LEAKEY FOUNDATION GRANT FINAL REPORT

"Evolutionary effects of light environment on nocturnal lemur color vision"

Carrie Cecilia Veilleux, University of Texas at Austin

Introduction

The primary goal of this dissertation project was to identify how habitat variation in ambient light environments affects the evolution of primate visual anatomy, specifically that related to color vision. Using nocturnal lemurs as a model, I first quantified the variation in nocturnal light environments available in different Malagasy habitats and then used population genetics to measure the type of selection acting on the S-opsin gene (coding for the production of short wavelength-sensitive retinal cones) in species endemic to different habitat types. In particular, I hypothesized that selection on the S-opsin gene to maintain dichromatic color vision in nocturnal lemurs is linked to the abundance of short wavelength (blues/violets: SW) light available in their habitats. I sought to address three primary questions: (1) how do light environments vary between lemur habitats? (2) does selection for dichromatic color vision vary between lemurs from different habitats? (3) Do differences in ambient light available in different habitats correlate with differential selection?

Project status

This project involves two research components: ecological fieldwork in Madagascar quantifying light environments and forest structure in lemur habitats and molecular work examining selection differences on the S-opsin gene for dichromacy. The field research, which was funded by the Leakey Foundation, as well as the Wenner-Gren Foundation, the American Philosophical Society, and the American Society of Mammalogists, was conducted from July 16 to October 22, 2009. Almost all ecological data have been analyzed and are currently being prepared for publication. The molecular research, which was funded by the National Science Foundation (DDIG #0752692), Sigma Xi, and the University of Texas at Austin, is still ongoing, with a projected completion date of August 2011. Because the molecular research is not complete, I will primarily report results of the field research funded by the Leakey Foundation.

I. Forest Light in Madagascar: How do light environments vary between lemur habitats?

Currently, little is known regarding the variation in nocturnal light environments available in natural habitats, despite its potential importance for studying nocturnal color vision in primates and other animals. The primary goal of my field research was to explore how nocturnal light environments vary within and between habitats and to identify important factors driving this variation. To achieve this goal, I quantified light environments and forest structure in two distinct habitat types in Madagascar: dry deciduous forest/succulent woodland at Kirindy Mitea National Park and humid rainforest at Ranomafana National Park. At Ranomafana, I worked at the montane forest Valohoaka site (Valo) and the lowland rainforest Talatakely site (Tala). I predicted that (1) open-canopied Kirindy Mitea will have higher proportions of SW light compared to closed-canopy Ranomafana sites; (2) at both sites, SW light intensity should increase with increasing canopy height; (3) SW light intensity should vary with lunar phase at both sites, with more full phases having greater proportions of SW light. Additionally, because the light environments nocturnal lemurs encounter may vary with their activity patterns (such as lunar philia/phobia), I tested the effects of nocturnal light intensity on vocalization frequency in two nocturnal lemur species at Kirindy Mitea National Park.

Methods

Foliage density

At all sites, I established nine 2x50m transects at a distance of 3-10m parallel to existing trails. Foliage density was measured at 10m intervals along each transect using hemispheric photography and at 5m intervals using an LAIL meter (Cournac et al., 2002). Photographs were taken with a digital camera and fisheye lens positioned at a height of 0.89m. Using Gap Light Analyzer v.2.0, I calculated % canopy openness for each location. LAIL measurements were taken between 10:30 h and 13 h on sunny days, with the LAIL apparatus held at 2 m height. Three measurements were taken per transect location and averaged for analysis. Following Emmons and Dubois (2003), I derived a dimensionless value LAI* using published estimates of *k* for dry deciduous Kirindy Mitea and rainforest Valo/Tala (*k* = 0.61 for tropical dry deciduous forests: Maass et al., 1995; *k* = 0.88 for humid rainforests: Cournac et al., 2002). At Kirindy Mitea, I established a tenth transect and quantified all stems with a diameter at breast height ≥ 2.5cm within each transect.

Light environments

I collected 532 nocturnal irradiance measurements, including 514 measurements from Kirindy Mitea, 8 from Valo, and 10 from Tala. All measurements were collected using an International Light IL1700 Research Radiometer and calibrated PMC271C photomultiplier detector (200-675 nm sensitivity range) positioned at a height of 0.89 m above the ground. To quantify spectral sensitivity, 12 narrow-bandpass interference filters were positioned in a filter wheel resting on the photomultiplier detector. During measurement, the photomultiplier detector was pointed directly up at the sky (90° zenith angle). Weather conditions (cloudy/clear) and time were recorded for each measurement. The time of data collection varied nightly but always began after astronomical twilight had ended when the sun no longer contributes to irradiance. Using the time and date of measurement and site location, the position of the moon in the sky (lunar altitude) and lunar phase (lunar fraction: 0 = new moon, 1.0 = full moon) were calculated for each measurement from data available at the United States Naval Observatory (USNO, 2010). At Kirindy Mitea, nocturnal irradiance was measured over 32 nights (29 July - 8 Sept 2009). Data were collected at 10m intervals along the nine transects. Each transect location was revisited approximately every 4 nights to sample locations across a lunar cycle. At Valo and Tala, nocturnal irradiance was measured on one night each. At Valo, data were collected for 8 transect locations on a clear crescent moon night (22 Sept 2009), while data were collected at Tala for 10 transect locations on a gibbous moon night (8 Oct 2009, 3 clear, 7 cloudy).

At all sites I also sampled diurnal irradiance at 10m intervals along each of the nine transects, resulting in 54 measurements per site. Diurnal irradiance was quantified using a light meter (LI-COR) and 13 narrow-bandpass interference filters. At Kirindy Mitea, I also sampled vertical light environment variation for four vertical transects. Using arborist tree climbing techniques, I measured light intensity at 2m vertical intervals (2-12m).

Calling behavior

During each nocturnal light measurement at Kirindy Mitea, I also collected vocalization data (presence/absence) for two nocturnal species: *Lepilemur ruficaudatus* and *Phaner pallescens*.

Statistical analyses

For nocturnal analyses, I quantified four aspects of nocturnal light environments: total flux, percent SW light (%SW), percent middle wavelength light (%MW); percent long wavelength light (%LW). The three spectral quality variables (%SW, %MW, and %LW) were calculated from measurements taken with the narrow bandpass interference filters. For %SW, I summed the measurements from filters between 400 and 460 nm and divided that value by the sum of measurements from all filters. %MW and %LW were calculated similarly (490-540 nm and 560-650nm filters, respectively). To identify how lunar altitude, lunar fraction, cloud cover, and canopy openness affect nocturnal light environments, I employed linear mixed effects models from the Kirindy Mitea dataset for each of the four aspects of light environments. Separate analyses were run for moonlight observations (lunar altitude > 0°, n=390) and starlight observations (lunar altitude < -12°, n=105). Measurement location and transect were treated as nested random effects to prevent spatial and temporal autocorrelation. I also developed nocturnal light spectra using raw data (uncorrected for detector sensitivity) by calculating the averaged proportion of irradiance for each filter (filter measurement/sum of all filter measurements) to qualitatively compare light environments under different conditions (phase, altitude, cloud cover, and canopy openness). Diurnal irradiance has not yet been analyzed.

Results

Foliage density.

Analyses of LAI* and hemispheric photographs support predictions that foliage density would be lower in the dry forest at Kirindy Mitea compared to the two rainforest sites. Using LAI*, Kirindy Mitea has lower foliage density than either Valo (Mann Whitney U=827, $p<0.001$) or Tala (U=805.5, $p<0.001$), while the two rainforest sites do not significantly differ (U=4350, $p>0.05$). Similarly, median canopy openness (Fig. 1) was significantly higher at Kirindy Mitea (38.09%) than Valo (16.61%; Wilcoxon rank sums W=2739, $p<0.0001$) and Tala (19.92%; W=2704.5, $p<0.0001$). However, unlike LAI*, median canopy openness at Tala was significantly higher than at Valo (W=2228.5, $p<0.0001$).

This study provides the first estimate of forest structure at Kirindy Mitea. The relative densities of stems for dbh classes suggest that the structure of the dry forest at Kirindy Mitea closely resembles the dry gallery forest at Beza Mahafaly (Sussman & Rakotozafy, 1994).

Nocturnal light environments

The results of the linear mixed effects models for Kirindy Mitea (Table 1) suggest that lunar fraction, lunar altitude, weather conditions, and canopy openness all have significant effects on the intensity (total flux) and spectral quality (%SW, %MW, %LW) of nocturnal light. Looking specifically at the proportions of shorter wavelengths (%SW), %SW in moonlight increases with increasing lunar fraction, canopy openness, and clear skies. Similarly, %SW in starlight only conditions is positively correlated with canopy openness. One of the most interesting results is the effect of lunar altitude on %SW (Fig. 2). In general, %SW is actually higher when the moon is relatively low in the sky (< 50°) compared to when it is directly overhead. The spectra derived from the raw, uncorrected PMT values support the model results (Fig. 3).

While many of the spectral comparisons between habitat types lack statistical significance, they do differ in the predicted directions (Fig. 3E, F). The lack of significance may reflect the small sample sizes. Comparing Valo and Kirindy Mitea in clear skies under a crescent moon, Kirindy Mitea has significantly higher %MW (W=56, $p<0.05$) and significantly lower %LW (W=6.5, $p<0.005$) than Valo (Fig. 3E). There is no significant difference in %SW between Kirindy Mitea and Valo (W=38, $p=0.29$), although the difference is in the predicted direction. In cloudy gibbous skies, Kirindy Mitea is richer in %SW (W=82, $p=0.11$), richer in %MW (W=54, $p=0.17$), and lower in %LW (W=23, $p=0.0599$) than Tala.

Calling behavior

The results of the generalized linear mixed effects models for calling presence/absence reveal that nocturnal light intensity significantly affects vocalizations in *Phaner pallescens* ($p=0.0046$) but not in *Lepilemur ruficaudatus* ($p=0.204$). *Phaner* thus appears lunar philic in vocalization behavior, increasing calling with increasing light intensity. *Lepilemur*, in contrast, exhibits lunar neutral behavior.

Summary

The results of the field analyses have thus far supported my predictions. The spectral quality of nocturnal light varies significantly under different conditions (Fig 3). Looking specifically at the proportions of shorter wavelengths of light (relevant for the SW-sensitive cone):

1. Cloud cover decreases ambient SW light.
2. The proportion of SW light increases with canopy openness both among microhabitats at Kirindy Mitea and between habitat types.
3. SW light increases with lunar fraction such that more full lunar phases are richer in SW light. Starlight is deficient in SW light and rich in LW (red) light.
4. The proportion of SW light varies with the height of the moon in the sky.

These results reveal that nocturnal light environments vary both temporally (over a single night with lunar altitude or clouds, over a lunar month with phase) and spatially (microhabitats and habitat). Nocturnal lemurs and other animals are thus encountering dramatically changing light environments that could have significant implications for their visual adaptations. The vocalization study further suggests that even sympatric nocturnal lemurs may encounter different light environments depending on their behavioral response to moonlight.

II. Selection on the S-opsin Gene in Nocturnal Lemurs:
Does selection for dichromatic color vision vary between lemurs from different habitats?

For the molecular component of this project, I sought to determine whether nocturnal lemur species are experiencing differential selection on the S-opsin gene for dichromatic color vision. Collaborating with Dr. Edward Louis of the Henry Doorly Zoo, I employed a sample of 112 nocturnal lemurs representing 19 species and 5 genera. In order to control for effects of phylogeny on selection, I used population genetic selection tests and lineage selection tests to compare selection on the S-opsin gene between congeneric populations endemic to different habitats (closed canopy rainforest, open canopy dry forest and spiny forest). I hypothesized that rainforest populations would exhibit evidence of relaxed selection on the gene while congeners endemic to spiny and dry forests would exhibit purifying selection to maintain dichromacy.

Methods

Gene amplification and sequencing

After designing lemur- and genus-specific polymerase chain reaction (PCR) primers, I amplified the S-opsin gene of each individual in two ~1700 base pair (bp) PCR products. I purified the PCR products and sequenced them at the University of Texas at Austin DNA Sequencing Facility. Several species of *Cheirogaleus, Lepilemur, Avahi,* and *Microcebus* included heterozygous individuals with insertions/deletions in one of the S-opsin gene alleles. This condition required cloning the PCR product to separate alleles for sequencing. For each individual requiring cloning, I used TOPO TA Cloning Kits (Invitrogen) and amplified clone colonies using S-opsin gene PCR primers. I sequenced at least 10 clone colonies per individual in order to achieve at least 3 copies of each allele. I am also collaborating with Shoji Kawamura and Yukiko Fukuyo to identify the spectral tuning of the nocturnal lemur S-opsin pigments.

Preliminary Results

I have completed population-based selection analyses (including comparisons of nonsynoymous and silent nucleotide diversity) for all *Cheirogaleus* and *Phaner*, as well as many *Lepilemur* and *Avahi* populations and partial *Microcebus* individuals. These preliminary results reveal substantial intrageneric variation in selection on the S-opsin gene, including evidence of potential S cone loss in one species. Among *Lepilemur*, comparisons of nonsynonymous and silent nucleotide diversity (Perry et al., 2007) reveal evidence of purifying selection on the dry forest and spiny forest species and evidence of relaxed selection on the rainforest species. In contrast, *Avahi* from both rainforest and dry forest populations exhibit evidence of purifying selection to maintain dichromacy.

The Cheirogaleidae results are particularly interesting. All *Phaner* exhibit a premature stop codon in exon 1 followed by a second start codon. It is currently unclear how missing the first 41 amino acids will affect the function of the S-opsin protein. None of the critical tuning sites or attachment sites for the chromophore are included in the missing 41 amino acids (Kawamura & Kubotera, 2004). If the second start codon is sufficient for S cone production, the dry forest species exhibits evidence of purifying selection while the rainforest species exhibits relaxed selection. Among *Cheirogaleus*, the rainforest species *C. major* appears to have a functional S-opsin gene under purifying selection. The dry forest species (*C. medius*), however, has two major alleles in their population that should affect S-opsin gene function. One allele has a 4bp insertion causing a frame shift and seven premature stop codons that I predict would result in a nonfunctional protein. The second allele has a 54bp insertion in exon 2. This insertion adds 17 amino acids to the protein, but the functional implications of this addition are unclear.

III. Preliminary Conclusions

While the final data of this project remain to be collected and analyzed, several interesting finds have already been made. My work is the first to identify differential selection for color vision within a genus (*Lepilemur, Cheirogaleus,* possibly *Phaner*). I also identified new and surprising effects on variation in nocturnal light environments in natural habitats. I offer the first evidence that nocturnal spectral quality varies significantly with the height of the moon in the sky and cloud cover. My study is also the first to quantify variation in spectral quality between lunar phases within natural habitats encountered by nocturnal animals. These results demonstrate that nocturnal light environments are dramatically variable on a temporal and spatial scale, possibly even more variable than diurnal light environments. These dramatically changing light environments may even offer an adaptive reason for maintaining dichromatic color vision in some nocturnal primates. Some researchers have proposed that nocturnal color vision offers a reliable way of identifying visual targets under changing light conditions (Kelber & Roth, 2006; Johnsen et al., 2006).

IV. Publications

The results of this research have been presented at several conferences (American Association of Physical Anthropologists 2010, 2011; American Society of Primatologists 2010; American Anthropological Association 2009; Sensory Ecology Course 2010 at Lund University, Sweden).

Manuscripts

Veilleux CC, Cummings ME. *in prep*. Variation in nocturnal light environments in Malagasy tropical forests: implications for nocturnal color vision. *J Exp Biol. To be submitted 7/2011.*

Published Abstracts from Research:

Veilleux CC. accepted. Effects of nocturnal light intensity on calling frequency in dry forest *Phaner* and *Lepilemur*. *Am J Primatol.*

Veilleux CC. 2011. Nocturnal light environments in Madagascar: implications for nocturnal primate color vision. *Am J Phys Anthropol* Suppl 52:299.

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V. References

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Figures for Veilleux Leakey Final Report

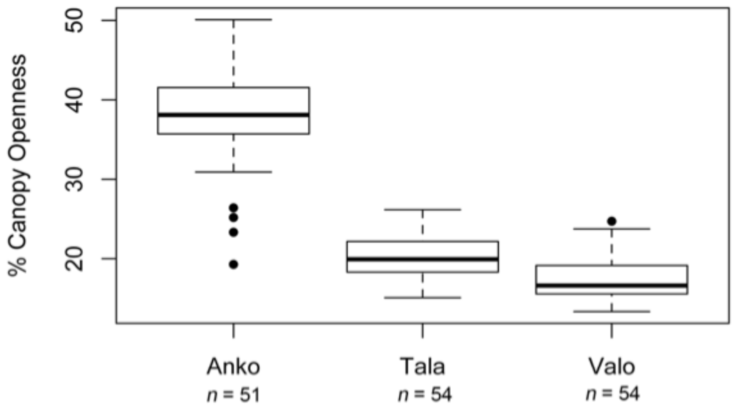

Fig. 1. Foliage Density Between Sites

Anko = Kirindy Mitea National Park, Tala and Valo = Ranomafana National Park

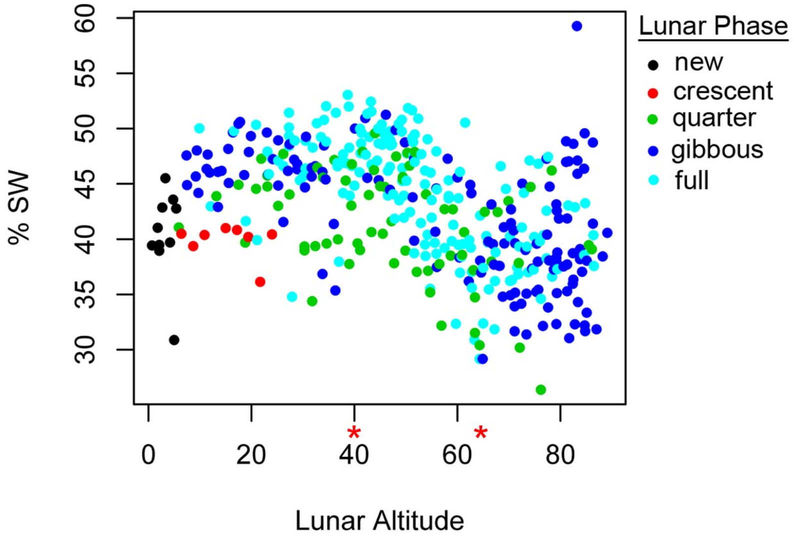

Fig. 2. %SW and Lunar Altitude.
Data from Kirindy Mitea (*n*=390). Points are colored by lunar phase to depict the interaction of altitude and fraction on %SW (see Table 1). Lunar phase includes grouped lunar fractions: new (0.00), crescent (0.01 to 0.15), quarter (0.40 to 0.69), gibbous (0.70 to 0.90), full (0.91 to 1.0). No moonlight cases were collected with fractions between 0.15 and 0.40.

The relationship between %SW and altitude is nonlinear. Stars along X-axis reflect points where the slope of the relationship appears to change. %SW increases with lunar altitude until ~40°, then decreases with increasing lunar altitude until ~65°.

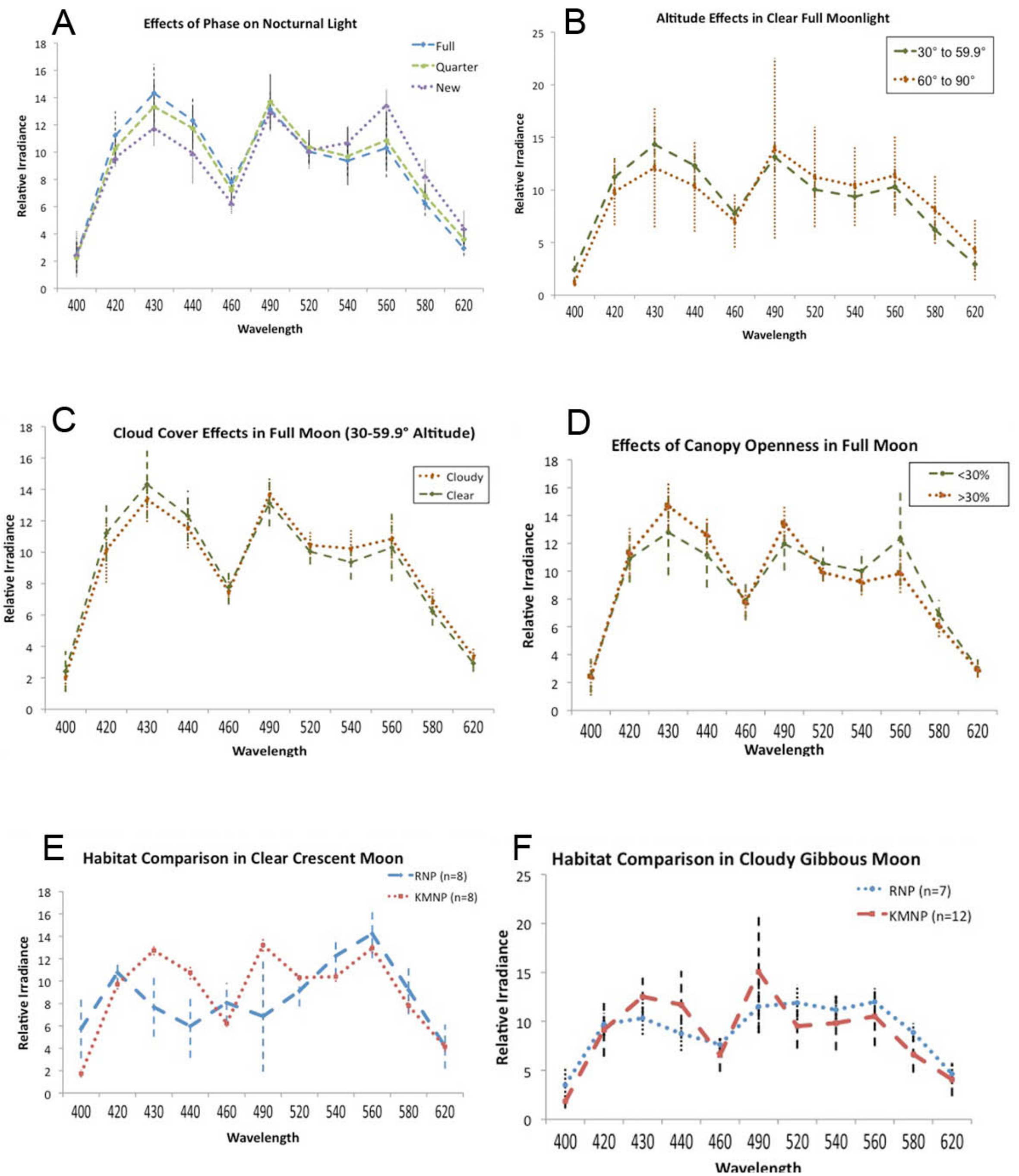

Fig. 3. Nocturnal light spectra under different conditions.

Bars represent one standard deviation. (A) Effects of lunar phase on spectral quality in clear nights. For full and quarter moon, lunar altitude is 30-60°. (B) Effects of lunar altitude on spectral quality in clear full moon nights. (C) Effects of cloud cover on spectral quality in full moon nights with lunar altitude at 30-60°. (D) Effects of canopy openness on spectral quality in clear full moon nights (lunar altitude 30-60°). (E) Spectral quality comparisons of Valo (*n*=8) and Kirindy Mitea (*n*=8) in clear crescent moon at 6-29°. (F) Spectral quality comparisons of Tala (*n*=7) and Kirindy Mitea (*n*=12) in cloudy gibbous moon at 30-60°.

Table 1. Mixed Models fit by REML for Moonlight (*n*=337 observations)

Response Variable	Explanatory Variable	Coefficient	Std. Error	t value	*P* value
Log Total Flux[a]	Altitude	0.002	0.0049	0.39	0.698
	Fraction	0.393	0.0050	2.02	0.044
	Weather	-0.371	0.1941	-2.39	0.018
	Canopy Openness	0.017	0.1553	3.14	0.002
	Altitude x Fraction	0.021	0.0049	4.39	0.000
	Altitude x Weather	0.006	0.0027	2.33	0.021
%SW[b]	Altitude	0.294	0.0832	3.55	0.000
	Fraction	15.757	2.1560	7.31	0.000
	Weather	2.451	0.5230	4.69	0.000
	Canopy Openness	0.491	0.0989	4.96	0.000
	Altitude:Fraction	-0.159	0.0533	-2.98	0.003
	Altitude:Canopy	-0.007	0.0017	-4.33	0.000
%MW[c]	Altitude	0.060	0.0076	7.91	0.000
	Fraction	-2.976	0.8217	-3.62	0.000
	Weather	-1.516	0.3627	-4.18	0.000
	Canopy Openness	0.036	0.0259	1.39	0.164
%LW[d]	Altitude	-0.356	0.0709	-5.02	0.000
	Fraction	-14.382	1.8390	-7.82	0.000
	Weather	-0.961	0.4458	-2.16	0.032
	Canopy Openness	-0.471	0.0849	-5.54	0.000
	Altitude:Fraction	0.209	0.0455	4.60	0.000
	Altitude:Canopy	0.006	0.0015	4.38	0.000

[a] 19.32% variation explained by random effects (transect and location)

[b] 0.17% variation explained by random effects (transect and location)

[c] 0% variation explained by random effects (transect and location)

[d] 0.71% variation explained by random effects (transect and location)