra	Talatakely	Valohoaka	Vatoharanana
Sahamalaotra	---	0.066	0.081	0.099
Talatakely	0.009	---	0.022	-0.008
Valohoaka	**0.002**	0.053	---	0.047
Vatoharanana	**0.001**	0.813	**0.003**	---

Table 3.7 Results of *M*-ratio tests for the population of red-bellied lemurs in Ranomafana National Park. *M* = observed average *M* calculated across all loci for the combined male-female, female-only, and male-only data sets. The percentage of *M* values falling below observed *M* are given for both $p_s = 0.78$ and $p_s = 0.90$.

	Full data set/Sahamalaotra excluded			**Female-only data set/Sahamalaotra excluded**			**Male-only data set/Sahamalaotra excluded**		
Theta	*M*	% falling below *M*		*M*	% falling below *M*		*M*	% falling below *M*	
		$p_s = 0.78$	$p_s = 0.90$		$p_s = 0.78$	$p_s = 0.90$		$p_s = 0.78$	$p_s = 0.90$
0.2	0.974	90.97/90.76	67.71/68.47	0.935	74.74/74.99	43.98/43.85	0.974	90.63/90.81	67.54/68.31
1	0.974	98.52/98.61	88.77/89.11	0.935	92.50/92.04	67.54/68.25	0.974	98.38/98.67	89.94/90.67
2	0.974	99.62/99.72	95.43/95.05	0.935	98.21/97.89	83.34/83.99	0.974	99.78/99.71	96.19/96.05
10	0.974	100	99.88	0.935	99.95/99.99	99.08/99.33	0.974	100/100	99.98/99.93
20	0.974	100	100/99.99	0.935	100	99.88/99.95	0.974	100/100	100/100

Values to the right of "/" were calculated using data sets with Sahamalaotra samples excluded. If no value is provided, values for both data sets are the same.

Chapter 4

Dichromacy may be adaptive for food foraging in red-bellied lemurs (*Eulemur rubriventer*)

ABSTRACT

Trichromatic color vision in primates has long been considered to be adaptive. Among multiple hypotheses proposed for the evolution of trichromacy, many highlight its importance for foraging on red food. Adaptive explanations accord well with the prevalence of routine or polymorphic trichromacy in diurnal haplorrhine species. In lemurs, polymorphic trichromacy occurs in some diurnal/cathemeral species, but this trait appears to have been lost in others. It is unknown what evolutionary mechanisms might lead to losses of polymorphic trichromacy, but variation in species' foraging ecologies might play a role.

Eulemur rubriventer in Ranomafana National Park (RNP) is dichromatic with a long-wavelength (L) opsin and likely lost the ancestral *Eulemur* condition of polymorphic trichromacy. Using reflectance spectra from 40 species (72 plant parts) consumed by *E. rubriventer* in RNP, this study quantified their chromaticities as perceived by trichromatic and dichromatic *Eulemur* phenotypes. Results indicate that red-green chromaticities (unavailable to dichromats) of many food items, particularly many ripe fruit, would be conspicuous to a trichromatic *Eulemur*, suggesting trichromacy should provide a theoretical foraging advantage. However, when blue-yellow and luminance contrasts were calculated for the two dichromatic phenotypes (L opsin vs. medium-wavelength opsin), luminance contrasts were significantly greater for dichromats with the L opsin. Results suggest *E. rubriventer* in RNP may use

luminance cues during foraging, which could lead to relaxed selection on trichromacy or selection against trichromacy in favor of dichromacy, because chromatic information potentially corrupts luminance vision. Fixation of the L opsin may represent directional selection and adaptation for maximizing luminance cues.

INTRODUCTION

Color vision refers to the ability to discriminate between stimuli based solely on chromaticity, as opposed to intensity or brightness (e.g., Jacobs 1981, 1993). Among eutherian mammals, primates in particular are highly variable in their color vision capacities (e.g., Jacobs 1981, 1993), and this variation results from small changes at the molecular level (e.g., Nathans et al. 1986; Jacobs and Neitz 1987; Neitz et al. 1991; Jacobs et al. 1993a; Tan and Li 1999). More specifically, color vision requires an organism to possess more than one functional cone photoreceptor tuned to different spectral sensitivities (although rods may contribute to color vision under some circumstances, e.g., Jacobs and Deegan 1993; Freitag and Pessoa 2012), as well as the neural mechanisms to compare the differences (e.g., Jacobs 1993; Dacey 2000; Kelber et al. 2003; Kelber and Roth 2006). The spectral tuning of cones is dependent on the photopigment, and this in turn is determined by variation in photopigment opsin proteins coded by opsin genes (e.g., Nathans et al. 1986; Jacobs and Neitz 1987; Neitz et al. 1991).

Most primates have one functional short-wavelength (S) opsin gene located on chromosome 7 that codes for short-wavelength sensitive cone photoreceptors (S cones) in the retina (Jacobs 1993; Jacobs 2013). S cones coupled with medium-long wavelength (M/L) sensitive cone photoreceptors, which are coded by a single M/L opsin gene on the X chromosome, results in dichromatic color vision (Jacobs 1993; Neitz et al. 1991). Primate

species with this type of color vision, including tarsiers and some lemuriforms (Tan and Li 1999; Tan et al. 2005; Melin et al. 2013b), have the potential to discriminate between colors reflecting in short-wavelengths (e.g., blue) and longer wavelengths (e.g., yellow), but have difficulty distinguishing between colors reflecting in the middle-wavelength range of light (e.g., green) and those reflecting longer wavelengths (e.g., red; Jacobs 1981, 1993). The latter ability is conferred to those primates with trichromatic color vision and is achieved through two molecular mechanisms (Jacobs 1981, 1993, 1998; Dulai et al. 1999).

Catarrhines and New World howling monkeys have two separate M/L opsin genes on the X chromosome (known as routine trichromacy) as the result of two independent gene duplication events (Nathans et al. 1986; Jacobs et al. 1996b; Kainz et al. 1998; Dulai et al. 1999; Jacobs and Deegan 1999; Nathans 1999). Each gene results in a spectrally distinct photopigment: one medium-wavelength (M) opsin and one long-wavelength (L) opsin, which, together with the S opsin, provides the potential for trichromatic color vision (Nathans et al. 1986; Jacobs et al. 1996b; Jacobs and Deegan 1999). All other New World monkeys for which data are available (with the exception of nocturnal owl monkeys: Jacobs et al. 1993b; Jacobs et al. 1996a), as well as some lemurs, achieve trichromatic color vision through allelic variation of a single M/L opsin gene (reviews in Surridge et al. 2003; Jacobs 2007, 2008; Kawamura et al. 2012). Under this scenario, species exhibit two or more M/L opsin alleles that code for two or more opsins varying in their medium-long wavelength sensitivities (e.g., Jacobs and Neitz 1987; Jacobs et al. 1993a; Jacobs 1998; Tan and Li 1999). Functional variation is primarily linked to changes in one to three amino acid sites (site 180 coded in exon 3; sites 277 and 285 coded in exon 5), and this mechanism leads to color vision variation both within and among species and populations (Jacobs and Neitz 1987; Neitz et al. 1991; Williams et al. 1992; Jacobs et al. 1993a; Tan and Li

1999; Bradley et al. 2009; Bradley et al. in prep). Because variation is X-linked, females that are heterozygous for the M/L opsin gene have the potential for trichromatic color vision, while homozygous females and all males are dichromatic (Jacobs and Neitz 1987; Neitz et al. 1991; Williams et al. 1992; Jacobs et al. 1993a; Tan and Li 1999).

The apparent ubiquitous presence of trichromatic color vision in catarrhines, as well as its seemingly near ubiquitous presence, through one mechanism or another, in platyrrhines suggests that trichromacy in primates is adaptive. Indeed, the fixation of M/L opsin gene duplications in two separate primate lineages suggests strong positive selection (Surridge et al. 2003), as does the apparent long-term maintenance of color vision polymorphisms in New World monkeys (Boissinot et al. 1998; Surridge and Mundy 2002; Surridge et al. 2003). However, the selective pressure favoring trichromatic color vision remains unknown.

Multiple hypotheses for adaptive advantages of trichromatic color vision have been proposed. These include advantages related to 1) detecting signals of conspecifics based on variation in skin or pelage coloration (Changizi et al. 2006; but see Fernandez and Morris 2007; Kamilar et al. 2013), 2) predator detection (Coss and Ramakrishnan 2000; Pessoa et al. 2014), and the most long-standing hypothesis, 3) food detection (e.g., Allen 1879; Mollon et al. 1984; Mollon 1989; Osorio and Vorobyev 1996; Lucas et al. 1998; Dominy and Lucas 2001; Osorio et al. 2004). The latter has received the most attention, and several nuanced versions have been proposed. These variations include (but are not limited to) 1) detecting red fruits against a green foliage background (e.g., Mollon 1989; Osorio and Vorobyev 1996; Sumner and Mollon 2000a; Osorio et al. 2004), 2) discriminating ripe from unripe fruit (e.g., Sumner and Mollon 2000b; Smith et al. 2003), and 3) detecting red young leaves against green foliage (e.g., Lucas et al. 1998; Dominy and Lucas 2001). Furthermore, advantages may be limited to particular detection

distances (e.g., Pàrraga et al. 2002; Bompas et al. 2013; Melin et al. 2014), and may be influenced by variation in light levels during foraging (e.g., Osorio et al. 2004).

Foraging hypotheses for color vision evolution have been examined in a number of primate species, with particular focus on polymorphic New World monkeys, because trichromats and dichromats are present within a single population, providing unique situations to address hypotheses under natural conditions (e.g., Janson 1983; Vogel et al. 2007; Hiramatsu et al. 2008, 2009; Melin et al. 2007, 2008, 2009). Interestingly, while trichromatic foraging advantages can be readily observed under experimental conditions in captivity (Caine and Mundy 2000; Smith et al. 2003), behavioral evidence for such advantages is limited in wild primate populations (e.g., Vogel et al. 2007; Melin et al. 2008, 2009; Hiramatsu et al. 2008; Bunce et al. 2011).

The most evidence in support of trichromatic foraging hypotheses comes from theoretical studies modeling food color conspicuousness to trichromatic and dichromatic phenotypes. Such studies have found that trichromats should be better able to detect many food items (fruit or young leaves) compared to dichromats under various conditions (long- and short-distances, high and low light levels; e.g., Osorio and Vorobyev 1996; Sumner and Mollon 2000a, b; Regan et al. 2001; Lucas et al. 2003; Osorio et al. 2004; Riba-Hernández et al. 2004; Melin et al. 2014). These results suggest that variation in color vision capacity should result in modified foraging behaviors, making it particularly surprising that evidence for advantages in wild populations remains limited.

In order to explain the dichotomy between theoretical support and empirical support in the wild, some have suggested that advantages may be 1) important for fitness-related tasks other than foraging (see alternative hypotheses above), 2) too subtle to detect with current sample sizes or methodologies, and/or 3) restricted to very particular foraging contexts that also may be

dependent on each species' socioecology (e.g., Bunce et al. 2011; Melin et al. 2013a; Bunce 2015). Although it is possible that trichromatic color vision evolved for tasks unrelated to foraging behavior, foraging hypotheses remain the most prominent, and comparatively well-supported, hypotheses explaining variation in color vision capacity across primates, suggesting the latter two scenarios may help resolve the lack of support in wild populations.

Another approach to understanding the role of foraging in color vision evolution is using a broader comparative context, which explores potential foraging advantages across primate species with variable color vision capacities. Among haplorhines, all species (for which data are available) appear to be trichromatic (routine or polymorphic; e.g., Jacobs and Williams 2001; reviews in Surridge et al. 2003; Jacobs 2007, 2008, 2009; Kawamura et al. 2012), with the exceptions of the two nocturnal taxa: *Aotus*, which is monochromatic (lacks color vision; Jacobs et al. 1993b; Jacobs et al. 1996a), and nocturnal tarsiers, which are dichromatic, having likely lost polymorphic color vision (Melin et al. 2013b). This pattern is consistent with enhanced color vision being an adaptation to foraging. Again, there is overwhelming theoretical support for trichromatic foraging advantages (either for fruit and/or young leaves) across multiple species of day-active New World monkeys and catarrhines (e.g., Osorio and Vorobyev 1996; Sumner and Mollon 2000a, b; Regan et al. 2001; Lucas et al. 2003; Osorio et al. 2004; Riba-Hernández et al. 2004; Melin et al. 2014), and in accordance with these results, trichromatic color vision appears to be maintained in these primate species/populations (Jacobs and Williams 2001; Surridge and Mundy 2002; Hiwatashi et al. 2010; Kawamura et al. 2012). For *Aotus* and tarsiers, on the other hand, there may be relaxed selection for enhanced color vision related to shifts from foraging activity during high or dim light levels to activity during lower light levels, when color vision may be less important (Wright 1989; Jacobs et al. 1996a; Melin et al. 2013b). However, foraging

behavior need not be the only fitness task for which selection may have been relaxed under nocturnal conditions, and as such, this species-level pattern in haplorhines provides limited support for the relationship between foraging advantages and color vision capacity.

Lemurs represent a relatively under-studied but interesting group of primates to explore questions related to foraging and color vision, because, unlike most New World monkeys that have maintained trichromatic color vision, allelic trichromacy has been potentially lost among multiple species/populations of lemurs (chapter 2). Furthermore, losses have occurred among species/populations for which enhanced color vision would be seemingly advantageous for foraging (chapter 2). For example, color vision variation has likely been lost multiple times among the genus *Eulemur*, and these losses may represent fixation of one opsin at the species- or population-level (Bradley et al. 2009; Bradley et al. in prep; chapter 2). The genus *Eulemur* is of particular interest, because species are cathemeral, so they are active during the night (low light levels) but also readily active during the day and dusk (high and mesopic light levels) (reviewed in Mittermeier et al. 2010). They are also highly frugivorous (reviewed in Mittermeier et al. 2010), which, under several foraging hypotheses, would be considered a classic ecological scenario favoring a trichromatic foraging advantage (e.g., Allen 1879; Mollon 1989; Osorio and Vorobyev 1996; Sumner and Mollon 2000a, b; but see Lucas et al. 1998; Dominy and Lucas 2001). Interestingly, among lemur species/populations that likely lost a color vision polymorphism, there is variation in the fixation of particular opsins. That is, some species/populations are monomorphic for the M opsin, while others are monomorphic for the L opsin (Tan et al. 1999; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Bradley et al. in prep; Veilleux et al. 2014; chapter 2).

Relaxed selection has been proposed to account for the loss of allelic trichromacy in some lemurs (Kawamura et al. 2012), with some suggestion that strepsirrhine primates may have reduced color vision acuity compared to haplorhines as a result of differences in their visual systems, such as lower cone densities and higher retinal summation (Jacobs and Deegan 2003; Kawamura et al. 2012). However, generally lower color vision acuity in strepsirrhines would not explain why allelic trichromacy originally evolved in this lineage and why it appears to be maintained in some day-active lemurs and not others, especially when such variation occurs within genera (although more subtle species- or population-level variation in lemur visual systems may play a role; e.g., Jacobs and Deegan 2003).

Another, albeit non-mutually exclusive, possibility, however, may be related to foraging ecology. For example, it has been suggested that fruits consumed (and dispersed) by lemurs (including some *Eulemur* species) in Madagascar are primarily "dull" in coloration (i.e., green and brown; Dew and Wright 1998; Birkinshaw 2001). Most adaptive hypotheses for trichromatic color vision in New World monkeys and catarrhines, however, suggest that foraging advantages are likely to occur when detecting food items (fruit or leaves) that reflect in longer wavelengths (i.e., yellow, orange, red; e.g., Mollon 1989; Dominy and Lucas 2001; Bunce 2011; but see Melin et al. 2014). If food items consumed by some lemur species/populations are not more conspicuous to trichromats than dichromats, then selection to maintain trichromatic color vision in a population may be relaxed.

Along similar lines, chromatic information may be less important to the foraging behavior of some lemurs. Rather, other cues, such as differences in luminance (Hiramatsu et al. 2008) or non-visual cues, such as olfactory information (Dominy et al. 2001; Hiramatsu et al. 2009; Melin et al. 2009), may play a larger role. The influence of the latter remains poorly

understood, and is beyond the scope of this study.[2] However, if some lemur species rely heavily on luminance vision, which may be useful for breaking camouflage under both high and low light levels (Livingstone and Hubel 1988; Morgan et al. 1992; Melin et al. 2007; Caine et al. 2010), there could be relaxed selection to maintain trichromatic color vision. At the same time, this foraging scenario could actually result in selection against trichromacy (Osorio et al. 1998; Osorio et al. 2004), given that chromatic information may actually corrupt luminance vision (Osorio et al. 1998), and could lead to loss of color vision variation through disruptive selection (Futuyma 1998).

The above scenarios suggest that color discrimination during foraging may be less important to some lemurs compared to other primates, indicating that variation in dichromacy (being either monomorphic for the M or L opsin) observed in lemurs would likely result from random allele loss. An alternative hypothesis is that, while loss of color vision variation may result from relaxed or disruptive selection, directional selection may also play a role in the distribution of M and L opsins observed in dichromatic lemurs. For example, the nocturnal genus *Avahi* has maintained dichromatic color vision (Veilleux et al. 2014), having potentially lost a color vision polymorphism (chapter 2), and appears to be monomorphic for the L opsin (Veilleux et al. 2014). When comparing the performance (using color modeling techniques) of different dichromatic phenotypes in detecting chromatic cues of *Avahi* food items (young leaves), dichromacy with the L opsin had superior performance compared to the M opsin, suggesting that the high frequency of the L opsin may be an adaptation to foraging on young leaves (Veilleux et al. 2014).

[2] Although several anatomical features suggest olfaction may play an important role in lemur foraging behavior (Martin 1990), experimental data indicate that lemurs do rely heavily on visual cues (Rushmore et al. 2012).

The overall goal of this study is to evaluate these various hypotheses related to foraging ecology for loss of allelic trichromacy and dichromatic color vision in the red-bellied lemur (*Eulemur rubriventer*) population in Ranomafana National Park (RNP), Madagascar. This population of *E. rubriventer* is monomorphic for the L opsin (chapter 2), which is unique among other *Eulemur* species/populations that are either polymorphic or monomorphic for the M opsin (Tan and Li 1999; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). This condition appears to represent loss of color vision variation from a polymorphic ancestor, but whether this represents variation at the population level or characterizes the species as a whole, is unknown (chapter 2). Importantly, this population may have experienced a recent genetic bottleneck, which provides a possible (not mutually exclusive) mechanism for loss of color vision variation and fixation of the L opsin (chapter 3), but alternative/additional adaptive explanations have yet to be explored.

This study uses reflectance spectra collected from food items consumed by red-bellied lemurs in RNP to address the following hypotheses:

Loss of a color vision polymorphism represents relaxed selection on the trichromatic phenotype during foraging. The expectation for this hypothesis is that the red-green chromaticities of dietary items viewed by a trichromatic phenotype will not be discriminable from those of a leaf background.

Fixation of the L opsin represents directional selection favoring the L allele over the M allele during foraging contexts. The expectation in support of this hypothesis is that the chromatic contrast of food items consumed by *E. rubriventer* will be greater for dichromats with the L opsin than dichromats with the M opsin.

MATERIALS AND METHODS

All methods were approved by and in compliance with institutional (Stony Brook IACUC: 2010/1803, 2011/1895) and national guidelines (Madagascar National Parks).

Study subjects and study site

Red-bellied lemurs (*Eulemur rubriventer*) are medium-sized, cathemeral lemurs that are endemic to Madagascar's eastern rainforests (Mittermeier et al. 2010). Previous research indicates they are highly frugivorous (≥ 70% of feeding time; Overdorff 1991; Durham 2003; Tecot 2008), with the majority of fruit feeding time comprising ripe fruit (Overdorff 1991; Durham 2003; Tecot 2008).

E. rubriventer live in small groups (2-6 individuals) that actively defend a home range (Overdorff 1991). Groups usually include an adult male-adult female pair and immature individuals (Overdorff 1991; Tecot 2008). Red-bellied lemurs are sexually dichromatic and exhibit individual variation in their pelage coloration/patterns. This variation allowed observers to identify individuals in this study.

Data were collected on the population of *E. rubriventer* in Ranomafana National Park (RNP), which is an area of 41,000 ha of montane rainforest in southeastern Madagascar (E47°18' - 47°37', S21°02' - 21°25'; Wright 1992). This study population has been shown to be monomorphic for the M/L opsin gene and exhibits only the L opsin allele with peak spectral sensitivity at 558 nm (chapter 2).

E. rubriventer food items

From the end of September 2012 through mid-May 2013, feeding/foraging data were collected on 3 groups of red-bellied lemurs from each of three localities within RNP (N = 9 groups; Table 4.1; Figure 4.1). During behavioral data collection, groups were followed from the time of group location through dusk until sunset when light levels precluded visual observation of animals. Sites were rotated monthly (every 10 days), with an attempt to follow each group 3 days/month (see Table 4.1 for N_{Days}).

During group follows, all occurrences (i.e., "bouts") of feeding/foraging were recorded. Bouts were defined as when at least one individual in the group entered a new tree to feed or forage or when feeding/foraging resumed in a tree after all individuals had stopped feeding for at least 10 minutes. During feeding/foraging bouts, the species of the food item consumed was recorded (using the local vernacular species names), along with the plant part consumed and the color of the plant part consumed when possible. Plant parts were defined as ripe and unripe fruit when visual color changes of the fruit allowed identification (i.e., the use of ripeness category in this study does not refer to quantified mechanical properties of food items). For some fruit, multiple color changes occur during ripening (e.g., green to yellow to red), in which case a category of "mid-ripe" was added *post hoc* based on the color of fruit consumed. For fruit that do not exhibit color changes during ripening (or color changes were unknown), items were defined broadly as "fruit". Additional plant parts included flower buds, flowers, leaf petioles, young leaves, mature leaves, galls, and mushrooms.

Reflectance data

Food items consumed by *E. rubriventer* were collected from known feeding trees when possible within 10 days of when a study group had been observed feeding. However, when necessary, food items were collected from trees of the same tree species within the site, but these trees had not been directly observed to be fed in by one of the study groups. Food items were collected directly from trees using an extendable tree pruner when possible. For many trees, however, the height of the tree precluded direct collection, and, in such cases, "fresh" samples (i.e., excluding any overripe or decaying fruit) were collected from the ground (Dominy 2004). Once collected, samples were placed into a cooler with ice packs and taken to the research station for spectral data collection within 14 hours of sample collection (Dominy and Lucas 2004).

Reflectance spectra of samples were measured using a USB2000+UV–VIS Miniature Fiber Optic Spectrometer (Ocean Optics, Dunedin, FL). All measurements were recorded using standard lighting conditions with a PX-2 Pulsed Xenon Light Source (Ocean Optics). Measurements were recorded relative to a diffuse reflectance standard (WS-1; Ocean Optics) using a reflection probe that was maintained at a fixed angle (45°) and distance (5 mm) from each sample using a probe holder (RPH-1; Ocean Optics). The spectrometer was frequently recalibrated during data collection to minimize drift. Depending on the size of the sample, multiple measurements were taken (1-5 measurements). In addition to food samples, mature leaves were collected from plant species when possible. One to three measurements were recorded for the upper and lower part of each leaf. All food items and leaves are represented by 1-8 individual samples, and mean reflectance was calculated for each item for data analysis.

Chromaticities of *E. rubriventer* food items

To address the question of whether trichromatic color vision would theoretically provide a foraging advantage, the chromaticities of food items as perceived by a trichromatic *Eulemur* were calculated. Previous research on New World monkeys and catarrhines suggests that theoretical trichromatic advantages exhibit a predictable pattern in which red-green chromaticities of food items are greater than the chromaticities of mature leaves but largely overlap in blue-yellow chromaticity and luminance (see Figure 4.2A; Hiramatsu et al. 2008; Sumner and Mollon 2000a; Regan et al. 2001). If red-green chromaticities are not discriminable from those of a leaf background (as predicted), the expectation is that red-green chromaticities of food items and mature leaves will largely overlap (Figure 4.2B).

Chromaticity was calculated using the quantum catch of cone photoreceptors for a trichromatic *Eulemur*: S = 413 nm, M = 543 nm, L = 558 nm (Tan and Li 1999; Bradley et al. 2009; Veilleux and Bolnick 2009; Carvalho et al. 2012; Bradley et al. in prep). Calculations followed Hiramatsu et al. (2008) in which the quantum catch (Q) of each cone photoreceptor i (i.e., S, M, and L) was based on the following formula:

$$Q_i = \int_{400}^{700} R(\lambda) I(\lambda) S_i(\lambda) d(\lambda)$$

The quantum catch was calculated across 400 nm – 700 nm, which represents the visual spectrum of primates (following Hiramatsu et al. 2008; Valenta et al. 2013). In this formula, λ refers to wavelength, R is the reflectance spectrum of the item, I is the spectrum of the illumination, and S is the spectral sensitivity of the cone photoreceptor (Hiramatsu et al. 2008).

Analyses were based on two illumination spectra referred to as “day” and “dusk” (Figure 4.3). Both illuminations were used because red-bellied lemurs were invariably active and feeding during daylight and dusk (low sun angles: 10° to below the horizon; Endler 1993) conditions (R.

Jacobs, unpublished data). The illumination representing "day" was collected in RNP on April 29, 2013 under light shade and overcast conditions at 1040 hours. The illumination representing "dusk" was collected at near sunset (1720 hours) on the same day under overcast conditions. Illumination spectra were measured with down-welling light (probe directed upward) through a cosine corrector (CC-3-DA; Ocean Optics) directly attached to the USB2000+UV–VIS Miniature Fiber Optic Spectrometer. Spectral sensitivity functions followed Hiramatsu et al. (2008) but did not include effects of macular pigment on the pre-receptoral filter. Rather, functions were calculated using methods for lemurs following Valenta et al. (2013) and included only the effects of the lens.

For a trichromatic *Eulemur*, red-green chromaticity (which is unavailable to dichromatic lemurs) was calculated as a ratio of the quantum catch for L cones to L and M cones (i.e., L/(L+M)). Blue-yellow chromaticity was calculated as the ratio of quantum catch for S cones to L and M cones (i.e., S/(L+M)). Relative luminance was calculated by dividing the quantum catch of L and M cones (L+M) by a hypothetical white surface that reflects 100% of the given illumination. Chromaticities and luminance of mature leaves were compared to those of food items using non-parametric Mann-Whitney U tests performed in SPSS Version 23.0 (IBM Corp.).

Chromatic and luminance contrast

To determine whether the L opsin would provide a theoretical advantage over the M opsin during foraging (see Figure 4.4), relative chromatic and luminance contrasts for food items consumed were calculated for each dichromatic phenotype (558 nm - L opsin only and 543 nm - M opsin only) under both illumination conditions. In this case, the contrast was calculated

between the food item consumed and its respective mature leaf background (for upper and lower leaf backgrounds). In cases where data for mature leaves of the same species were unavailable, mean leaf background (using all mature leaves in the data set) was used in calculations.

The chromaticities of each food item and mature leaf background were calculated following methods outlined above with the following modifications for dichromatic lemurs. For dichromats with the L (558 nm) opsin, blue-yellow chromaticities were calculated as the relative quantum catch of S cones to L cones (S/L), and for dichromats with the M (543 nm) opsin, blue-yellow chromaticities were calculated as the relative quantum catch of S cones to M cones (S/M). Luminance was calculated as the relative quantum catch of L cones and M cones to that of a hypothetical white surface (as above) for dichromats with the L opsin and dichromats with the M opsin, respectively.

Blue-yellow chromatic contrast was then calculated as $\left|\ln\left(Q^{f}_{i_{M/L}}\right) - \ln(Q^{b}_{i_{M/L}}\right| - \left|\ln(Q^{f}_{S}) - \ln(Q^{b}_{S})\right|$. Luminance contrast was calculated as $\left|\ln\left(Q^{f}_{i_{M/L}}\right) - \ln(Q^{b}_{i_{M/L}}\right|$. Q is the quantum catch of L or M cones ($i_{M/L}$) for each dichromatic phenotype and S cones (S) for each food item (*f*) and mature leaf background (*b*). All calculations followed Hiramatsu et al. (2008) and were performed in Matlab R2014b using code and methods generously provided by Drs. Amanda Melin and Chihiro Hiramatsu (Washington University, St. Louis; Kyushu University, Japan).

In order to determine if relative chromatic or luminance contrast is greater for dichromats with the L opsin compared to dichromats with the M opsin, Wilcoxon Signed-Rank Tests were used on full data sets for upper and lower leaf backgrounds for both illumination conditions. Given that previous research has identified ripe fruit as comprising the majority of fruit feeding time in *E. rubriventer*, tests were also performed on a reduced ripe-fruit only data set. Statistical

analyses were performed in SPSS Version 23.0 (IBM Corp.). All tests were two-tailed with significance set at $p < 0.05$.

RESULTS

E. rubriventer food items

A total of 2,924 foraging bouts on plant material (excluding soil and millipedes) were recorded during the study period. Table 4.2 lists all species and plant parts consumed, as well as the percentage of foraging bouts for each plant taxon. Overall, *E. rubriventer* fed on 115 plant taxa. Fruit feeding/foraging accounted for the majority of bouts (1,947; 66.59%), followed by flowers/flower buds (480; 16.42%), and leaves (399; 13.75%). For the fruit foraging bouts for which ripeness of food items consumed could be determined ($N = 58$ species), unripe fruit accounted for 56.40% of bouts.

Chromaticities of *E. rubriventer* food items

Reflectance data for 40 species, which includes 72 plant parts, consumed by red-bellied lemurs in RNP were included in chromaticity analyses (see Table 4.2). The 40 species represent 75.21% of foraging bouts. Figure 4.5 illustrates reflectance spectra for three plant species and six plant parts consumed. Chromaticity plots for a trichromatic *Eulemur* under "day" and "dusk" conditions are illustrated in Figure 4.6. Red-green (L/(L+M)) vs. blue-yellow (S/(L+M)) chromaticity plots reveal that most food items have greater red-green chromaticity compared to mature leaves under both illumination conditions, which is consistent with the pattern identified for a theoretical foraging advantage for trichromats (Figure 4.2A). Descriptive statistics are

provided in Table 4.3. Overall, mean red-green chromaticities of food items ("day" = 0.523, SE = 0.003; "dusk" = 0.527, SE = 0.003) was greater than upper ("day" = 0.497, SE = 0.000; "dusk" = 0.504, SE = 0.000) and lower leaves ("day" = 0.505, SE = 0.001; "dusk" = 0.510, SE = 0.001). Results of Mann-Whitney U Tests reveal that red-green chromaticities of food items ("day": Median = 0.515; "dusk": Median = 0.518) are significantly greater than mature leaves under both "day" (Median upper leaf = 0.497, Median lower leaf = 0.503) and "dusk" (Median upper leaf: 0.504, Median lower leaf = 0.508) illuminations ("day": upper leaf: $U = 3$, $p < 0.001$; lower leaf: $U = 346$, $p < 0.001$; "dusk": upper leaf: $U = 50$, $p < 0.001$; lower leaf: $U = 444$, $p < 0.001$).

Observations of chromaticity plots (Figure 4.6) suggest that many food items with greater red-green chromaticities are ripe fruit. To explore this further, a *post hoc* Mann-Whitney U Test was performed to determine if ripe fruit ($N = 21$) has significantly greater red-green chromaticity than all other food items ($N = 61$). Results indicate that the red-green chromaticity of ripe fruit is greater than that of other food items under both "day" (Median ripe fruit = 0.531; Median other food items = 0.510; $U = 229.5$, $p < 0.001$) and "dusk" (Median ripe fruit = 0.533; Median other food items = 0.515; $U = 254$, $p < 0.001$) conditions.

Luminance (L+M) vs. blue-yellow chromaticity plots indicate that most food items largely overlap in luminance as well as in blue-yellow chromaticity. A similar pattern of overlap in luminance and blue-yellow chromaticity for a trichromatic lemur is found in both dichromatic phenotypes (Figure 4.7). Despite the large amount of overlap apparent from the chromaticity plots, blue-yellow chromaticity is significantly greater for food items ("day": Median = 0.137; "dusk": Median = 0.151) compared to mature leaves under both "day" (Median upper leaf = 0.105, Median lower leaf = 0.101) and "dusk" (Median upper leaf: 0.111, Median lower leaf = 0.107) illuminations ("day": upper leaf: $U = 659$, $p < 0.01$; lower leaf: $U = 731$, $p < 0.05$; "dusk":

upper leaf: U = 623, p <0.01; lower leaf: U = 692, p < 0.01). Luminance was also significantly greater for food items ("day": Median = 3.330; "dusk": Median = 3.286) compared to mature leaves under some conditions ("day": Median upper leaf = 2.919, Median lower leaf = 3.282, upper leaf: U = 226, p <0.001; lower leaf: U = 838, p = 0.122; "dusk": Median upper leaf: 2.894, Median lower leaf = 3.248, upper leaf: U = 254, p <0.001; lower leaf: U = 869, p = 0.189).

Chromatic and luminance contrast

Chromatic and luminance contrasts of food items for both dichromatic phenotypes are illustrated for upper and lower leaves in Figures 4.8 and 4.9, respectively. Compared to the expectation of greater chromatic contrasts for dichromats with the L opsin (Figure 4.4), chromatic contrasts appear to be greater for dichromats with the M opsin. Results of Wilcoxon Signed-Rank Tests confirm that chromatic contrasts are significantly greater for dichromats with the M opsin compared to dichromats with the L opsin under both "day" (N = 72; upper leaf: Z = -4.113, p < 0.001; lower leaf: Z = -5.572, p < 0.001; see Table 4.4 for descriptive statistics) and "dusk" conditions (N = 72; upper leaf: Z = -5.140, p < 0.001; lower leaf: Z = -6.033, p < 0.001). Results are similar when only ripe fruit contrasts are included in analyses (N = 21; "day": upper leaf: Z = -2.728, p < 0.01; lower leaf: Z = -3.215, p < 0.01; "dusk": upper leaf: Z = -3.111, p <0.01; lower leaf: Z = -3.250, p < 0.01).

Luminance contrasts, on the other hand, are significantly greater for dichromats with the L opsin compared to dichromats with the M opsin under "day" (N = 72; upper leaf: Z = -6.296, p < 0.001; lower leaf: Z = -3.165, p < 0.01; see Table 4.5 for descriptive statistics) and "dusk" illumination (N = 72; upper leaf: Z = -6.178, p < 0.001; lower leaf: Z = -3.547, p < 0.001). Results hold under upper leaf conditions using a ripe fruit only data set (N = 21; "day": upper

leaf: Z = -2.972, p <0.01; lower leaf: Z = -1.025, p = 0.305; "dusk": upper leaf: Z = -2.763, p < 0.01; lower leaf: Z = -1.547, p = 0.122).

DISCUSSION

Results of this study suggest that while *E. rubriventer* has likely lost the *Eulemur* ancestral condition for polymorphic trichromatic color vision, trichromacy (i.e., the ability to discriminate between green and red hues) would provide a theoretical advantage to foraging on many food items. Specifically, red-green chromaticities of many food items consumed by *E. rubriventer*, and in particular ripe fruit, are greater than those of upper and lower leaves, but largely overlap in luminance and dichromatic chromaticity. A similar pattern has been identified among food items consumed in polymorphic New World monkeys (Regan et al. 2001; Hiramatsu et al. 2008) and routinely trichromatic catarrhines and howling monkeys (Sumner and Mollon 2000a; Regan et al. 2001). This result does not support an ecological scenario in which food items consumed by *E. rubriventer* are largely "dull" in coloration and likely less conspicuous in red-green chromaticity, which could potentially result in relaxed selection to maintain polymorphic trichromacy in this taxon.

That said, despite the large overlap in luminance and dichromatic chromaticities, both red-green and blue-yellow chromaticities of food items were significantly greatly than mature leaves under both illumination conditions. Luminance of food items was also significantly greater than mature leaves under some conditions. Therefore, it appears that many food items consumed by *E. rubriventer* would be conspicuous to dichromats, which could result in relaxed selection on the trichromatic phenotype.

In order to assess whether fixation of the L opsin in the *E. rubriventer* population in RNP may result from directional selection favoring the L opsin over the M opsin, this study examined chromatic and luminance contrasts of food items from their leaf backgrounds. Results indicate that chromatic contrasts of food items (all food items, as well as ripe fruit only) are not greater for dichromats with the L opsin than dichromats with the M opsin. Rather, chromatic contrasts are significantly greater for dichromats with the M opsin. On the other hand, luminance contrasts are greater for dichromats with the L opsin compared to dichromats with the M opsin.

Given this result, it is possible that chromatic information is less important to *E. rubriventer* during foraging, and rather luminance may be a more important cue to *E. rubriventer*. This scenario could also lead to relaxed selection on polymorphic trichromacy or even selection against trichromacy (disruptive selection), as chromatic information has been shown to corrupt luminance vision (Osorio et al. 1998), which has been argued to account for dichromatic primates exhibiting greater foraging efficiency than trichromats on some camouflaged food items (Melin et al. 2007, 2010; Caine et al. 2010). At the same time, if luminance cues are important to *E. rubriventer* during foraging, there may have been directional selection favoring the L opsin, which results in greater luminance contrast compared to the M opsin. Therefore, dichromacy for the L opsin in *E. rubriventer* may be adaptive for foraging using luminance cues.

This latter scenario hypothesizes that *E. rubriventer* color vision may be the result of relaxed or disruptive selection on trichromacy combined with directional selection favoring the L opsin, but it is important to note that there may be alternative/additional explanations. First, random allele loss due to either relaxed or disruptive selection alone could account for fixation of the L opsin in *E. rubriventer*. Although luminance contrast is greater for the L opsin compared to

the M opsin, it remains unknown the extent to which luminance cues are important during *E. rubriventer* foraging. Studies examining the influence of chromatic and luminance cues on *E. rubriventer* foraging efficiency, similar to those conducted on New World monkeys (e.g., Hiramatsu et al. 2008, 2009; Melin et al. 2007, 2008), will be necessary to evaluate these alternative hypotheses.

It is also important to note that, although chromatic distances are overall greater for dichromacy with the M opsin, there are some food items (e.g., some *Ficus* fruit) that are more chromatically conspicuous (i.e, greater chromatic distances) to a dichromat with the L opsin compared to dichromats with the M opsin. This study does not consider "preference" of food items, which would require data on foraging behavior in relation to food availability. It is possible then that fixation of the L opsin represents directional selection to maximize chromatic cues of particular (i.e., "preferred") food items, rather than maximizing luminance cues overall, as has been hypothesized for another lemur species, *Avahi* (Veilleux et al. 2014). Future studies should take into account foraging preferences in *E. rubriventer* and other taxa with different color vision phenotypes to address this alternative explanation.

Finally, previous research has found that this population of *E. rubriventer* may have experienced a recent genetic bottleneck (chapter 3). A recent population crash could result in a greater influence of genetic drift and account for fixation of the L opsin rather than directional selection (Futuyma 1998). Along similar lines, population bottlenecks can also result in loss of advantageous alleles, such that polymorphic trichromacy may provide an advantage, but was lost through nonadaptive mechanisms (Futuyma 1998). To better understand color vision evolution in this taxon, it will be necessary to determine opsin allele frequencies in additional populations, as

it is presently unknown whether dichromacy for the L opsin is a characteristic of *E. rubriventer* as a whole or is specific to the population in RNP (chapter 2).

Although this study evaluates potential foraging hypotheses for loss of polymorphic trichromacy and fixation of the L opsin in one lemur taxon, it is important to keep in mind that multiple lemur species have polymorphic trichromatic color vision or are strictly dichromatic with the M opsin (Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Interestingly, variation can be found within sites among sympatric lemurs, and RNP is no exception (Bradley et al. 2009; Bradley et al. in prep). There are four species of day-active frugivorous lemurs in RNP: *Propithecus edwardsi*, *Varecia variegata*, *Eulemur rufifrons*, and *Eulemur rubriventer*. Polymorphic trichromacy has been documented in the former three taxa with variation in M and L allele frequencies across species (Bradley et al. 2009; Bradley et al. in prep). All frugivorous species exhibit a large amount of overlap in food items consumed (Overdorff 1991; Wright et al. 2011; Razafindratsima et al. 2014), although some plant taxa may be consumed by only a single lemur species (Wright et al. 2011).

Accounting for color vision variation from an ecological perspective in light of the results of this study is difficult without simultaneous data on sympatric frugivorous lemurs, but it may very well be that different lemur species preferentially feed on plant taxa or plant parts that vary in their chromatic or luminance cues, which could result in different selective pressures to maintain polymorphic trichromacy. For example, red-green chromaticity may be particularly advantageous for detecting ripe fruit, and while previous research suggests that *E. rubriventer* primarily consumes ripe fruit, results of this study suggest unripe fruit accounts for more foraging bouts compared to ripe fruits when ripeness stage could be assessed. In one of the few

studies examining feeding behavior in sympatric *Eulemur* in RNP, Overdorff (1991) found that *E. rufifrons* spent more time feeding on ripe fruit compared to *E. rubriventer*. Such differences in feeding ecology might account for differences in color vision status observed between the two species.

Another factor that might account for variation in color vision capacities across sympatric species may be activity pattern. *Propithecus* and *Varecia* are generally considered to be diurnal (but see Rea et al. 2014 on *Varecia* cathemerality), while *E. rubriventer* and *E. rufifrons* are considered to be cathemeral. It is possible, given primary activity during the day, that the former two taxa rely more heavily on color vision compared to luminance vision, whereas *E. rubriventer* relies more heavily on luminance vision as a compromise between its diurnal and nocturnal activity. Such a general difference would not explain polymorphic trichromacy in *E. rufifrons* (and other polymorphic cathemeral *Eulemur*), but it is possible that these sympatric cathemeral species also exhibit variation in their nocturnal activity patterns. Overdorff (1996) suggests that *E. rufifrons* is actually more active at night compared to *E. rubriventer*, and the pattern of peak activity varied. That is, *E. rubriventer* exhibited a more crepuscular pattern (peak activity in late evening and early morning), while *E. rufifrons* was less active at these times with a peak in activity during the middle of the night (ca 2400 hours) that was not observed in *E. rubriventer*. However, the results from Overdorff (1996) were based on very small sample sizes and other variables (e.g., variation in moonlight during nocturnal follows) might account for these observed differences. Furthermore, chromatic and luminance contrasts under nocturnal light environments were not evaluated in the present study, making interpretations difficult at this time. That said, more detailed studies on cathemeral activity patterns that also take into account

nocturnal light environments may provide additional insights into color vision variation in *Eulemur* and other cathemeral taxa.

In sum, this study has found that trichromatic color vision would be theoretically advantageous in detecting many food items consumed by *E. rubriventer*, but many food items may also be conspicuous to dichromats based on blue-yellow chromaticities and luminance. Furthermore, loss of color vision variation could be due to a greater reliance on luminance vision in this taxon. Given that luminance contrasts of food items are greater for the L opsin compared to the M opsin, there may have been directional selection favoring the L opsin, suggesting a potential adaptive role of dichromacy for food foraging in this taxon. Therefore, this study provides additional support that variation in primate color vision may result from different selective pressures related to foraging. Given *E. rubriventer*'s cathemeral activity pattern, one interesting avenue for future research would be to evaluate chromatic and luminance contrasts under nocturnal light environments to determine if the pattern of greater luminance contrast holds. Additional studies on foraging behavior in lemur species that vary in their color vision capacities will help further elucidate the evolutionary mechanisms underlying color vision variation in this lineage.

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Figure 4.1 Study sites for behavioral data collection within RNP. This figure was modified with permission from Baden (2011).

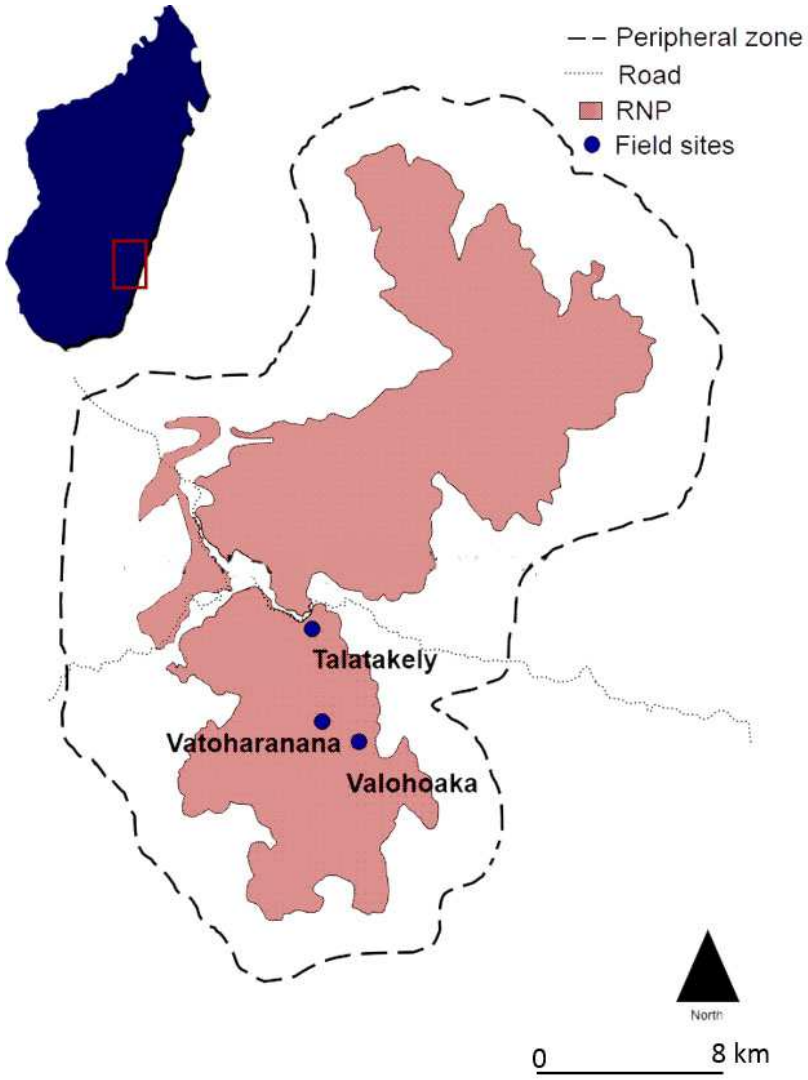
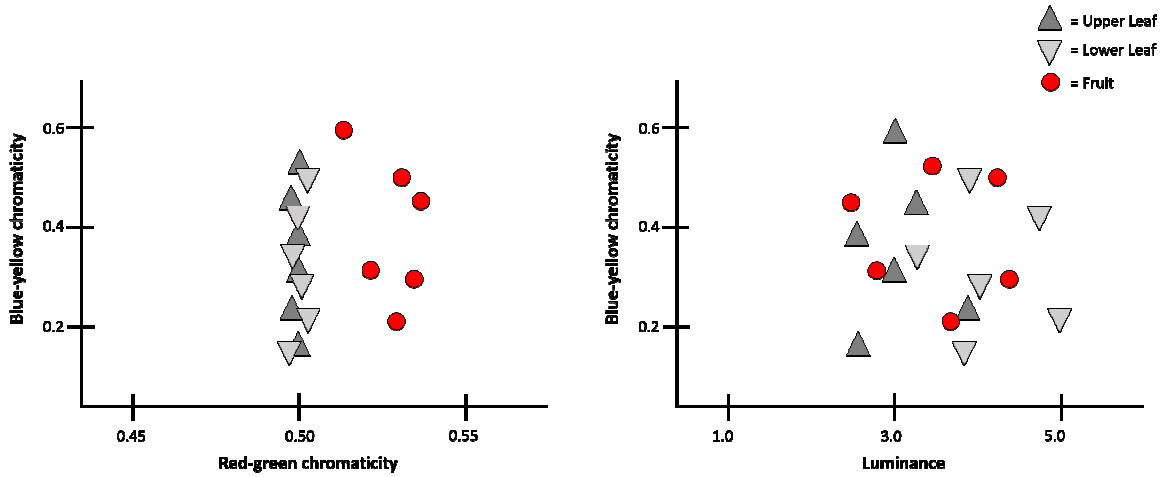
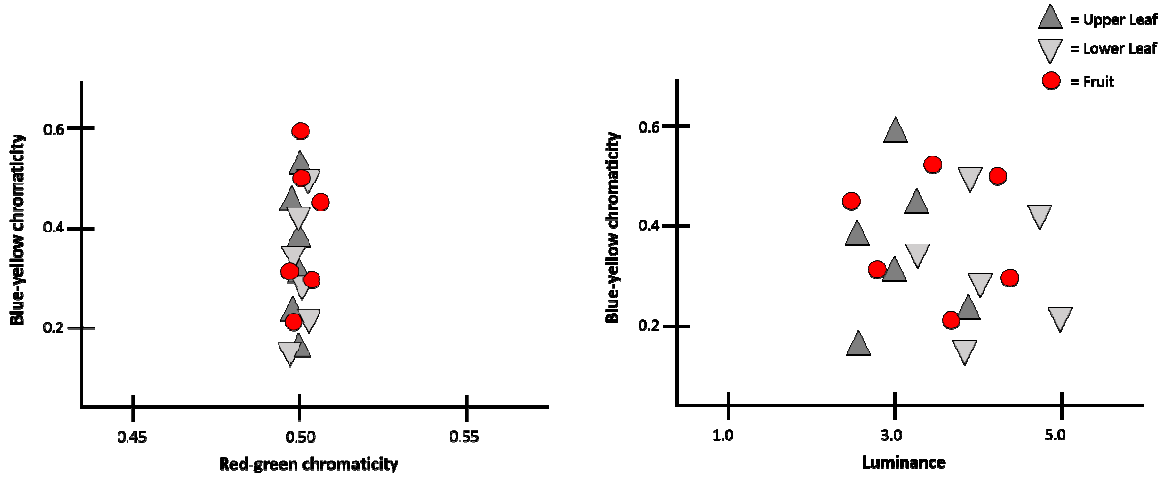


Figure 4.2 Illustration of hypothetical chromaticity and luminance plots for **A)** a theoretical trichromatic foraging advantage, and **B)** the expectation if trichromatic color vision does not provide a theoretical foraging advantage. Note that red-green chromaticities of food (i.e., "Fruit": red circles) are greater than those of mature leaves (grey triangles) in **A** but overlap in **B**. Blue-yellow chromaticities and luminance of food and mature leaves largely overlap under both conditions.



A.



B.

Figure 4.3 Illumination spectra measured in RNP.

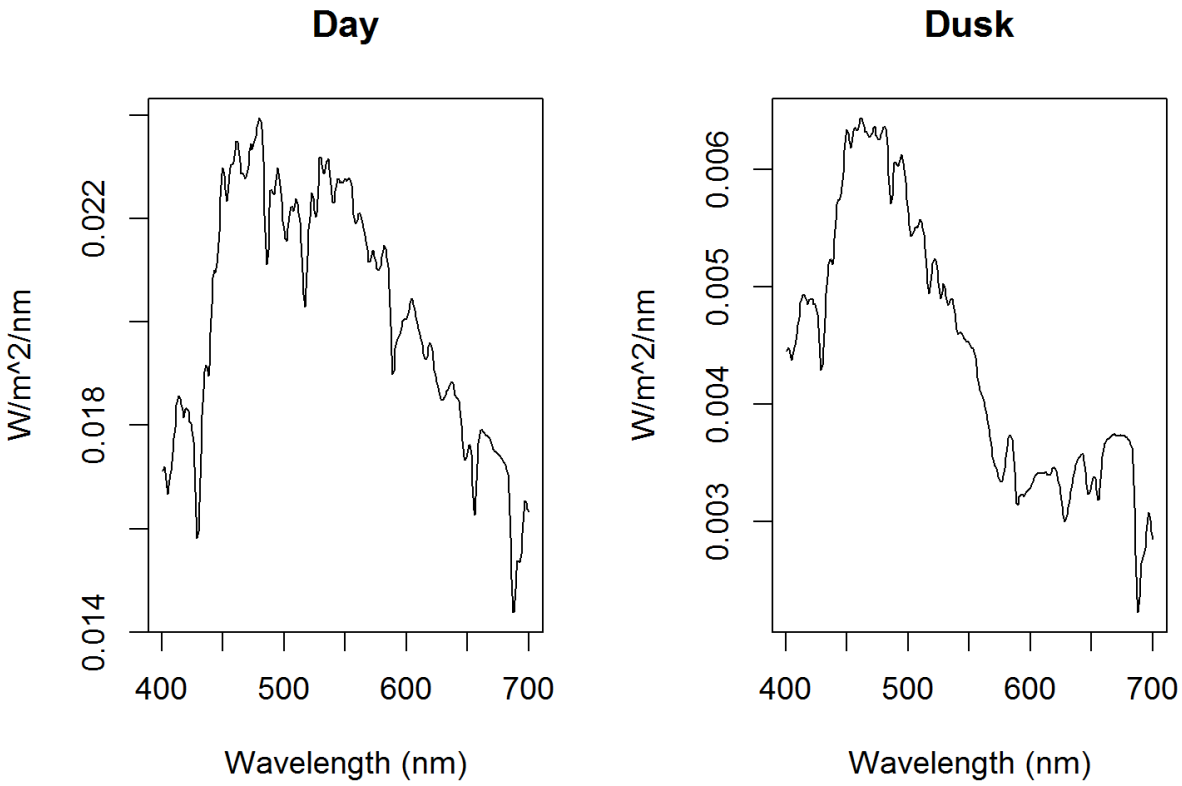


Figure 4.4 Illustration of hypothetical chromatic and luminance contrasts for the prediction that chromatic contrasts of food items (i.e., "Fruit" illustrated here) will be greater for dichromats with the L opsin compared to dichromats with the M opsin.

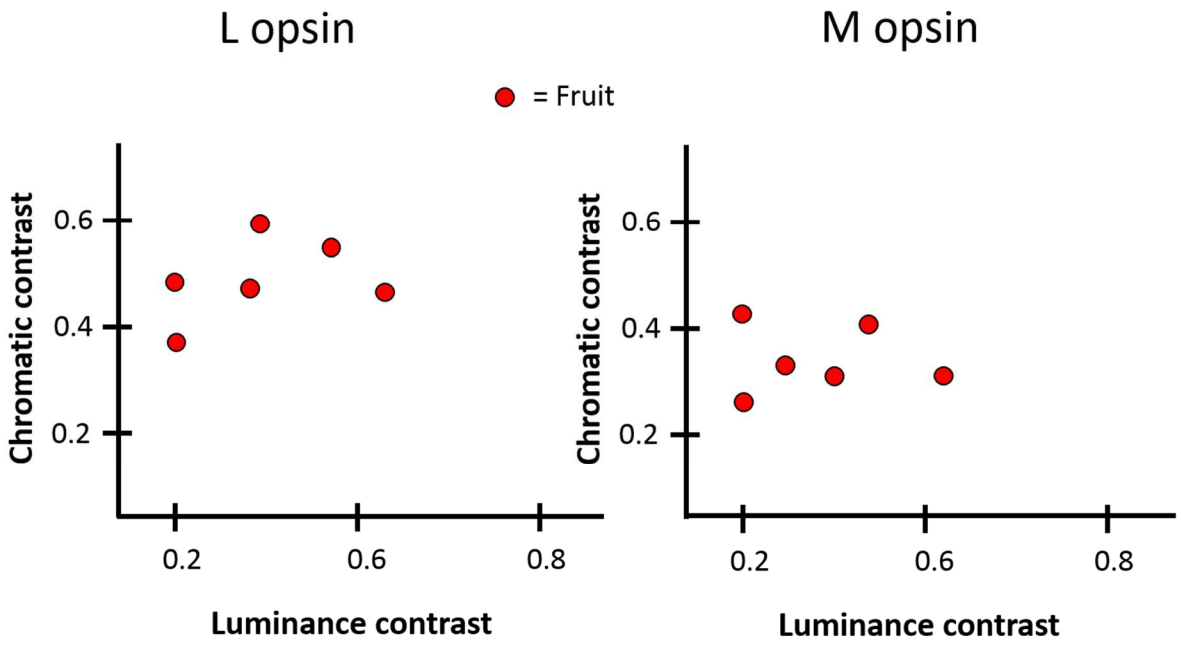


Figure 4.5 Example reflectance spectra, representing mean reflectance for each plant part, for three plant species and six plant parts consumed by *E. rubriventer* in RNP. (Photo credits: Guava: Natalee Phelps; Tsirika and Natojabo: Joseph Falinomenjanahary)

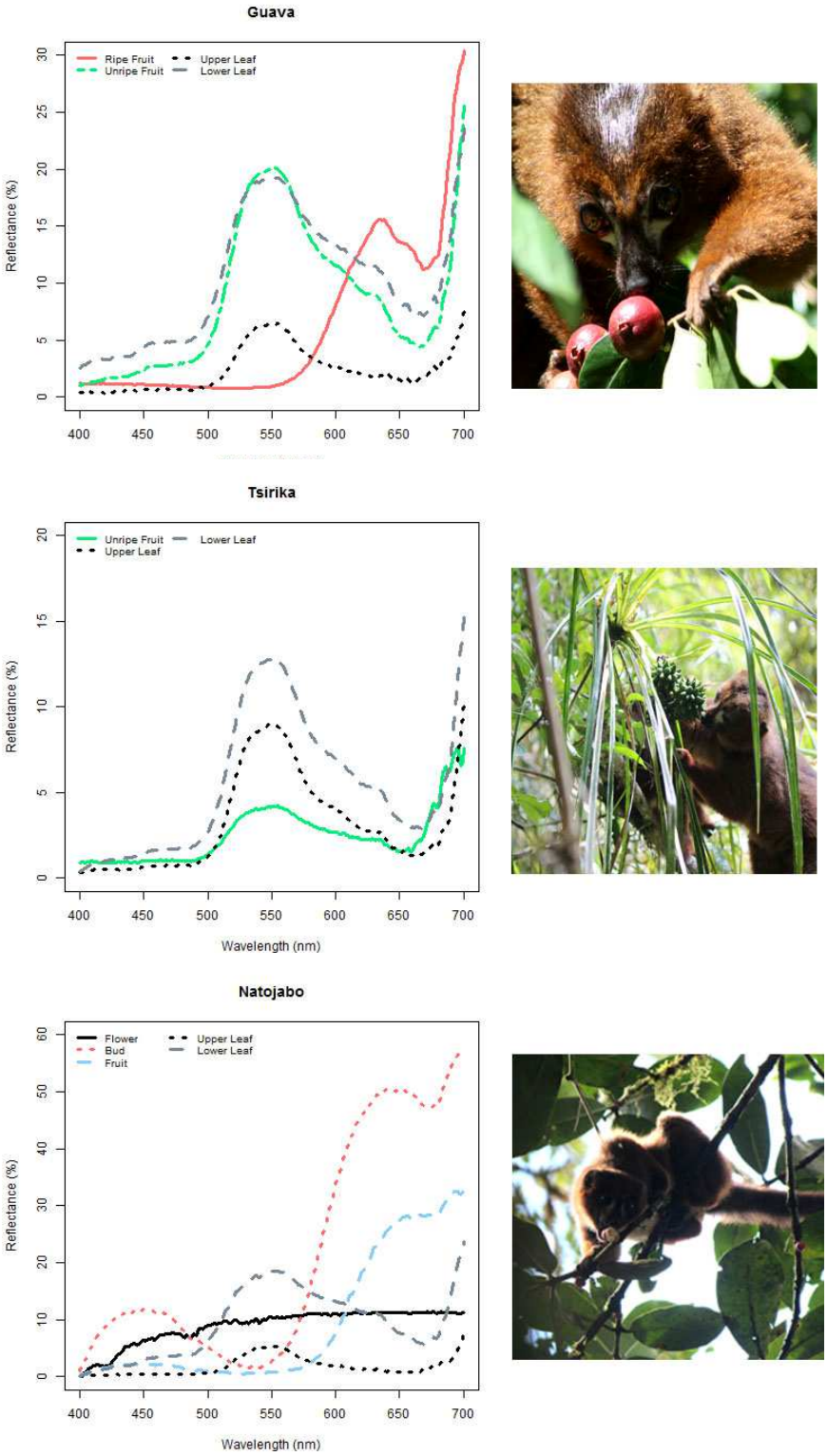


Figure 4.6 Chromaticity and luminance plots under "day" and "dusk" illumination for 72 plant parts from 40 plant species consumed by *E. rubriventer* in RNP. Mean value is plotted for each plant part. Left: Red-green (L/(L+M)) chromaticity vs. blue-yellow (S/(L+M)) chromaticity plots; Right: Luminance (L+M) vs. blue-yellow (S/(L+M)) chromaticity plots.

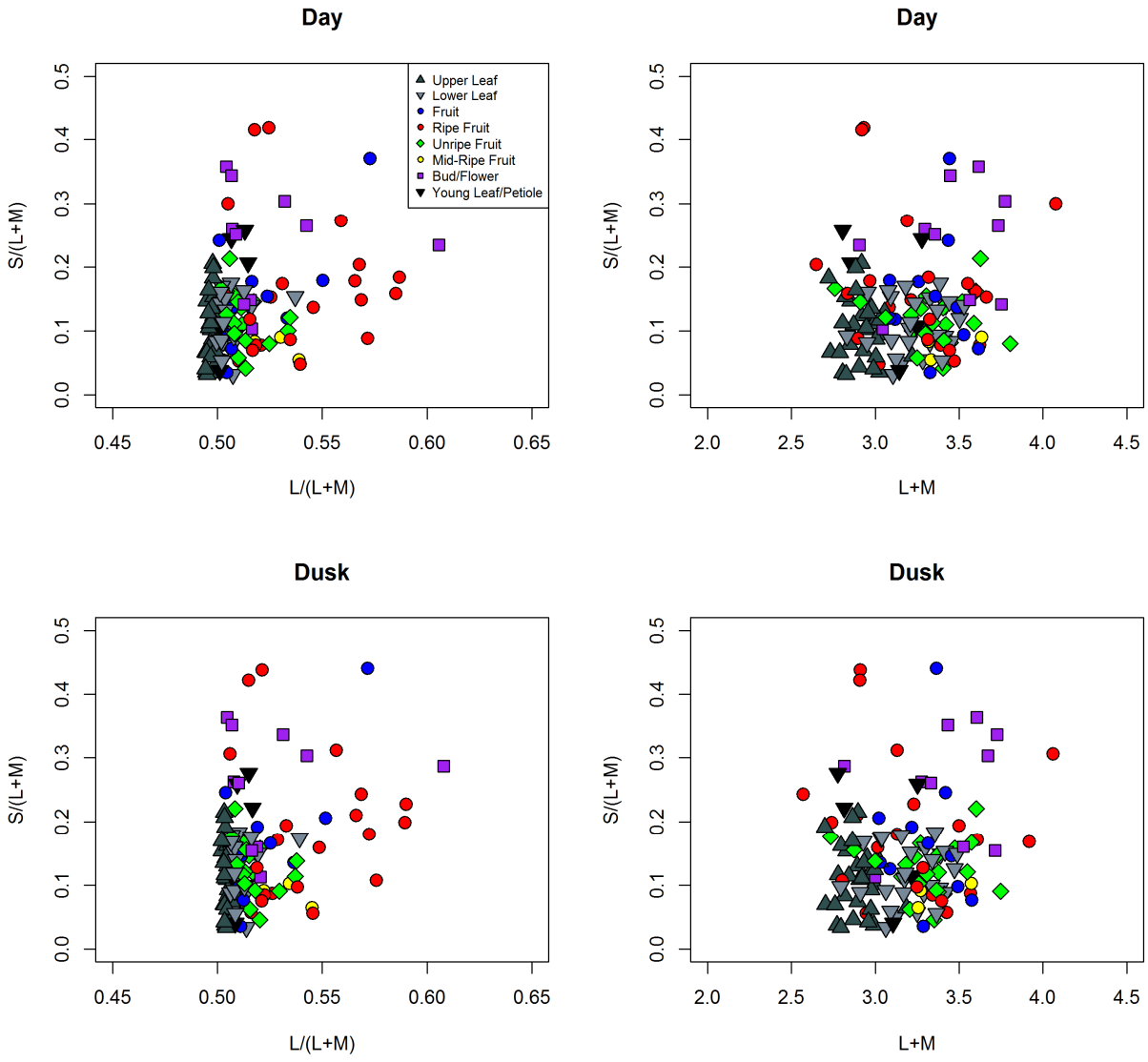


Figure 4.7 Chromaticity vs. luminance plots under "day" and "dusk" illumination for 72 plant parts from 40 plant species consumed by *E. rubriventer* in RNP. Mean value is plotted for each plant part. Luminance (L or M) vs. blue-yellow (S/(L or M)) plots are given for each dichromatic phenotype. Top: Dichromatic with the L opsin; Bottom: Dichromatic with the M opsin.

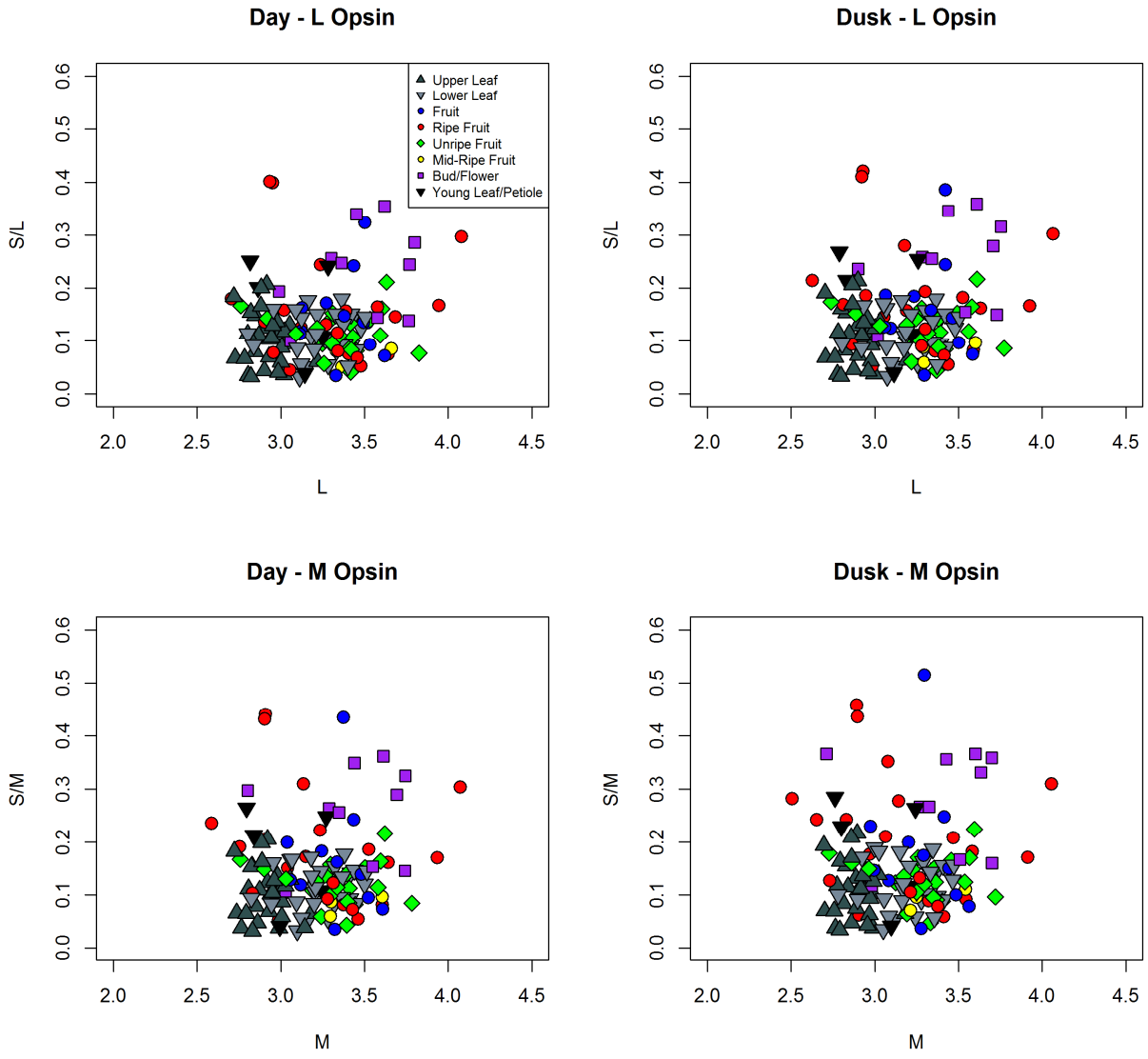


Figure 4.8 Chromatic (blue-yellow) and luminance contrasts under "day" and "dusk" illumination for 72 plant parts from 40 plant species consumed by *E. rubriventer* in RNP. Mean value is plotted for each plant part and contrasts were calculated using the upper side of mature leaves as background. Plots are given for each dichromatic phenotype. Left: Dichromatic with the L opsin; Right: Dichromatic with the M opsin. Top: Contrasts under "day" illumination; Bottom: Contrasts under "dusk" illumination.

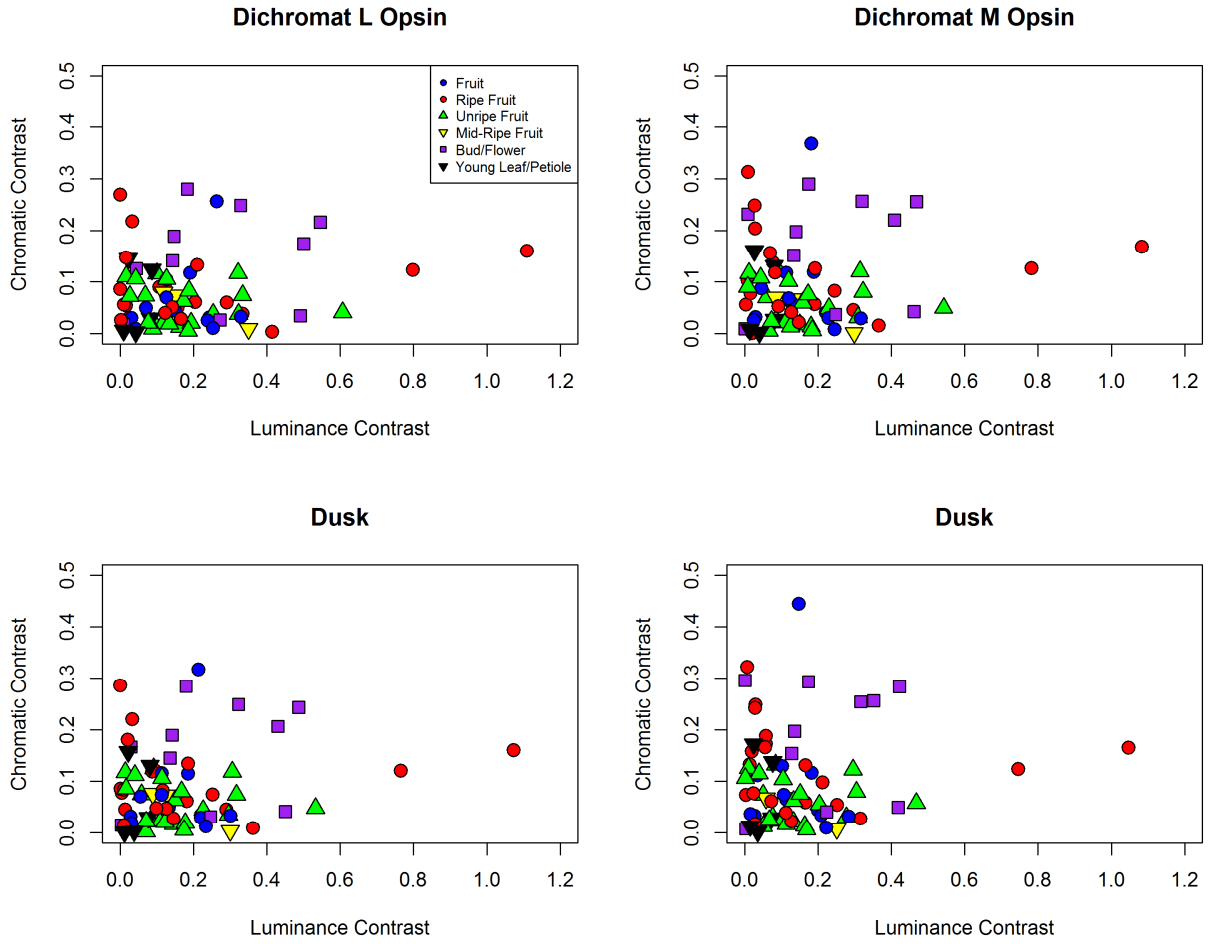


Figure 4.9 Chromatic (blue-yellow) and luminance contrasts under "day" and "dusk" illumination for 72 plant parts from 40 plant species consumed by *E. rubriventer* in RNP. Mean value is plotted for each plant part and contrasts were calculated using the lower side of mature leaves as background. Plots are given for each dichromatic phenotype. Left: Dichromatic with the L opsin; Right: Dichromatic with the M opsin. Top: Contrasts under "day" illumination; Bottom: Contrasts under "dusk" illumination.

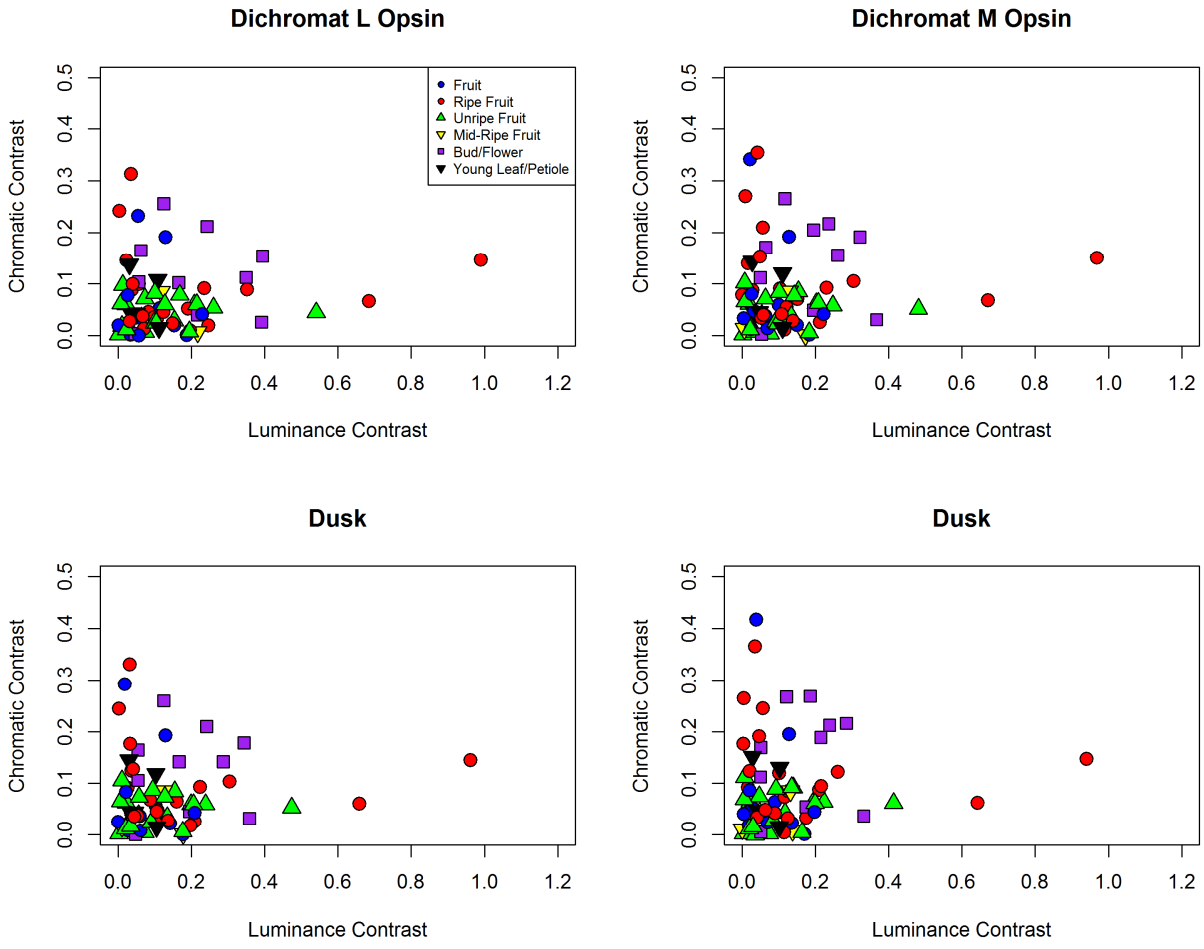


Table 4.1 Red-bellied lemur study groups, compositions, and number of days followed during behavioral data collection.

Group	Locality	$N_{Adult\ Males}$	$N_{Adult\ Females}$	$N_{Immature\ Males}$	$N_{Immature\ Females}$	$N_{Infants}$	$N_{Total\ Individuals}$	N_{Days}
TK3	Talatakely	1 (1)*	1	1	1	0	4-2	18
TK4	Talatakely	1	1	0	2	0	4	14
TK5	Talatakely	1	1	0	1	0	3	20
VT3	Vatoharanana	1	1	0	0	1	3	16
VT5	Vatoharanana	1	1	1	1	0	4	12
VT7	Vatoharanana	1	1	1	1	0	4	14
VL1	Valohoaka	1	1	1	2	0	5	15
VL5	Valohoaka	1	1	0	1	0	3	18
VL9	Valohoaka	1	1	1	2	0	5	22
		10	9	5	11	1	36	149

* Between September and October 31, 2012, TK3 included 4 individuals as listed in the table. The original male was evicted (reported by a tourist guide who witnessed the event) from the group at the end of October and a new male was present during group follows in November. During December follows to the end of the study in May 2013, the group only included one adult male and one adult female. The immature male and female were no longer in the group.

Table 4.2 List of plant species and plant parts foraged and fed on by *E. rubriventer* between September 2012 and May 2013 at three sites in RNP. Table includes the percentage of foraging bouts accounted for by each plant taxon overall (% All) and for each site (TK = Talatakely, VL = Valohoaka, VT = Vatoharanana). Plant parts/species included in color modeling analyses are highlighted in grey. Ripeness of fruit is identified in bold if it was consumed in >50% of fruit foraging bouts when both unripe and ripe were consumed. Plant parts identified with "?" were present during a bout, but had not been directly observed to be consumed. (FR = Fruit, RFR = Ripe Fruit, MR = Mid-Ripe Fruit, UFR = Unripe Fruit, BD = Flower Bud, FL = Flower, YL = Young Leaf, ML = Mature Leaf)

Family	Genus	Species	Vernacular	Plant part	% TK	% VL	% VT	% All	Spectral Data
Acanthaceae	*Ruellia*	*Sp*	Velatra	ML, YL	1.809	1.928	2.030	1.915	
Anacadiaceae	*Abrahamia*	*Sp*	Sandramy	FR, YL	0.201	0.526	1.523	0.684	FR
	Abrahamia	*Sp*	Sandramy fotsy	FR	0.000	0.263	0.000	0.103	
	Abrahamia	*Sp*	Sandramy mena	FR	0.000	0.526	0.127	0.239	
Annonaceae	*Ambavia*	*capuronii*	Ramiavotoloho	FR	0.000	0.351	1.269	0.479	
Aphloiaceae	*Aphloia*	*theiformis*	Fandramanana	RFR, **UFR**	2.010	0.175	0.000	0.752	RFR, UFR
Apocynaceae	*Carissa*	*Sp*	Fantsy	RFR, **UFR**	0.603	0.351	1.396	0.718	
	Plectaneia	*stenophilla*	Vahiakondro	UFR, ML, YL	1.106	0.438	0.381	0.650	
	Landolphia	*sp*	Vahiherotra	FR, ML, YL	1.910	1.315	0.381	1.265	
Arecaceae	*Dypsis*	*nodifera*	Sira	UFR	2.714	0.701	0.508	1.334	UFR
Asteraceae	*Vernonia*	*tanala*	Maranitratoraka	ML, YL	0.101	0.175	0.635	0.274	
Bignoniaceae	*Colea*	*lantziana*	Disohasaka	UFR	0.101	0.000	0.000	0.034	
Burseraceae	*Canarium*	*madagascariensis*	Ramy	FR	0.302	0.351	1.142	0.547	FR
Clusiaceae	*Haroungana*	*madagascariensis*	Harongana	RFR, MR, **UFR**	0.101	0.438	0.508	0.342	RFR, MR, UFR, ML
	Symphonia	*sp*	Kimba sp. 2 (small leaves)	ML, YL	0.000	0.088	0.127	0.068	
	Symphonia	*sp*	Kimba sp. 3	FL	0.000	0.000	0.127	0.034	
	Symphonia	*gymnoclada*	Kimba	FL, BD	0.000	1.490	1.396	0.958	FL, BD, ML
	Mammea	*bongo*	Natojabo	FL, BD, FR	0.000	11.656	15.863	8.824	FL, BD, FR, ML
	Mammea	*angustifolia*	Natovoraka	FR	0.201	0.000	0.381	0.171	FR
	Garcinia	*mangoriensis*	Tsikimbakimba	UFR	0.000	0.000	0.127	0.034	

	Garcinia	*aphanophlebia*	Voamalambotaholahy	UFR	0.000	0.088	0.127	0.068	
	Garcinia (Rheedia)	*megaphylla*	Voasavora	FL	0.000	0.000	0.381	0.103	
Cucurbitaceae	*Raphidocystis*	*Sp*	Vahimbarongy	ML, YL	0.503	1.315	1.142	0.992	YL, ML
	Ampelosicyos	*humblotii*	Voatakaboka	FR, ML	0.804	0.088	0.000	0.308	
Cunoniaceae	*Weinmannia*	*humblotii*	Sisitra	FL	0.000	0.175	0.127	0.103	
Dichapetalaceae	*Dichapetalum*	*chlorinum*	Vahindavenona	**RFR**, UFR	0.201	0.789	0.000	0.376	
Ebenaceae	*Diospyros*	*gricilipes*	Hazomainty	RFR?, UFR, FL	0.201	0.175	0.127	0.171	
Erythroxylaceae	*Erythroxylum*	*sphaerathum*	Malambovony	ML	0.000	0.000	0.127	0.034	
Euphorbiaceae	*Antidesma*	*petiolare*	Tsirivodrivotra	**RFR**, UFR	0.101	0.613	0.635	0.445	
	Drypetes	*madagascariensis*	Tsivalandrano	FR	0.101	0.263	0.127	0.171	
Fabaceae	*Strongylodon*	*craveniae*	Vahimberana	FL, YL	1.106	9.553	0.381	4.207	FL
	Albizia	*guimmifera*	Volomborona	FR	0.000	0.088	0.000	0.034	
Gentianaceae	*Anthocleista*	*amplexicolis*	Dendemy	FR, FL[1]	0.905	0.351	0.761	0.650	FR
Lauraceae	*Potameia*	*chartacea*	Sary	UFR	0.000	0.000	0.127	0.034	
	Cryptocarya	*sp*	Tavolo	UFR, Gall	0.201	0.438	0.254	0.308	UFR
	Cryptocarya	*acuminate*	Tavolo malady	RFR, UFR	1.206	0.175	0.508	0.616	
	Cryptocarya	*ovalifolia*	Tavolo manitra	FR, FL, YL	0.000	0.613	0.888	0.479	
	Cryptocarya	*thouvenotii*	Tavolo pina	FL, Gall	0.000	0.000	0.508	0.137	
	Cryptocarya	*ovalifolia*	Tavolo rano	**RFR**, UFR?	2.010	0.000	0.000	0.684	RFR, UFR
	Ocotea	*sp*	Varongy	UFR	0.101	0.263	0.000	0.137	
	Ocotea	*racemose*	Varongy fotsy	BD?	0.000	0.000	0.127	0.034	
	Ocotea	*sp*	Varongy mainty	UFR	0.000	0.000	0.127	0.034	
	Ocotea	*sp*	Varongy vazaha	UFR, BD?	0.000	0.175	0.000	0.068	
Laurentaceae	*Bakerella*	*clavata*	Tongolahy	FR, FL, YL, ML	1.608	0.701	1.777	1.300	
Malvaceae	*Grewia*	*apetala*	Hafipotsy	FR	1.005	1.665	1.015	1.265	FR, ML
	Dombeya	*sp*	Hafitra	FL	0.000	0.000	0.127	0.034	
	Grewia	*bredifolia*	Hafitrataikalalao	FR	0.201	2.892	0.000	1.197	FR, ML
Melastomataceae	*Medinilla*	*sp*	Kalamasimbaraka	RFR, YL, ML	0.402	0.000	0.635	0.308	
Memecylaceae	*Memecylon*	*roseum*	Tomenjy	RFR?, UFR	0.000	0.175	0.127	0.103	

Mendonciaceae	*Mendocia*	*sp*	Vahivoraka sp. 1	**RFR**, UFR, YL, ML	1.709	3.067	3.934	2.839	RFR, ML
	Mendocia	*sp*	Vahivoraka sp. 2	FR	0.101	0.088	0.000	0.068	
Monimiaceae	*Tambourissa*	*thouvenotii*	Ambora	ML	0.201	0.175	0.000	0.137	
	Tambourissa	*purpurea*	Amboralahy	YL, ML	0.101	0.088	0.127	0.103	
Moraceae	*Ficus*	*lutea*	Amontana	FR	0.704	0.000	0.000	0.239	FR
	Streblus	*mauritianus*	Apaly	BD	0.000	0.088	0.000	0.034	
	Ficus	*pachyclada*	Apana	**RFR**, MR, UFR, YL	1.508	4.645	7.995	4.480	RFR, MR, UFR, ML
	Treculia	*africana*	Avoha	FR	0.000	0.088	0.635	0.205	
	Ficus	*politoria*	Famakilela	**RFR**, UFR, ML	1.307	1.840	1.396	1.539	RFR,UFR, ML
	Streblus	*dimepate*	Mahanoro	RFR?, UFR	0.402	0.000	0.000	0.137	
	Ficus	*rubra*	Nonoka	FR[2]	1.206	8.940	12.690	7.319	RFR, UFR, ML
	Ficus	*rubra*	Vahinonoka	RFR	0.201	0.175	0.000	0.137	
	Ficus	*tilifolia*	Voara	RFR, UFR	3.819	0.876	0.127	1.676	RFR
	Ficus	*tilifolia*	Voara be	RFR, UFR	0.603	0.351	0.635	0.513	RFR, UFR, ML
	Ficus	*tilifolia*	Voara tenany	RFR, UFR	0.905	0.526	0.254	0.581	RFR, UFR, ML
	Ficus	*botryoides*	Voararano	**RFR**, UFR, YL, Gall	2.714	0.701	1.269	1.539	RFR, UFR, YL, ML
Myrsinaceae	*Oncostemum*	*nervosum*	Kalafambakaka	**RFR**, ML, YL, BD, FL	5.327	2.980	2.919	3.762	RFR, BD, FL, YL, ML
	Oncostemum	*spp*	Kalafana Unspecified[3]	RFR, **UFR**, BD, FL, YL, ML	2.613	3.856	1.396	2.770	
	Oncostemum	*botryoides*	Kalafana Big	UFR, FL, BD, YL, Petiole, ML	0.101	3.944	2.411	2.223	BD, FL, Petiole, ML
	Oncostemum	*leprosum*	Kalafana Small	RFR, **UFR**, BD, YL, ML	2.111	1.928	1.015	1.744	RFR, UFR, ML, YL
	Embellia	*sp*	Kalamasina	UFR, BD, YL?, ML	0.201	0.175	0.635	0.308	
Myrtaceae	*Psidium*	*cattleianum*	Guava	RFR, **UFR**	21.407	0.000	0.000	7.285	RFR, UFR, ML
	Syzygium	*danguyanum*	Rotra	UFR, FL, BD, YL	0.402	0.613	0.761	0.581	UFR, ML
	Eugenia	*louvelii*	Voabe	RFR	0.101	0.000	0.000	0.034	

	Syzygium	*jambos*	Zamborozano	FL	0.101	0.000	0.000	0.034	
Oleaceae	*Noronhia*	*grandifolia*	Solaitra fotsy	UFR	0.000	0.000	0.127	0.034	
Pandanaceae	*Pandanus*	*leptopodus*	Tsirika	RFR, **UFR,** FL	8.442	0.263	0.000	2.975	UFR, FL, ML
	Pandanus	*sp*	Vakoana	RFR	0.201	0.000	0.127	0.103	
Passifloraceae	*Passiflora*	*edulis*	Kilelaka	RFR, **UFR**	0.000	0.000	1.015	0.274	
	Adenia	*sp*	Vahimavo	FR, YL, ML	1.106	0.438	0.888	0.787	
Rhamnaceae	*Gouania*	*mauritiana*	Vahimpisorona	FR, FL	1.106	0.351	0.635	0.684	
Rubiaceae	*Mussaenda*	*arcuate*	Anambahy	RFR, UFR, ML	2.010	0.263	0.127	0.821	UFR, ML
	Gaertnera	*phyllostachya*	Bararata	UFR	0.603	0.000	0.000	0.205	
	Psychotria	*mandrarensis*	Fanorafa	RFR, UFR	0.603	0.438	2.030	0.923	
	Canthium	*spp*	Fatsikahitra Unspecified[3]	FR, UFR, FL	1.608	3.330	2.792	2.599	
	Canthium	*sp*	Fatsikahitra sp. 1	FR	2.915	0.964	1.396	1.744	FR, ML
	Canthium?	*sp?*	Fatsikahitra sp. 2	FR	0.000	0.000	0.761	0.205	
	Canthium?	*sp?*	Fatsikahitra sp. 3	FR	0.201	0.000	0.000	0.068	
	Canthium?	*sp?*	Fatsikahitra sp. 4	FR	0.000	0.088	0.000	0.034	
	Canthium?	*sp?*	Fatsikahitra sp. 5	FR	0.000	0.000	0.761	0.205	
	Canthium?	*sp?*	Fatsikahitra sp. 6	FR	0.302	0.175	0.127	0.205	
	Canthium?	*sp?*	Fatsikahitra sp. 7	**RFR**, UFR	0.101	0.000	0.888	0.274	RFR, UFR
	Canthium?	*sp?*	Fatsikahitra sp. 8	RFR, UFR	0.402	0.175	0.127	0.239	
	Canthium?	*sp?*	Fatsikahitra sp. 9	FR	0.302	0.088	0.888	0.376	
	Canthium?	*sp?*	Fatsikahitra sp. 10	FR	0.101	0.000	0.000	0.034	
	Psychotria	*sp*	Fohaninasity	**RFR**, MR, UFR	1.608	0.000	0.635	0.718	RFR, MR, UFR, ML
	Danais	*rhamnifolia*	Vahitamboro	RFR, UFR	0.503	0.088	0.127	0.239	
	Gyrostipula	*foveolata*	Valotra	FR	0.201	0.438	0.127	0.274	
	Breonia	*sp*	Voakringy	FR	0.000	0.000	0.254	0.068	
Rutaceae	*Toddalia*	*asiatica*	Anakatsimba	RFR, UFR	0.101	0.613	0.000	0.274	RFR, UFR, ML
Salicaceae	*Ludia*	*scolopioides*	Faritraty	RFR, UFR?	0.101	0.263	0.000	0.137	RFR, UFR
Sapindaceae	*Allophylus*	*cobe*	Dikana	**RFR**, UFR	0.101	2.191	1.015	1.163	RFR, UFR, ML

	Tina	*striata*	Lanary	RFR?, UFR, FL	0.000	0.263	1.396	0.479	
	Plagioscyphus	*louvelii*	Lanary mainty	FR	0.402	0.088	0.000	0.171	
Sapotaceae	*Sideroxylon*	*betsimisarakum*	Nato sp.	UFR, FL	0.000	0.088	0.381	0.137	
	Chrysophyllum	*boivinianum*	Rahiaka	FR	0.603	0.964	1.650	1.026	FR, ML
Smilacaceae	*Smilax*	*craussiana*	Roindambo	RFR, **UFR**, YL	0.704	0.351	0.127	0.410	RFR, UFR, ML
Violaceae	*Sauvagesia*	*erecta*	Hazotana	RFR, UFR?	1.106	0.701	0.000	0.650	
	Sauvagesia	*sp*	Hazotana small	RFR	0.201	0.000	0.000	0.068	
Vitaceae	*Cissus*	*pileata*	Vahirano	RFR, UFR	4.020	9.904	4.442	6.430	RFR, UFR, ML
Unknown			Mushroom	Mushroom	0.603	0.613	0.761	0.650	
Unknown			Vahi sp. 1	FR	0.000	0.175	0.000	0.068	
Unknown			Vahi sp. 2	FR	0.101	0.000	0.000	0.034	
Unknown			Vahi sp. 3	ML	0.000	0.088	0.000	0.034	
Unknown			Voamasoandro	FR	0.000	0.000	0.127	0.034	
Unknown			Zahatsifady	FR	0.000	0.088	0.000	0.034	

[1]Dendemy flowers were observed to be tasted (placed into the mouth) but not consumed.
[2]During behavioral data collection, ripeness of fruit consumed could not be accurately determined for this species. However, when collected ripe fruit could be distinguished from unripe fruit based on differences in size and color.
[3]During the study, the terms "Fatsikahitra" and "Kalafana" were used to describe multiple plant taxa. Once this was noted, distinctions were made. Therefore, prior to the distinctions, these terms may include multiple taxa. However, "Fatsikahitra Unspecified" is likely overrepresented by Fatsikahitra sp. 1, Fatsikahitra sp. 7, and Fatsikahitra sp. 8. "Kalafana Unspecified" is likely overrepresented by Kalafana Small.

Table 4.3 Descriptive statistics of chromaticities and luminance of food items and mature leaves (upper and lower leaves) for a trichromatic *Eulemur*. Data are presented for both illumination conditions.

	Red-Green Chromaticity							Blue-Yellow Chromaticity						Luminance					
Day																			
	N	**Mean**	**SD**	**SE**	**25th%**	**Mdn**	**75th%**	**Mean**	**SD**	**SE**	**25th%**	**Mdn**	**75th%**	**Mean**	**SD**	**SE**	**25th%**	**Mdn**	**75th%**
Food items	72	0.523	0.024	0.003	0.506	0.515	0.533	0.157	0.090	0.011	0.089	0.137	0.199	3.321	0.280	0.033	3.127	3.330	3.515
Upper leaves	29	0.497	0.002	0.000	0.496	0.497	0.498	0.103	0.049	0.009	0.063	0.105	0.133	2.920	0.108	0.020	2.837	2.919	2.980
Lower Leaves	29	0.505	0.007	0.001	0.502	0.503	0.507	0.109	0.040	0.007	0.083	0.101	0.145	3.249	0.174	0.032	3.114	3.282	3.392
Dusk																			
Food items	72	0.527	0.023	0.003	0.511	0.518	0.536	0.171	0.096	0.011	0.099	0.151	0.221	3.280	0.294	0.035	3.094	3.286	3.472
Upper leaves	29	0.504	0.001	0.000	0.503	0.504	0.505	0.109	0.051	0.010	0.067	0.111	0.140	2.891	0.106	0.018	2.807	2.894	2.968
Lower Leaves	29	0.510	0.006	0.001	0.507	0.508	0.512	0.115	0.043	0.008	0.088	0.107	0.152	3.215	0.176	0.033	3.077	3.248	3.360

Table 4.4 Descriptive statistics of chromatic contrasts of food items against their leaf backgrounds (upper and lower leaves) for the two dichromatic phenotypes for *Eulemur*. Data are presented for both illumination conditions and for the data set including all food items as well as the reduced ripe fruit only data set

Background	**Dichromat L Opsin**						**Dichromat M Opsin**					
	Mean	**SD**	**SE**	**25th %**	**Median**	**75th %**	**Mean**	**SD**	**SE**	**25th %**	**Median**	**75th %**
Day												
All Food Items (*N* = 72)												
Upper leaves	0.079	0.069	0.008	0.027	0.058	0.117	0.092	0.082	0.010	0.028	0.069	0.125
Lower Leaves	0.069	0.067	0.008	0.020	0.045	0.096	0.081	0.079	0.009	0.028	0.058	0.104
Ripe Fruit (*N* =21)												
Upper leaves	0.087	0.067	0.015	0.039	0.060	0.128	0.108	0.079	0.017	0.049	0.103	0.147
Lower leaves	0.082	0.077	0.017	0.031	0.051	0.099	0.102	0.087	0.019	0.041	0.078	0.145
Dusk												
All Food Items (*N* = 72)												
Upper leaves	0.086	0.075	0.009	0.029	0.073	0.120	0.102	0.091	0.011	0.031	0.074	0.137
Lower Leaves	0.076	0.072	0.008	0.025	0.055	0.105	0.090	0.087	0.010	0.032	0.063	0.121
Ripe Fruit (*N* =21)												
Upper leaves	0.097	0.070	0.015	0.046	0.082	0.127	0.123	0.083	0.018	0.057	0.123	0.169
Lower leaves	0.092	0.079	0.017	0.036	0.064	0.126	0.116	0.090	0.020	0.045	0.092	0.162

Table 4.5 Descriptive statistics of luminance contrasts of food items against their leaf backgrounds (upper and lower leaves) for the two dichromatic phenotypes for *Eulemur*. Data are presented for both illumination conditions and for the data set including all food items as well as the reduced ripe fruit only data set

Background	Dichromat L Opsin						Dichromat M Opsin					
	Mean	**SD**	**SE**	**25th %**	**Median**	**75th %**	**Mean**	**SD**	**SE**	**25th %**	**Median**	**75th %**
Day												
All Food Items (*N* = 72)												
Upper leaves	0.188	0.191	0.022	0.050	0.143	0.253	0.170	0.182	0.022	0.044	0.127	0.229
Lower Leaves	0.140	0.162	0.019	0.039	0.098	0.194	0.132	0.154	0.018	0.033	0.101	0.184
Ripe Fruit (*N* =21)												
Upper leaves	0.204	0.280	0.061	0.016	0.123	0.251	0.185	0.273	0.060	0.019	0.083	0.219
Lower leaves	0.182	0.240	0.052	0.039	0.097	0.216	0.174	0.234	0.051	0.046	0.108	0.215
Dusk												
All Food Items (*N* = 72)												
Upper leaves	0.169	0.179	0.021	0.044	0.124	0.225	0.153	0.170	0.020	0.036	0.113	0.208
Lower Leaves	0.129	0.153	0.018	0.034	0.092	0.177	0.121	0.145	0.017	0.033	0.090	0.169
Ripe Fruit (*N* =21)												
Upper leaves	0.185	0.269	0.059	0.012	0.099	0.219	0.169	0.261	0.057	0.027	0.058	0.189
Lower leaves	0.170	0.232	0.051	0.036	0.103	0.204	0.162	0.226	0.049	0.038	0.101	0.193

Chapter 5

Color vision in *Eulemur rubriventer* and its implications for understanding color vision evolution in lemurs

The overall goal of this dissertation was to identify potential mechanisms underlying color vision evolution in lemurs. In so doing, this dissertation used molecular methods to characterize color vision in a population of red-bellied lemurs (*Eulemur rubriventer*) in Ranomafana National Park (RNP), southeastern Madagascar, and then explored potential nonadaptive and adaptive explanations to account for the type of color vision observed in this population (chapters 2-4).

Results of this dissertation indicate that the color vision of *E. rubriventer* in RNP is unique among other species/populations of *Eulemur*, for which data are available (Tan and Li 1999; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep; chapter 2). Specifically, red-bellied lemurs in RNP (N = 87 individuals) exhibit a single M/L opsin gene variant (based on sequence variation at site 285 located in exon 5), indicating they are strictly dichromatic. All individuals yielded amino acid threonine at site 285, which suggests the peak spectral sensitivity of the M/L opsin in this population is 558 nm (identified as the long-wavelength or L opsin; chapter 2). Dichromacy with the L opsin contrasts with other *Eulemur* species/populations, which appear to be either dichromatic with the medium-wavelength or M opsin (peak spectral sensitivity at 543 nm), or polymorphic, having both M and

L opsins present within a population (Tan and Li 1999; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep).

Populations that are polymorphic for the M/L opsin are identified as having polymorphic trichromatic color vision (e.g., Mollon et al. 1984; Jacobs and Neitz 1987; Jacobs et al. 1993; Tan and Li 1999). That is, allelic variation of the X-linked M/L opsin gene provides some females with the potential for trichromatic color vision, while all males and other females are dichromatic (e.g., Mollon et al. 1984; Jacobs and Neitz 1987; Jacobs et al. 1993; Tan and Li 1999). This condition is also found among most New World monkeys (e.g., see Jacobs 2007 and Kawamura et al. 2012 for reviews; de Lima et al. 2015) and has long been thought to be adaptive (e.g., Mollon et al. 1984; Surridge and Mundy 2002; Surridge et al. 2003). Results of this dissertation suggest that polymorphic color vision was likely the ancestral *Eulemur* condition, and *E. rubriventer* in RNP likely lost the polymorphism (chapter 2), begging the question of why a potentially advantageous trait would be lost from a population.

This dissertation provides two possible, non-mutually exclusive, explanations for loss of color vision variation and fixation of the L opsin in *E. rubriventer*. First, chapter 3 explored the potential for a recent genetic bottleneck in the population of red-bellied lemurs in RNP. Past and ongoing threats to lemurs in Madagascar, such as habitat loss and hunting (e.g., Harper et al. 2007; Schwitzer et al. 2014), have led to large-scale population declines, and genetic bottlenecks have been reported for a number of lemur species (e.g., Fredsted et al. 2007; Olivieri et al. 2008; Craul et al. 2009; Brenneman et al. 2012; Parga et al. 2012; Holmes et al. 2013). Genetic bottlenecks provide a potential nonadaptive mechanism through which genetic variation can be lost, as the impact of genetic drift increases in small populations and can even result in loss of advantageous alleles (Futuyma 1998).

Using genotypes for 7 variable microsatellite loci from 55 adult red-bellied lemurs, this study found mixed support for a genetic bottleneck in the RNP population. Specifically, results indicate that this population exhibits significant heterozygosity excess, which is potentially indicative of a genetic bottleneck, but does not exhibit significantly low *M* ratios (i.e., ratio of number of alleles to range in allele size), which is not indicative of a genetic bottleneck. Given mixed results, the potential for a genetic bottleneck cannot be rejected, suggesting that polymorphic trichromatic color vision may have been lost through nonadaptive mechanisms in the population of *E. rubriventer* in RNP. Under this scenario, fixation of the L opsin would likely represent random allele loss due to genetic drift.

Chapter 4 explored potential adaptive explanations for dichromatic color vision in red-bellied lemurs. In particular, this study focused on foraging hypotheses that could result in relaxed or potentially even disruptive selection on polymorphic trichromacy and/or directional selection favoring the L opsin in red-bellied lemurs.

Trichromatic color vision has long been thought to be adaptive to foraging on many food items, particularly red food items, such as ripe fruit and young leaves (e.g., Mollon 1989; Osorio and Vorobyev 1996; Dominy and Lucas 2001; Bunce 2011; but see Melin et al. 2014). Using a color modeling approach, chapter 4 explored the possibility that food items consumed by *E. rubriventer* are primarily "dull" in coloration (e.g., green and brown), as had been suggested for many lemur species (Dew and Wright 1998; Birkinshaw 2001). If trichromacy offers little advantage in detecting many food items, this could result in relaxed selection to maintain polymorphic trichromatic color vision. The results indicate that trichromatic color vision would offer a potential advantage in detecting many food items consumed by *E. rubriventer*,

particularly many ripe fruits, but many food items are also chromatically conspicuous to dichromats.

This study also found that dichromatic color vision with the L opsin may be adaptive for foraging using luminance cues. Specifically, when color and luminance contrasts were compared between the two dichromatic phenotypes (L opsin vs. M opsin), luminance contrasts were significantly greater for dichromats with the L opsin. This result is interesting, as it has been suggested that chromatic information can actually interfere with luminance vision (Osorio et al. 1998). Accordingly, if *E. rubriventer* relies heavily on luminance cues during foraging, there may have been relaxed selection to maintain polymorphic trichromacy (or even selection against trichromatic color vision), which would lead to loss of color vision variation. At the same time, fixation of the L opsin may be adaptive for maximizing luminance contrast and may have been driven to fixation through directional selection, suggesting a potential adaptive explanation for color vision in *E. rubriventer*.

In sum, this dissertation has explored color vision evolution in red-bellied lemurs in RNP and has identified potential adaptive and nonadaptive mechanisms to explain loss of polymorphic trichromacy and fixation of the L opsin in this population. Ultimately, by using *E. rubriventer* as a case study, this research provides multiple scenarios for understanding color vision evolution in lemurs, but in the end, it has generated more questions than answers. Therefore, this dissertation concludes with suggestions for future research.

Understanding color vision evolution in lemurs

Comparative analyses. The red-bellied lemur population in RNP represents one datum within a lineage that exhibits a large amount of variation in color vision capacities (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Although color vision has been characterized for multiple lemurs (see chapter 2), they represent less than a third of the potential lemur species currently identified (Mittermeier et al. 2010). Furthermore, many samples represent single populations or captive individuals (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). These factors are highly relevant, because color vision variation observed in lemurs occurs across and within families, genera, and species (Tan and Li 1999; Jacobs et al. 2002; Jacobs and Deegan 2003; Tan et al. 2005; Bradley et al. 2009; Leonhardt et al. 2009; Veilleux and Bolnick 2009; Bradley et al. in prep). Moving forward, it will be important to characterize color vision in additional taxa from natural populations, as many remaining questions may be better addressed using a broad comparative framework.

For example, in chapter 2, this study used ancestral state estimations to evaluate color vision evolution in lemurs and demonstrated that polymorphic trichromacy was likely the ancestral condition for the genus *Eulemur*. Results also indicate that polymorphic trichromacy may have arisen earlier in lemurs, which, overall, suggests multiple losses of polymorphic trichromacy. This latter result remains more equivocal and further analysis will benefit from a larger sample size. As it stands, an early evolution of polymorphic trichromacy in lemurs would suggest this trait may have evolved under conditions seemingly less compatible with trichromatic

color vision (i.e., nocturnality; Santini et al. 2015). That said, multiple factors have been identified as potential important evolutionary pressures on primate color vision that include activity pattern, but also diet and habitat, among others (e.g., Mollon 1989; Jacobs et al. 1996; Osorio and Vorobyev 1996; Melin et al. 2013; Veilleux et al. 2013). Examining the evolutionary histories of these traits across lemurs and how they relate to the evolutionary history of color vision may help identify broad patterns associated with the variety of color vision capacities observed today and potentially require reevaluation of long-held hypotheses for color vision evolution in primates (e.g., Melin et al. 2013).

At the same time, this dissertation has identified a potential caveat in comparative analyses. Previous research on lemur color vision has found that different populations of the same species can vary in their color vision capacities (Bradley et al. 2009; Bradley et al. in prep), and the evolutionary mechanisms acting on these populations have and will continue to vary. At present, this dissertation has identified *E. rubriventer* as dichromatic and monomorphic for the L opsin, but this is based on M/L opsin allele frequencies from a single population (chapter 2). Furthermore, results from chapter 3 suggest that this population may have experienced a recent genetic bottleneck. Therefore, it is possible that loss of polymorphic trichromacy and fixation of the L opsin is the result of genetic drift rather than adaptive processes. To further clarify the roles of nonadaptive and adaptive mechanisms in this taxon, as well as other lemur species, it will be necessary to characterize color vision in multiple populations and consider their different evolutionary histories that might impact observed genetic variation.

Although nonadaptive mechanisms may play a role in color vision evolution, this dissertation does not downplay the potential role of adaptive processes. In accordance with the long-standing hypothesis that color vision variation represents adaptations to foraging (e.g.,

Mollon et al. 1984; Mollon 1989; Osorio and Vorobyev 1996; Dominy and Lucas 2001; Melin et al. 2007; Veilleux et al. 2014), chapter 4 found that dichromatic color vision in *E. rubriventer* may be adaptive for foraging using luminance cues. A reliance on luminance vision during foraging could lead to relaxed selection or disruptive selection on polymorphic trichromacy and ultimately result in loss of allelic variation. Consequently, monomorphism for the L opsin may still result from random allele loss due to either relaxed or disruptive selection, but chapter 4 also found that luminance contrasts are significantly greater for the L opsin compared to the M opsin. This suggests that there may have been directional selection favoring the L opsin for foraging using luminance vision.

Here again, disentangling the roles of selection and drift could benefit from comparative analyses. For example, dichromatic color vision with the L opsin is found in other lemur taxa, such as *Avahi*, *Hapalemur*, and *Microcebus* (Tan and Li 1999; Tan et al. 2005; Bradley et al. 2009; Veilleux et al. 2014; Bradley et al. in prep). It has been suggested that color vision in *Avahi* may be adaptive for foraging using chromatic cues, given chromatic contrasts were greater for the L opsin compared to the M opsin for many food items consumed by *Avahi* (Veilleux et al. 2014). However, luminance contrasts were also greater for the L opsin (Veilleux et al. 2014), and similar studies have yet to be published on species that are dichromatic with the M opsin for comparison. Quantifying foraging cues for other taxa with different color vision phenotypes may help clarify potential adaptive roles of color vision across lemurs.

Experimental studies. Ultimately, there are limitations to what the above avenues for future research can tell us about color vision evolution in lemurs, because, in the end, such

studies can only provide patterns of association. Given these limitations, it will be important to address many color vision questions in experimental settings.

First and foremost, it is important to acknowledge that color vision is fundamentally a behavioral phenomenon. This dissertation and many studies of primate color vision rely on the assumption that color vision genotype reflects color vision phenotype. That is to say that primates identified genetically as monochromatic, dichromatic, and trichromatic can actually make (or not make) the chromatic discriminations implied from these characterizations. Although many controlled experimental studies suggest this assumption is warranted, most studies demonstrating the link between genotype and phenotype have been conducted on haplorhine primates (e.g., *Macaca*: Devalois et al. 1974; Bowmaker et al. 1980; *Saimiri*: Jacobs 1984; Jacobs and Blakeslee 1984; *Saguinus*: Jacobs et al. 1987). Research on lemurs remains limited and in one early study of *Lemur catta*, it was found that dichromats actually made chromatic discriminations suggestive of trichromatic color vision (Blakeslee and Jacobs 1985). This result was attributed to the potential contribution of rod signals to color vision (Jacobs and Deegan 1993), which is an area of research that is not well-understood. However, if rods regularly contribute to color vision in some lemurs, it may very well be that polymorphic trichromacy provides no additional advantages in these taxa, which could result in relaxed selection without sacrificing potential advantages of trichromatic color vision.

Along similar lines, in the only experimental study examining the influence of trichromatic color vision on foraging behavior in lemurs, results were equivocal (Leonhardt et al. 2009). Trichromatic red ruffed lemurs (*Varecia rubra*) retrieved red food faster than green food against a green background, but a similar pattern was not found in the single trichromatic sifaka (*Propithecus coquereli*) tested, and dichromatic collared brown lemurs (*Eulemur collaris*) were

the most efficient foragers overall (Leonhardt et al. 2009). Additional experimental studies that evaluate color vision behavior in lemurs will be important to verify the genotype-phenotype link that has been demonstrated in many New World monkeys and catarrhines.

Color vision research will also benefit from a greater understanding of sensory cues used during fitness-related tasks. For example, while foraging hypotheses are commonly invoked to explain color vision variation (e.g., Mollon et al. 1984; Mollon 1989; Osorio and Vorobyev 1996; Dominy and Lucas 2001; Melin et al. 2007; Veilleux et al. 2014), the sensory cues used during foraging are likely complex and are not well-understood in primates, lemurs being no exception (e.g., Dominy et al. 2001). For example, in haplorhines, foraging advantages (i.e., increased foraging efficiency) using color cues have been found in trichromatic individuals (Caine and Mundy 2000; Smith et al. 2003), but in wild primate populations, there is only limited support for the influence of chromatic cues on foraging behavior (Hiramatsu et al. 2009; Melin et al. 2009) and some support for the potential importance of achromatic cues (Melin et al. 2007; Hiramatsu et al. 2008, 2009). However, studies in wild populations may be confounded by additional factors, such as olfactory cues, which have also been shown to be important to foraging primates (Hiramatsu et al. 2009). Similar studies examining the influence of sensory cues on foraging efficiency in wild lemur populations have yet to be published.

Experimental research on lemurs, however, suggests that visual cues (including chromatic cues) may be important during foraging, but olfactory cues may also be important to some lemurs (Siemers et al. 2007; Pipe et al. 2008; Rushmore et al. 2012; Valenta et al. 2013). Furthermore, variation in reliance on particular sensory cues may be related to variation in species' foraging ecologies (Rushmore et al. 2012). Chapter 4 of this dissertation suggests that luminance cues may be more important to *E. rubriventer* than are chromatic cues during

foraging, but ultimately, this must be tested in controlled experimental settings to determine if this taxon can and does make foraging decisions based on luminance cues. Analyses that also examine how multiple cues interact (e.g., chromatic, luminance, and olfactory cues) will be informative for identifying specific foraging conditions that might favor different color vision capacities under natural conditions.

Molecular studies. Additional molecular analyses will provide further insight into the evolutionary history of color vision in lemurs. For example, as stated above, chapter 2 evaluated the evolutionary history of polymorphic trichromacy across lemurs, but ancestral state estimations were based on the assumption that M opsins and L opsins identified across taxa are homologous. Results, though equivocal, suggested that polymorphic trichromacy may have a single early origin in lemurs, and although such a result would represent a more parsimonious scenario than multiple origins, it is possible that polymorphic trichromacy arose independently in different lineages. To further differentiate between these hypotheses, it will be important to look more broadly at sequence variation across intron and exon regions of the M/L opsin gene. For example, Melin et al. (2013) used sequence variation of the M/L opsin gene to infer the ancestral color vision state of crown tarsiers. The color vision capacities of three tarsier species have been identified as dichromatic, but species vary in the spectral sensitivities of their M/L opsins (Melin et al. 2013). That is, similar to many lemurs, the L opsin appears to be fixed in some species (*Tarsier tarsier*, *T. syrichta*), while the M opsin appears to be fixed in another (*T. bancanus*; Melin et al. 2013). Phylogenetic trees based on variation in M/L opsin intron regions and synonymous sites of M/L opsin exons from each species were in accordance with currently accepted phylogenetic relationships of tarsiers, while non-synonymous sites of exons grouped

the L opsin of more distantly related *T. tarsier* with that of *T. syrichta*, suggesting polymorphic trichromacy was the ancestral condition of crown tarsiers (Melin et al. 2013). Similar studies in lemurs may help identify whether the M and L opsin distribution across taxa represents a single origin of polymorphic trichromacy or independent evolutionary events.

Molecular studies of New World primates have also identified signatures of balancing selection to maintain polymorphic trichromatic color vision in some species (Hiwatashi et al 2010; Kawamura et al. 2012). Such studies compared sequence variation of M/L opsin genes to sequence variation of neutral references and identified signatures of positive selection on M/L opsin genes in some species (Hiwatashi et al 2010; Kawamura et al. 2012). Similar studies across lemur populations would help evaluate whether or not M/L opsin variation is under positive selection, providing further insight into the potential roles of adaptive and nonadaptive mechanisms in the evolution of color vision in lemurs.

Finally, and relevant to the discussion above regarding comparative analyses, it is important to recognize that the diversity of lemurs present today does not represent the full range of diversity present just a couple thousand years ago. At least 17 species of lemur have recently gone extinct (Goodman and Jungers 2014). These taxa appear to be largely diurnal but exhibit variation in their behavioral ecologies (e.g., diet; Godfrey et al. 2006). Advances in molecular techniques have made it possible to obtain genomic information from subfossil remains (e.g., Kistler et al. 2015), which may ultimately allow characterization of color vision capacity in extinct taxa. Including subfossil lemurs in a comparative framework would provide a more complete picture of association patterns between color vision and potential selective pressures, such as diet and activity pattern, across lemurs.

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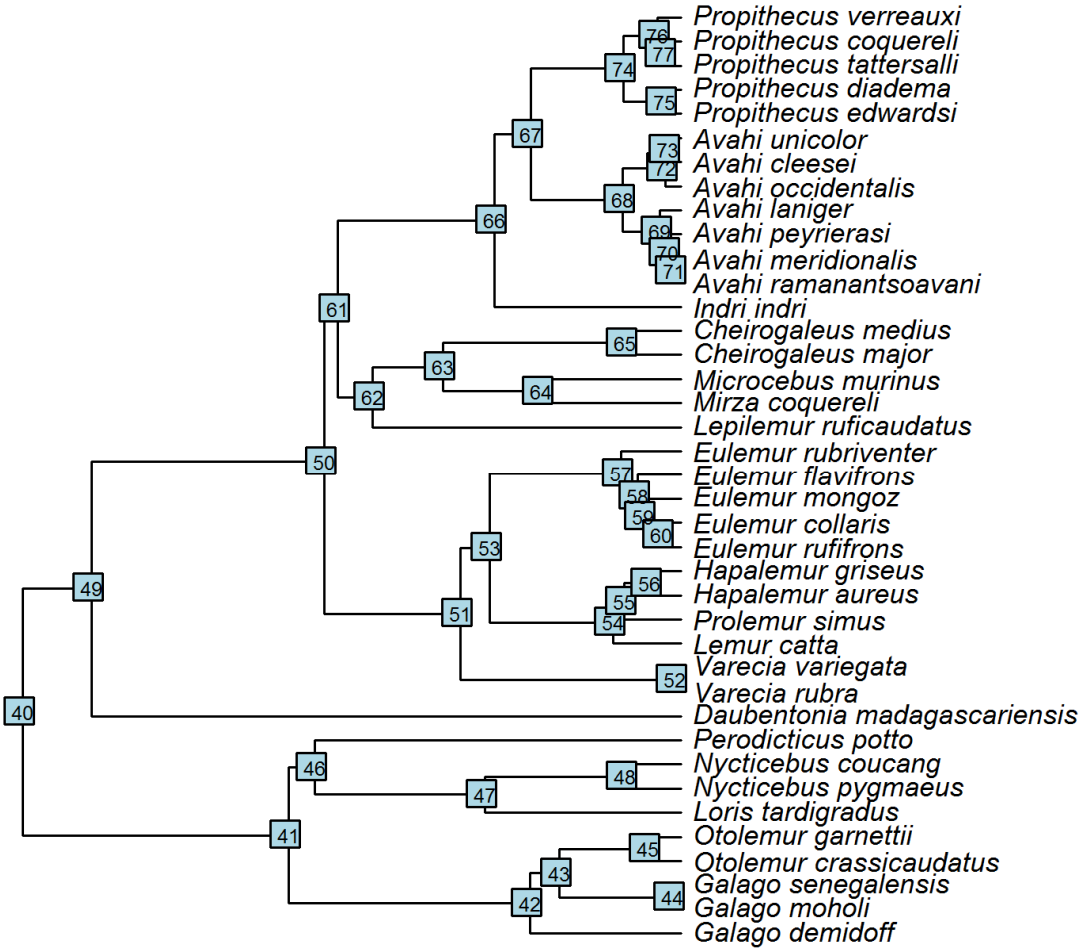
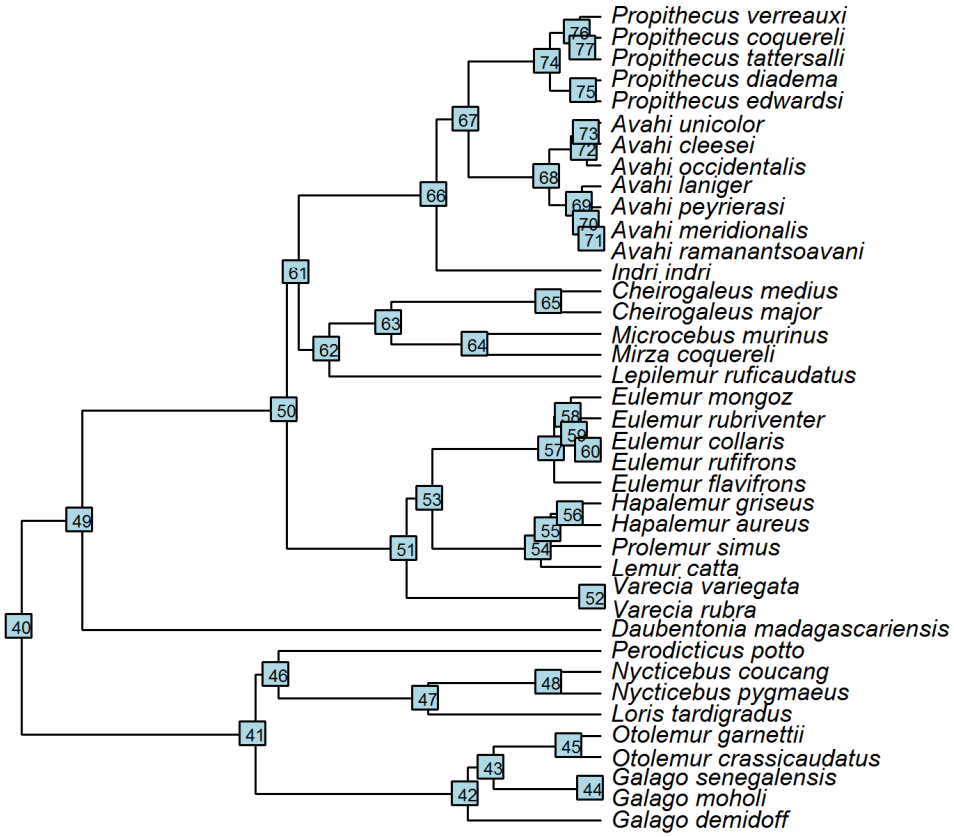
Appendix 1. SEA phylogeny used in maximum likelihood analyses of ancestral color vision phenotype in strepsirrhines. Nodes are labeled in blue boxes. The table below the phylogeny includes the relative proportions for each color vision state (1 = monomorphic for the M opsin, 2 = polymorphic, 3 = monomorphic for the L opsin) at each node for the ordered equal rates (ER) and better-fit ordered all-rates different (ARD) model**.** The state with the highest relative proportion is highlighted in gray.



	Color vision state											
	Ordered ER						Ordered ARD					
	L. catta coded as 1			*L. catta* coded as 2			*L. catta* coded as 1			*L. catta* coded as 2		
Node	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
40	0.510	0.326	0.165	0.583	0.314	0.102	0.000	0.464	0.535	0.000	0.521	0.479
41	0.804	0.175	0.021	0.879	0.115	0.006	0.858	0.113	0.030	0.868	0.110	0.022
42	0.957	0.043	0.001	0.978	0.022	0.000	0.962	0.036	0.002	0.966	0.033	0.001
43	0.973	0.027	0.000	0.987	0.013	0.000	0.963	0.036	0.001	0.967	0.032	0.001
44	1.000	0.000	0.000	1.000	0.000	0.000	0.998	0.002	0.000	0.998	0.002	0.000
45	0.996	0.004	0.000	0.998	0.002	0.000	0.981	0.019	0.000	0.984	0.016	0.000
46	0.818	0.164	0.018	0.890	0.105	0.005	0.856	0.118	0.026	0.869	0.112	0.019
47	0.894	0.101	0.005	0.940	0.058	0.001	0.913	0.077	0.010	0.919	0.073	0.008
48	0.984	0.015	0.000	0.992	0.008	0.000	0.967	0.032	0.000	0.971	0.029	0.000
49	0.474	0.340	0.187	0.539	0.343	0.118	0.000	0.364	0.636	0.000	0.436	0.564
50	0.229	0.474	0.297	0.201	0.589	0.210	0.000	0.118	0.882	0.000	0.207	0.793
51	0.115	0.503	0.382	0.062	0.597	0.340	0.000	0.140	0.860	0.000	0.199	0.801
52	0.000	0.999	0.001	0.000	0.999	0.000	0.000	0.998	0.002	0.000	0.998	0.002
53	0.091	0.492	0.417	0.040	0.562	0.397	0.000	0.137	0.863	0.000	0.181	0.819
54	0.033	0.336	0.631	0.001	0.244	0.755	0.000	0.152	0.848	0.000	0.091	0.909
55	0.002	0.114	0.884	0.000	0.061	0.939	0.000	0.038	0.962	0.000	0.023	0.977
56	0.000	0.013	0.987	0.000	0.005	0.995	0.000	0.004	0.996	0.000	0.003	0.997
57	0.056	0.679	0.265	0.031	0.704	0.265	0.000	0.499	0.501	0.000	0.543	0.457
58	0.109	0.876	0.015	0.072	0.917	0.011	0.000	0.946	0.054	0.000	0.950	0.050
59	0.251	0.746	0.002	0.216	0.783	0.001	0.000	0.986	0.014	0.000	0.986	0.014
60	0.285	0.715	0.000	0.244	0.756	0.000	0.000	0.999	0.001	0.000	0.999	0.001
61	0.227	0.480	0.293	0.199	0.596	0.206	0.000	0.126	0.874	0.000	0.217	0.783
62	0.264	0.472	0.265	0.242	0.571	0.187	0.000	0.229	0.771	0.000	0.320	0.680
63	0.233	0.468	0.299	0.217	0.544	0.239	0.000	0.293	0.707	0.000	0.372	0.628
64	0.023	0.230	0.747	0.014	0.220	0.766	0.000	0.080	0.920	0.000	0.113	0.887
65	0.951	0.049	0.001	0.963	0.036	0.000	0.944	0.055	0.002	0.953	0.046	0.001
66	0.065	0.546	0.389	0.043	0.664	0.292	0.000	0.193	0.807	0.000	0.273	0.727
67	0.025	0.536	0.439	0.014	0.639	0.347	0.000	0.194	0.806	0.000	0.262	0.738
68	0.000	0.028	0.972	0.000	0.025	0.975	0.000	0.011	0.989	0.000	0.014	0.986
69	0.000	0.001	0.999	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
70	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
71	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
72	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
73	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
74	0.006	0.962	0.031	0.003	0.979	0.018	0.000	0.914	0.086	0.000	0.926	0.074
75	0.001	0.998	0.001	0.000	0.999	0.001	0.000	0.995	0.005	0.000	0.996	0.004
76	0.000	0.999	0.001	0.000	1.000	0.000	0.000	0.997	0.003	0.000	0.997	0.003
77	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.999	0.001	0.000	0.999	0.001

Appendix 2. SMK phylogeny used in maximum likelihood analyses of ancestral color vision phenotype in strepsirrhines. Nodes are labeled in blue boxes. The table below the phylogeny includes the relative proportions for each color vision state (1 = monomorphic for the M opsin, 2 = polymorphic, 3 = monomorphic for the L opsin) at each node for the ordered equal rates (ER) and better-fit ordered all-rates different (ARD) model**.** The state with the highest relative proportion is highlighted in gray.

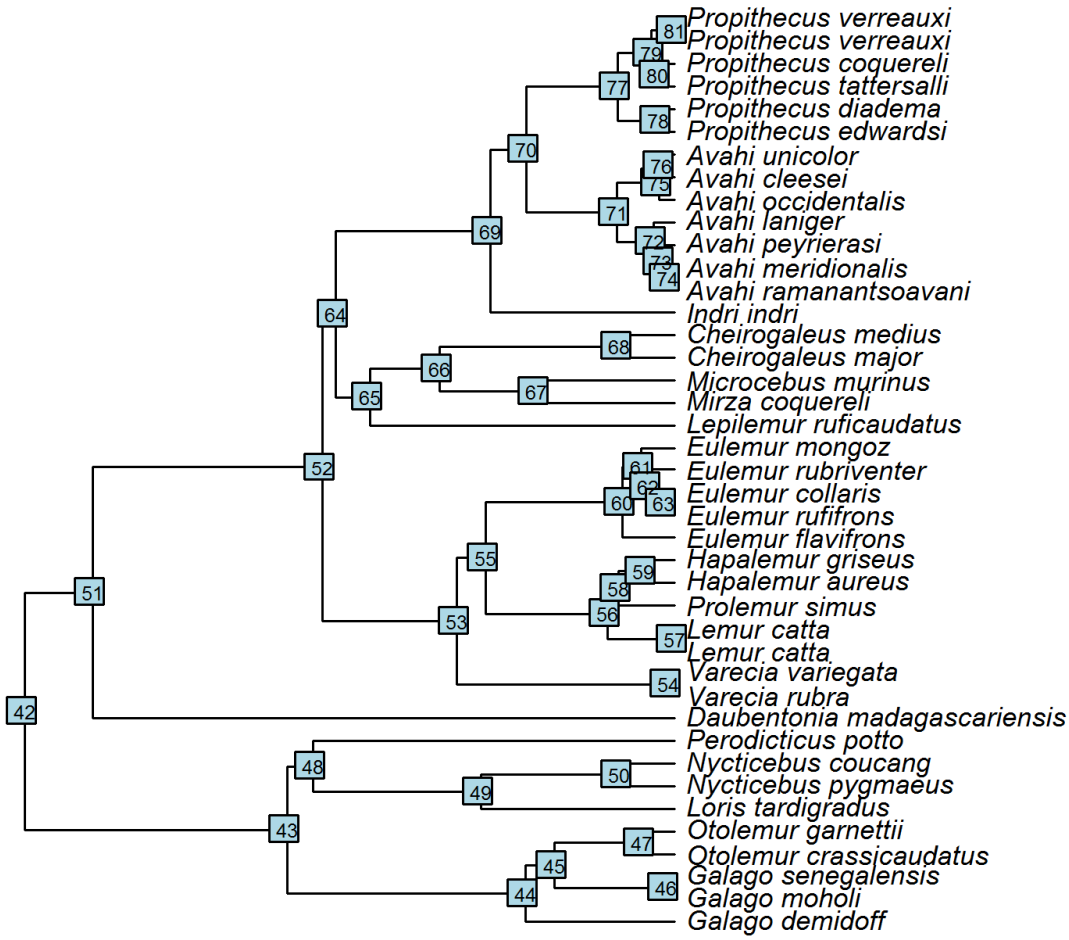


	Color vision state											
	Ordered ER						Ordered ARD					
	L. catta coded as 1			*L. catta* coded as 2			*L. catta* coded as 1			*L. catta* coded as 2		
Node	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
40	0.508	0.324	0.168	0.600	0.308	0.092	0.000	0.496	0.504	0.000	0.535	0.465
41	0.792	0.184	0.024	0.883	0.111	0.006	0.872	0.098	0.030	0.880	0.097	0.023
42	0.952	0.047	0.001	0.979	0.021	0.000	0.966	0.032	0.002	0.969	0.030	0.002
43	0.970	0.030	0.000	0.988	0.012	0.000	0.966	0.033	0.001	0.970	0.030	0.001
44	1.000	0.000	0.000	1.000	0.000	0.000	0.998	0.002	0.000	0.998	0.002	0.000
45	0.996	0.004	0.000	0.998	0.002	0.000	0.983	0.017	0.000	0.985	0.015	0.000
46	0.806	0.173	0.021	0.894	0.101	0.005	0.872	0.101	0.027	0.882	0.098	0.020
47	0.886	0.109	0.006	0.942	0.057	0.001	0.921	0.067	0.012	0.926	0.064	0.009
48	0.983	0.017	0.000	0.992	0.007	0.000	0.970	0.029	0.001	0.973	0.027	0.000
49	0.476	0.336	0.188	0.558	0.337	0.105	0.000	0.403	0.597	0.000	0.457	0.543
50	0.257	0.467	0.277	0.224	0.610	0.167	0.000	0.184	0.816	0.000	0.264	0.736
51	0.150	0.536	0.314	0.078	0.684	0.238	0.000	0.223	0.777	0.000	0.278	0.722
52	0.000	0.999	0.001	0.000	0.999	0.000	0.000	0.998	0.002	0.000	0.998	0.002
53	0.125	0.546	0.329	0.053	0.676	0.271	0.000	0.237	0.763	0.000	0.276	0.724
54	0.041	0.365	0.594	0.001	0.287	0.712	0.000	0.216	0.784	0.000	0.122	0.878
55	0.002	0.129	0.868	0.000	0.071	0.929	0.000	0.074	0.926	0.000	0.040	0.960
56	0.000	0.015	0.985	0.000	0.006	0.994	0.000	0.011	0.989	0.000	0.006	0.994
57	0.087	0.889	0.023	0.045	0.943	0.013	0.000	0.907	0.093	0.000	0.920	0.080
58	0.121	0.868	0.011	0.072	0.922	0.006	0.000	0.951	0.049	0.000	0.956	0.044
59	0.083	0.892	0.025	0.050	0.934	0.016	0.000	0.929	0.071	0.000	0.934	0.066
60	0.131	0.869	0.000	0.087	0.913	0.000	0.000	0.998	0.002	0.000	0.998	0.002
61	0.253	0.472	0.276	0.220	0.614	0.165	0.000	0.194	0.806	0.000	0.274	0.726
62	0.287	0.461	0.252	0.263	0.582	0.155	0.000	0.302	0.698	0.000	0.376	0.624
63	0.249	0.461	0.290	0.233	0.554	0.213	0.000	0.373	0.627	0.000	0.432	0.568
64	0.026	0.238	0.736	0.015	0.227	0.758	0.000	0.134	0.866	0.000	0.160	0.840
65	0.950	0.050	0.001	0.965	0.035	0.000	0.954	0.045	0.001	0.959	0.040	0.001
66	0.073	0.539	0.388	0.046	0.684	0.269	0.000	0.236	0.764	0.000	0.304	0.696
67	0.029	0.531	0.441	0.014	0.659	0.327	0.000	0.239	0.761	0.000	0.294	0.706
68	0.000	0.029	0.971	0.000	0.025	0.975	0.000	0.020	0.980	0.000	0.022	0.978
69	0.000	0.001	0.999	0.000	0.000	1.000	0.000	0.001	0.999	0.000	0.001	0.999
70	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
71	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
72	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
73	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
74	0.007	0.960	0.033	0.003	0.980	0.017	0.000	0.904	0.096	0.000	0.918	0.082
75	0.001	0.998	0.001	0.000	0.999	0.001	0.000	0.994	0.006	0.000	0.995	0.005
76	0.000	0.999	0.001	0.000	1.000	0.000	0.000	0.996	0.004	0.000	0.996	0.004
77	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.999	0.001	0.000	0.999	0.001

Appendix 3. SEA phylogeny used in maximum likelihood analyses of ancestral color vision phenotype in strepsirrhines. Two populations of *P. verreauxi* and *L. catta* are include in the phylogeny to examine the influence of population-level variation in color vision status on ancestral state estimations. Nodes are labeled in blue boxes. The table below the phylogeny includes the relative proportions for each color vision state (1 = monomorphic for the M opsin, 2 = polymorphic, 3 = monomorphic for the L opsin) at each node for the ordered equal rates (ER) and better-fit ordered all-rates different (ARD) model**.** The state with the highest relative proportion is highlighted in gray.

	Color vision state					
	Ordered ER			Ordered ARD		
Node	1	2	3	1	2	3
42	0.397	0.334	0.269	0.000	0.342	0.658
43	0.634	0.274	0.092	0.829	0.130	0.042
44	0.885	0.109	0.006	0.955	0.043	0.002
45	0.921	0.077	0.002	0.954	0.045	0.001
46	0.999	0.001	0.000	0.997	0.003	0.000
47	0.988	0.012	0.000	0.976	0.024	0.000
48	0.647	0.267	0.086	0.819	0.142	0.038
49	0.770	0.199	0.031	0.895	0.093	0.012
50	0.956	0.043	0.001	0.961	0.039	0.000
51	0.373	0.336	0.291	0.000	0.229	0.771
52	0.236	0.361	0.403	0.000	0.025	0.975
53	0.156	0.372	0.471	0.000	0.043	0.957
54	0.001	0.997	0.001	0.000	0.998	0.002
55	0.131	0.370	0.500	0.000	0.036	0.964
56	0.027	0.259	0.714	0.000	0.033	0.967
57	0.577	0.423	0.000	0.000	1.000	0.000
58	0.003	0.113	0.884	0.000	0.005	0.995
59	0.000	0.019	0.981	0.000	0.001	0.999
60	0.103	0.619	0.278	0.000	0.352	0.648
61	0.180	0.793	0.026	0.000	0.934	0.066
62	0.320	0.674	0.006	0.000	0.981	0.019
63	0.361	0.639	0.001	0.000	0.998	0.002
64	0.236	0.365	0.399	0.000	0.028	0.972
65	0.266	0.373	0.361	0.000	0.103	0.897
66	0.244	0.390	0.366	0.000	0.150	0.850
67	0.045	0.250	0.704	0.000	0.023	0.977
68	0.920	0.077	0.002	0.919	0.079	0.002
69	0.105	0.405	0.489	0.000	0.091	0.909
70	0.056	0.411	0.533	0.000	0.094	0.906
71	0.000	0.040	0.960	0.000	0.003	0.997
72	0.000	0.002	0.998	0.000	0.000	1.000
73	0.000	0.000	1.000	0.000	0.000	1.000
74	0.000	0.000	1.000	0.000	0.000	1.000
75	0.000	0.001	0.999	0.000	0.000	1.000
76	0.000	0.000	1.000	0.000	0.000	1.000
77	0.025	0.908	0.067	0.000	0.908	0.092
78	0.003	0.993	0.004	0.000	0.995	0.005
79	0.014	0.984	0.003	0.000	0.996	0.004
80	0.003	0.996	0.001	0.000	0.999	0.001
81	0.123	0.877	0.000	0.000	1.000	0.000

Appendix 4. SMK phylogeny used in maximum likelihood analyses of ancestral color vision phenotype in strepsirrhines. Two populations of *P. verreauxi* and *L. catta* are include in the phylogeny to examine the influence of population-level variation in color vision status on ancestral state estimations. Nodes are labeled in blue boxes. The table below the phylogeny includes the relative proportions for each color vision state (1 = monomorphic for the M opsin, 2 = polymorphic, 3 = monomorphic for the L opsin) at each node for the ordered equal rates (ER) and better-fit ordered all-rates different (ARD) model**.** The state with the highest relative proportion is highlighted in gray.



	Color vision state					
	Ordered ER			Ordered ARD		
Node	1	2	3	1	2	3
42	0.400	0.333	0.266	0.000	0.442	0.558
43	0.635	0.273	0.091	0.864	0.100	0.036
44	0.886	0.108	0.006	0.962	0.035	0.003
45	0.921	0.076	0.002	0.963	0.036	0.001
46	0.999	0.001	0.000	0.998	0.002	0.000
47	0.988	0.012	0.000	0.981	0.019	0.000
48	0.649	0.266	0.085	0.861	0.107	0.032
49	0.771	0.198	0.031	0.915	0.071	0.014
50	0.957	0.043	0.001	0.967	0.032	0.001
51	0.378	0.335	0.287	0.000	0.332	0.668
52	0.247	0.364	0.389	0.000	0.092	0.908
53	0.172	0.392	0.436	0.000	0.125	0.875
54	0.001	0.998	0.001	0.000	0.998	0.002
55	0.145	0.401	0.454	0.000	0.134	0.866
56	0.012	0.227	0.761	0.000	0.075	0.925
57	0.188	0.812	0.000	0.000	1.000	0.000
58	0.001	0.094	0.905	0.000	0.023	0.977
59	0.000	0.016	0.984	0.000	0.004	0.996
60	0.142	0.808	0.051	0.000	0.895	0.105
61	0.183	0.793	0.025	0.000	0.945	0.055
62	0.129	0.829	0.042	0.000	0.924	0.076
63	0.191	0.808	0.001	0.000	0.998	0.002
64	0.246	0.368	0.387	0.000	0.100	0.900
65	0.275	0.374	0.351	0.000	0.203	0.797
66	0.250	0.391	0.359	0.000	0.277	0.723
67	0.046	0.252	0.702	0.000	0.081	0.919
68	0.921	0.077	0.002	0.944	0.055	0.002
69	0.108	0.408	0.484	0.000	0.156	0.844
70	0.057	0.414	0.529	0.000	0.167	0.833
71	0.000	0.040	0.960	0.000	0.012	0.988
72	0.000	0.002	0.998	0.000	0.001	0.999
73	0.000	0.000	1.000	0.000	0.000	1.000
74	0.000	0.000	1.000	0.000	0.000	1.000
75	0.000	0.001	0.999	0.000	0.000	1.000
76	0.000	0.000	1.000	0.000	0.000	1.000
77	0.025	0.908	0.066	0.000	0.897	0.103
78	0.003	0.993	0.004	0.000	0.994	0.006
79	0.013	0.984	0.003	0.000	0.996	0.004
80	0.013	0.984	0.001	0.000	0.999	0.001
81	0.123	0.877	0.000	0.000	1.000	0.000