Discussion

Study Hypothesis

Prior to this study, no research has compared cortisol levels of free-living primates in habitats of varying quality. In order to establish a means for comparison in this study, a stress index was created to quantify differences in colobus habitat patches in Diani. The stress index was constructed from 6 habitat factors, and correlation with both mean total and mean basal cortisol levels is moderate. The fact that the index accounts for more than one-third of the variation in adult male cortisol levels in Diani is meaningful, but it is also important to note that other factors do play a role in cortisol variation. Correlation with the Stress Index was slightly higher for basal cortisol levels than for total cortisol levels. Basal levels may be more appropriate for comparison because the categories chosen for the Stress Index are primarily permanent features of the forest patches (basal levels may predict long-term stress exposure rather than spike values).

The sources of variation in cortisol levels that the Stress Index does not account for are more difficult to determine. Several possible factors include weather, behavior, and colobus group dynamics. Weather variations were not accounted for in the Stress Index in this study. Rain is very patchy along the coast, and there were days in the study period when the 5 habitat patches received differing amounts of rain. Colobus behavior varies with weather quality, and they tend to be less active during rain (personal observation). Also, a more detailed behavior record of all of the study troops is crucial for more complete comparisons. Without detailed information from each study group, it is impossible to rule out

non-behavioral explanations for spike levels. Finally, colobus group dynamics are not well understood. Each study troop faced different environments. Adult male cortisol levels may not be the most accurate summarization of these differences. Future studies should incorporate both environmental and behavioral factors in a stress index in addition to comparing cortisol values with those found in this study.

Stress Index

Mean male cortisol values show a general agreement with the stress index: colobus do appear to show higher mean total cortisol levels in more stressed habitat patches. The main anomaly in the data set is that the Colobus Cottage site, ranked as the least stressful site, did not have the lowest mean cortisol values or the fewest spike values of any group. Instead, mean male values from the Colobus Cottage site are similar to mean values in the TW2 site, another large group (n=9) with two adult males. It is possible that the presence of two males within a group is actually more stressful for each male than are other habitat factors. Another explanation for this discrepancy could be that Stress Index factors were not correctly weighted. Although Colobus Cottage has the largest area/individual, it also has more frequent encounters with baboons, sykes, and vervet monkeys than a habitat patch like Mosin. Finally, CCA and CCB did have the greatest difference between basal and peak levels. Using mean values for comparison in this study may not be an accurate measure of stress for the stress index. Mean values do not account for this variation, a factor that is important to consider in terms of overall stress. TW1, an

individual with very little difference between basal and spike levels, is considerably more stressed than either Colobus Cottage male.

Similar to total cortisol values, higher mean basal cortisol levels show a positive correlation with the stress index. In this case, low-stress habitat patches are clustered around 8 μg/dl, whereas other habitat patches are considerably higher (14 μg/dl). Since both alpha and beta males are included in this test, the analysis shows both TW2a, (whose values look more like the values of CC, W, and M) and TW2b (whose values are more similar to TW1. In total however, there is a general agreement with the study hypothesis for cortisol values, based on adult male data.

Adult Males

The fact that adult males were used for this study is potentially misleading on two accounts. First, without comparing values from other age categories among habitat patches, it is not clear whether other group members experience similar changes in cortisol levels. Second, the roles of alpha and beta males in colobus groups have been observed to be different (Kaspers, 1999). Therefore, comparisons of adult males may be affected by the fact that CCB and TW2b are sub-dominant males. To this end, it may be useful to look for similarities between CCA & CCB and TW2a & TW2b. In the Colobus Cottage group, CCB was the instigator of territorial defenses against baboons and vervet groups in the troop's forest region, while CCA was only seen to be the primary aggressor in encounters with a satellite sub-adult male who would sometimes infiltrate the group (Kaspers, 1999). Cortisol levels also reflect this differentiation after a baboon raid in the Colobus Cottage group (CCB's

sample showed extremely high cortisol but CCA's cortisol level remained at his mean basal level).

Although CCA and CCB have similar mean cortisol levels and numbers of spike values, TW2a and TW2b have very different mean levels. This difference could be a result of less well defined roles in the Colobus Cottage group. Because TW2 shares a forest patch with TW1, male roles in TW2 might be more polarized than in the Colobus Cottage group. At the Colobus Cottage patch, baboon and vervet groups are infrequent disturbances and the satellite male does not infiltrate the group as often as TW1 interacts with TW2. In support of this idea, TW1 and TW2b have similar mean cortisol levels, while TW2a has surprisingly low mean levels. It is possible that interactions between TW2b and TW1 do not involve TW2a. Additionally, TW2a's elevated social status in his own group may present fewer stressful interactions.

When the five alpha males' data were assessed apart from the two beta males, the results were similar for mean cortisol values. However, correlation with the Stress Index was less well defined than with all seven males. Five values does not give a very detailed picture in this case, and TW2a's role as an alpha male is suspect, since TW2b has been seen interacting more frequently with TW1 (personal observation). Further investigation of role differentiation is necessary before answering these questions in more depth.

While the direct mechanism of environmental stress is unknown in Diani, a record of group behavior does show a strong qualitative correlation between stressful events and spike cortisol levels. Spike cortisol levels were often found two

days after potential stressful events: groundskeepers working in forest patches, baboon displacement of colobus groups, birth of infants, and mating behaviors. In 21 samples that are from stressed or non-stressed individuals (based on behavior records), these values were significantly different. The ability to match behavioral record events with spike values was an initial focus of this study. Samples from juveniles and sub-adults were collected to test the impact of socializing, fighting, and other behaviors on cortisol values. Due to time constraints, analyses of these samples were not done. Since a connection between behavior and cortisol appears to exist, future studies that compare stressful behaviors over time would be important to understand this connection. Studies which compare cortisol responses from a colobus group to several stressful encounters (baboons, new infants, intruder male colobus) over time could help determine the nature of colobus/stressor interactions among group members.

Adult females

Adult female peak levels may also be correlated with stressful events. Mamom gave birth to an infant on 7/17 or 7/18, and the first sample collected from her, on 7/20, showed a cortisol level of 64 µg/dl. Additional cortisol levels from Mamom are significantly higher than those of MAF. During pregnancy, circulating levels of progesterone and cortisol increase (Miller, 1989) because of a rise in amount of cortisol binding globulin (CBG) (Schimmer, 1996). Progesterone binds to CBG with greater affinity than cortisol (Schimmer, 1996). However, the rate of increase

in CBG as compared to the rate of natural cortisol rise during pregnancy is unknown. High postpartum stress values predicted low maternal success rates in gorillas (Bahr *et al.*, 1998) and could be used in Diani to understand the relationship between habitat area and infant mortality.

It is interesting to note that cortisol levels in MAF were also highest on 7/20. All colobus females carry new infants for the first 4 months of their life. Small sample size (n=7 from 7/16-8/20) prevents a clear understanding of whether MAF's levels increase and plateau or whether they decrease again as the group adjusts to having a new member. However, investigation of this phenomenon would be very interesting, since colobus group behavior seems to change after an infant is born, especially with respect to intruders (personal observation).

Total Group Cortisol Levels

Whitten *et al.* (1998) found spike levels in their study of captive chimpanzees at approximately 8 nanograms/g wet weight. Due to transportation and storage constraints, this study used dry fecal samples for analysis, and spike levels were near 2 μg/g dry weight. The water content of colobus feces is unknown, and it is therefore difficult to compare study values to literature values. Based on animal size alone, colobus values should be lower than chimpanzee levels, but stressors in the wild might cause a more dramatic cortisol rise than those in the chimpanzee lab study.

Colobus cortisol levels do show evidence for some dependence on time of day, but they do not appear to increase over the course of the dry season. Male

cortisol values show more spike levels in the afternoon than in the morning, with specific clusters in the 11:00-12:59 and 3:00-7:00 PM blocks. Analyzing the data in this way is representative of colobus behavior. Colobus generally defecate during periods of movement and feeding. During the day, these periods are from 7:00-8:00 AM, 9:00-10:30 AM, 11:30-1:00 PM 2:00-3:00 PM, and 4:00-6:00 PM, and remained relatively constant throughout the study period (personal observation). My categorization of values in two-hour time blocks in Figure 9 is an accurate, and reflects uniform sampling throughout the day (n = 11, 10, 13, 9, 10, 10 for each block, respectively).

The blocks that include most of the spike levels are in the afternoon; times that are generally during periods of resting/digestion for colobus. Disturbances from this resting state may be more stressful for colobus than disturbances during feeding or moving in the morning. However, only adult colobus samples were analyzed, and sub-adult and juvenile data could highlight more variation in these areas.

Sampling for this study was done randomly. Originally, cortisol levels in all troop members were to be analyzed to give a picture of stress in Diani. As a result, random sampling of adult males and small study size (5 groups) could be the limiting factor in understanding the dynamics of environmental stress. Future studies which look at more concentrated questions of stressful events might prove to be more insightful. Based on the successes of this study, future studies could be used to make explicit policy regulations on habitat sizes and the baseline cortisol values could be the background for a study of translocation stress in colobus.

Ketones and Food Stress

Introduction

Ketones

Ketones are produced when the body metabolizes its own fat reserves to produce energy. The production of ketones in response to fat metabolism is a widespread phenomenon amongst mammals (Robinson *et al.*, 1980). When daily intake of food does not meet metabolic needs for an animal, their bodies must turn to internal reserves for energy. An estimate of food quality and abundance in a primate's habitat can be obtained from a simple urinalysis strip-test in the field (Knott, 1998). Studies of orangutans in Borneo, an island which has seasonal variation in food abundance, showed marked seasonal differences in individual's urinary ketone levels in the two seasons (Knott, 1998). During the non-masting season, when no fruit is available, the orangutans burn fat reserves stored up from the masting season (a time when significant proportions of trees produce fruit in synchrony).

Ketone testing will help to interpret whether variations in stress levels are in response to food or non-food stimuli, in addition to providing another comparison for habitat quality. The hypothesis for this study is that colobus in smaller, less diverse habitats would metabolize more fat than their counterparts with larger, food species-diverse territories during the dry season.

Food Stress

There are two dry seasons for the coastal region of Kenya: January to mid-March and June to October. This study was conducted in the middle of the second dry season. Historical weather patterns during these months show relatively light rainfall (Hawthorne, 1993). However, in 1998, the coast received more than average rainfall during these months (likely on account of El Niño). Despite this extra rain, trees seem to have exhibited normal fruit, seed, and leaf production. The first year's data from a two-year study of 12 colobus food-tree species along the coast can be seen in **Appendix A** (Anderson *et al.*, 1999). These data allow us to see food availability with respect to species in each habitat area (**Figure 3**).

Herbivores are rarely seen to eat more than a small portion of the plants considered available to them, and thus the idea that the supply or quality of food could be limiting their numbers or affecting behavior has largely been rejected (Moreno-Black and Bent, 1982). Equatorial regions may not produce extreme periods of food stress for low-quality foragers like colobus because the seasonality of the forest as a whole is less marked. However, there may be periods when food items are of relatively low nutrient quality which could affect the colobus' physiological state (Baranga, 1983). While colobus can eat mature leaves when young leaves, seeds, and fruits are not available, mature leaves are much more difficult for colobus to digest and provide an inadequate replacement protein source (Dasilva, 1992). Thus, without sufficient reserves young of leaves, seeds, or fruits,

habitat-wide fat metabolism could be observed in Diani. This analysis of habitat ketone levels will provide some insight on these questions.

Methods

Field Collection

Urine samples were taken randomly from 7/6/98 to 8/20/98 and analyzed for the presence of ketones. Urine was collected in conjunction with fecal sample collections. Urine collections were made by placing a plastic sheet on the ground below the urinating animal or by removing urine from broad-leaf ground vegetation with a 5 mL syringe. Urine that fell directly onto leaf litter on the forest floor was not used because of the potential for contamination from ground sources. In all, 86 samples were analyzed using Bayer Multistix® 10 SG reagent strips (Bayer Corporation, Elkhart, IN). The plastic reagent strips have a color pad which registers negative (0 mg/dl), trace (5 mg/dl), small (15 mg/dl), moderate (40 mg/dl), large (80 mg/dl), and large + (160 mg/dl). However, it was also possible to judge colors that were in-between two values. Ketone tests ranged from negative to small+, and the following value numbers were assigned to the results: Negative = 0; Negative+ = 1; Trace = 2, Trace+ = 3, Small = 4, and Small+ = 5.

Urine collection was not possible on rainy days. Water droplets on the vegetation and falling from the canopy interfere with urine concentration, compromising the accuracy of the test. Urine collection was done as non-invasively as possible. However, the opportunity to sample from some groups was lost as a

result of waiting until the group moved. Often, individuals urinate in the same region, and samples mix before they can be tested.

Food Stress Index

An index was created to assess the potential food stress faced by individuals in each of the study troops. The index is based on four categories, listed here in descending order of importance: number of feeding tree species per individual, number of feeding trees per individual, forest area/individual, and number of resting trees per individual. See **Table 3** for a summary of ranks and category information.

Habitat Area	Forest Area/Ind. (0.1)	Feed Trees/Ind. (0.3)	Feed Species per habitat (0.5)	Rest Trees/Ind. (0.1)	Food Stress Index
Colobus Cottage	1	2	1	1	**1.3**
Warandale	2	1	4	3	**2.8**
Tradewinds 2	5	4	2	2	**2.9**
Tradewinds 1	4	3	4	4	**3.7**
Mosin	3	5	3	5	**3.8**

Table 3. Summary of Food Stress Index rankings and components. Habitat patches are listed from least to most stressful with respect to food availability.

The number of feeding tree species per individual was chosen to be the most important factor in food stress because of its measure of the dietary diversity present for colobus (**Factor = 0.5**). The number of feeding trees per individual is also an important measure of habitat suitability (**Factor = 0.3**). The amount of forest area per individual and the number of resting trees per individual are both characteristics of colobus habitat which define the spatial segregation from other habitat areas and overall habitat quality (**Factor = 0.1** for both).

Statistical Analysis

Statistical analyses of data were done with Microsoft Excel 98, JMP 3.2, and Prism. Chi-Squared tests were used to compare male and female data, and Non-parametric regressions, Wilcoxon, and Tukey-Kramer HSD tests were used for comparisons of habitat areas and correlation with the Food Stress Index.

Results

Urine was sampled from all ages of colobus. Values of 0 or 1 were both counted as non-positive tests (with a value of 0). 13 positive tests (values 2-5) were recorded out of 86 samples. However, 3 of the positive tests can be explained on the basis of physiological state unrelated to environmentally induced food stress. The two infants in the TW2 group were weaning from their mothers when tested (both Trace +, or 3's), and would likely be burning fat as they begin more active foraging and lose fat stored from nursing. In addition, a sample taken from one of the pregnant females in the Mosin group can be disregarded (sample was Small +, or a 5). The energy costs of pregnancy and lactation increase energy expenditures by factors of at least 1.25 and 1.5, respectively (Dasilva, 1992), which would likely cause such females to metabolize fat. After these exclusions, 10/83 positive tests remain.

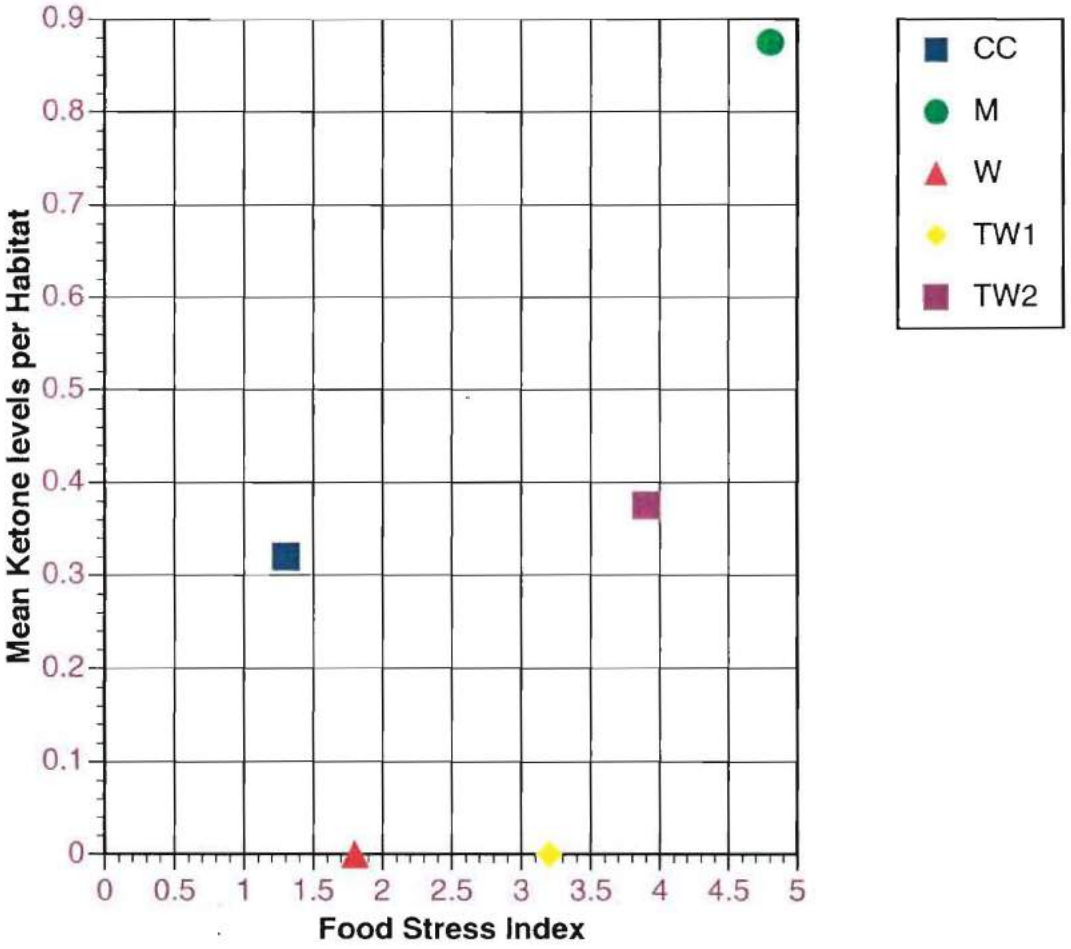
The mean ketone levels for each habitat area for males and females within each habitat area are displayed in **Table 4**. There were no significant differences

between the percentage of positive tests for males and females within each habitat, nor were there significant differences in male and female ketone levels in each habitat area (Chi-square ~0.35). When mean total, male, and female ketone levels per habitat were regressed against the Food Stress Index, no statistically significant correlations were found (total: $r = 0.75$, d.f. = 4, $p = 0.14$; male: $r = 0.77$, d.f. = 4, $p = 0.12$; female: $r = 0.74$, d.f. = 4, $p = 0.14$) (**Figure 12**). However, a general increasing trend can be seen between ketone levels and Food Stress Index, with r-values that are moderate. All but one of the positive values are from adult colobus. Finally, the 10 positive ketone values occurred from 7/8 to 8/18, without a significant clustering during any time in the dry season.

Habitat Patch	Mean Total Ketone	Mean Male Ketone	Mean Female Ketone	Food Stress Index
Colobus Cottage	0.32	0.25	0.35	1.3
Warandale	0	0	0	1.8
Tradewinds 1	0	0	0	3.2
Tradewinds 2	0.375	0.35	0.42	3.9
Mosin	0.875	1.17	0.77	4.8

Table 4. Ketone Levels in each Habitat Patch. Habitat patches are listed in ascending order of stress index. Mean levels are not significantly different between habitat patches, or between males and females within each patch.

Figure 12. Mean Ketone Levels per habitat With Respect to Food Stress Index (r = 0.65).



Discussion

In my study, 10/83 positive values points to some amount of food stress in Diani during these months. However, it is not clear whether food stress in the samples is a result of temporary behavioral changes (such as mating), weather-induced periods of inactivity, or from low food availability because of dry season or stress factors. Without wet season values (when colobus food resources are more prevalent in Diani) it is not possible to assign causes for these positive values. Since detectable levels of ketones in the urine are an indication of considerable fat metabolism, the individuals with positive values show extreme food stress. Ten positive values in Diani presents a very different picture from Knott's (1998) study of Borneo orangutans. In that study, 336 urine samples were tested and 257 positive tests were recorded. All of the positive tests occurred during the fruit-poor season.

The fact that positive values were only found in adults in this study, who are often less active during the day, is an interesting result. Juveniles and infants are very active in the late afternoon. These social times often involve bouts of chasing and wrestling, but urine levels did not reflect fat metabolism from these times. Adult males and females are generally move focussed on feeding and when younger colobus are playing. It is possible that adult behaviors (mating or territory monitoring) could prevent adult colobus from eating for extended periods of time.

There were no positive ketone levels in the smallest groups, Warandale and Tradewinds 1 (n=4 and 5, respectively). In all of the Food Stress Index categories, these two habitat patches rank in the middle of the 5 habitat areas. It is possible that

the small group size does not create as much food stress as larger groups, despite the apparently poor forest conditions.

Food stress in this study appears to be patchy throughout the duration of the study. The study hypothesis predicted food stress would be more apparent as the dry season progressed, but this was not the case. Elevated urinary ketone levels are found throughout the season, and may therefore be more closely linked to weather patterns (since colobus are relatively inactive during heavy rain) or some other, non-seasonal factor.

The evidence of coral and plaster eating by colobus in this study brings up additional questions about diet choices and behavioral adaptations in relation to ketone levels. Could colobus adapt to worse habitat areas by simply buffering their diet or replace minerals with non-plant resources? Unlike other coast primates, colobus are not tempted by human foods. However, colobus did eat pieces of baked clay (gray clay that is sold as a human diet supplement and is often eaten by pregnant women) (Wakuluzu Trust, 1998). These two phenomena suggest that colobus in Diani could be nutrient (calcium) deficient or need to buffer their diet of mature leaves. A chemical analysis of colobus food sources would highlight areas of mineral deficiency or toxic plant defenses.

Behavioral adaptations are often seen as animals overcome food stress (Milton, 1997). Similar to coral eating by colobus in Diani, charcoal is eaten by Zanzibar red colobus. When analyzed for its ability to absorb secondary toxins in leaves from the two main food sources, *Terminalia catappa* and *Mangifera indica* (two food trees also utilized by colobus in Diani), the study found that the charcoal

eaten by the colobus is effective at absorbing these toxins (Cooney, Struhsaker, 1997). This buffering capability may allow Zanzibar colobus to eat poorer food sources (such as mature leaves, which are higher in these toxins) effectively. Zanzibar colobus have the highest non-human anthropoid density ever recorded in areas that have charcoal present. Areas of the island without charcoal reserves support significantly fewer colobus (Struhsaker, Cooney, Siex, 1997). The question could then be raised; do colobus in Diani eat coral more frequently in poorer habitats to counteract the toxins in low-quality foodstuffs in their diet? This is a question to be answered with additional research.

Total food availability does not seem to be a problem in any of the study habitats. Instead, data from this study and observations by the Wakuluzu Trust in the past 3 years suggest that colobus may be adapting behaviorally to account for inadequacies in their diet. A comparison of ranging behaviors in the wet and dry seasons and a study of coral eating in response to diet characteristics would be important new directions for research.

Fecal samples were also of varying consistency in the study, which may be an indication of food stress. On 7/17, CCA had a very watery stool sample, and a urine test on this day was a Trace. Colobus do not drink water, and their stools are generally solid (a result of the high fiber content of their diet). Mosin group members also had loose stools throughout the study period, and Warandale Group members had large quantities of undigested seeds in their samples. Variations in diet quality may be evident from such differences. A more in-depth evaluation of diet among Diani colobus might discover key nutrient differences or other effects. It

might also be possible figure out maximum colobus per habitat patch by finding the ratio of protein to fiber in mature foliage. This parameter has been found to accurately predict the biomass of colobines in these forests (Oates, 1996). A study that determines this ratio for colobus in Diani would also be important.

Conclusion

The total picture of habitat suitability, considering combined evidence from cortisol and ketone levels, does not suggest that food stress is the main cause of spike cortisol levels during the dry season. **Figure 13** compares cortisol values with ketone values from the same collection day. Since only six positive urine tests were found for study individuals, (3 from adult males in Diani, and 3 from Mamom and MAF), there are very few positive values from which to compare low and high ketone values to cortisol results. As a result, Figure 12 only highlights long-term food stress events. An elevated cortisol level that appears two days following ketone presence in an individual's urine would be necessary to connect food stress with habitat stress (since fecal cortisol is a measurement of the physiological state of

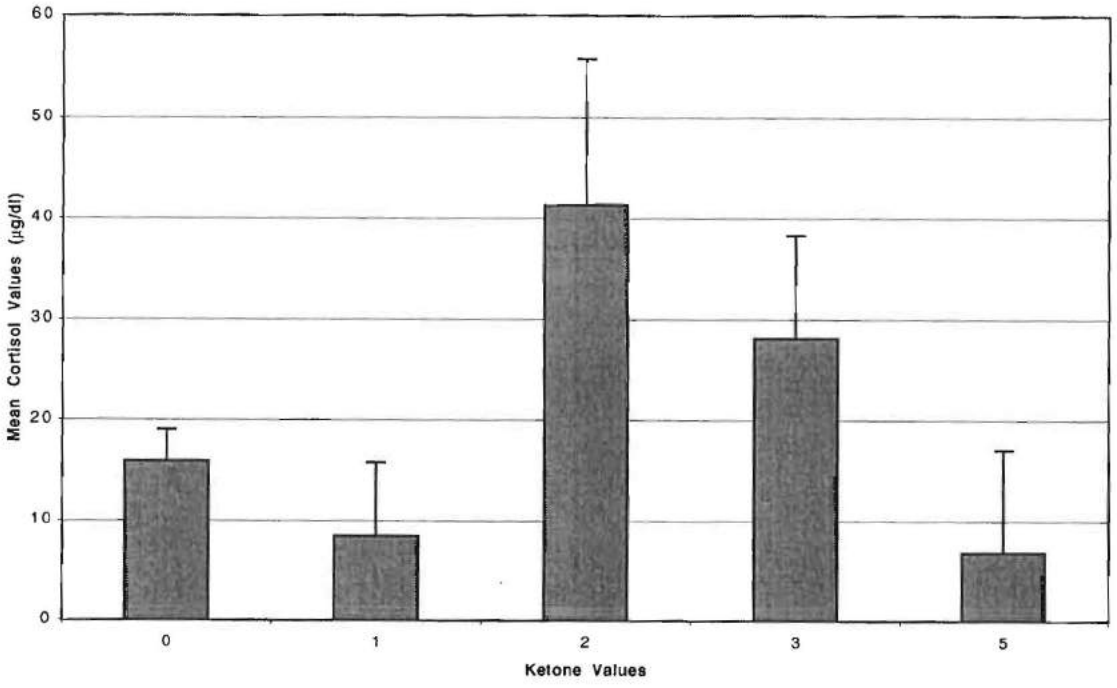


Figure 13. Cortisol levels with respect to Ketone Values for all individuals sampled on the same day. (n = 30, breakdown: **0** = 21, **1** = 4, **2** = 1, **3** = 2, **5** = 2)

the colobus 48 hours before the sample is collected). No values exist for comparison of ketone and cortisol levels from approximately the same time. The goal of my ketone testing was to ascertain (via random sampling of all group members) whether habitat-wide or Diani-wide food stress was occurring during the dry season. Studies that employ these techniques in the future should focus on coordinating sample collection for this purpose.

The laboratory procedures used in this study were time efficient and accurate, and should be used when studying fecal hormones in colobus in the future. The collection methods did not interfere with colobus behavior, and sample collections from specific individuals were most often possible in Diani. Sample collections from more wild troops or in forest patches with more dense understory would not be effective for future studies. Radioimmunoassay is also a viable technique for quantifying cortisol concentrations, and the specificity of the kits used in this study is certainly appropriate for detailed data analysis.

Based on the data in this study, it is likely that colobus experience both habitat and food stress in the dry season in Diani. However, a direct connection between habitat factors and behavior can not be made with spike cortisol levels or with elevated ketone values. Conclusions from this study offer new, narrower directions for future research in colobus habitat stress studies: investigation of specific behavior patterns and cortisol response, examination of adult females before and after infant births, and for the comparison of dry-season values to wet-season values. Furthermore, the values in this study form a 1998 dry-season baseline. Future studies that compare groups of colobus or compare Angolan colobus groups

should consult this data. The Wakuluzu Trust is currently involved with research and development of translocation protocol for colobus monkeys. The cortisol values recorded in this study are an ideal starting point for an investigation of translocation stress.

Today, development and farming on the south coast of Kenya threaten to take more of the remaining forest patches. In order to avoid a situation where coastal developments force the last remaining Kenyan colobus into unsuitable habitat sizes and types, it is essential to understanding the connection between habitat suitability and species fitness. An effective management scheme for the survival and recovery of colobus in Kenya will have to take steps to understand this relationship, including monitoring cortisol and ketone levels in Diani habitat patches.

Appendices

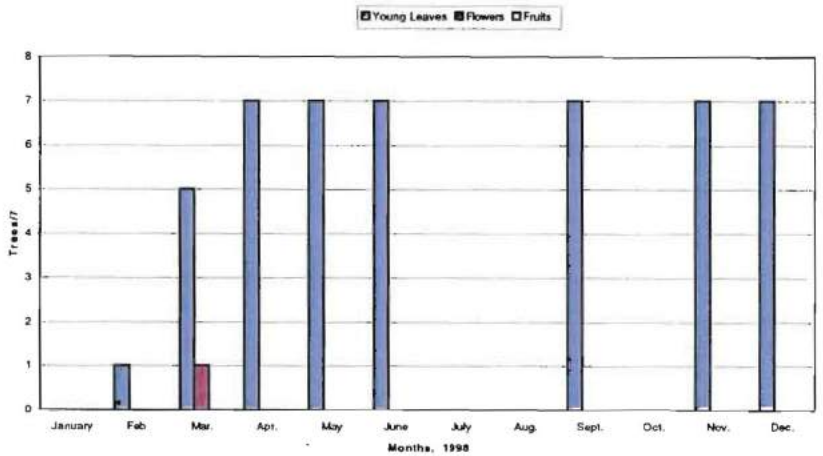
A. Colobus food availability. Data from Anderson *et al.*, 1999. Young leaves, flowers, and fruit availability in 12 colobus feeding tree species for January 1998 to January 1999.

B. Maps of Study Sites in Diani.

C. Chronological progression of laboratory procedures.

D. Works Cited.

Suregadi zanzibariensis



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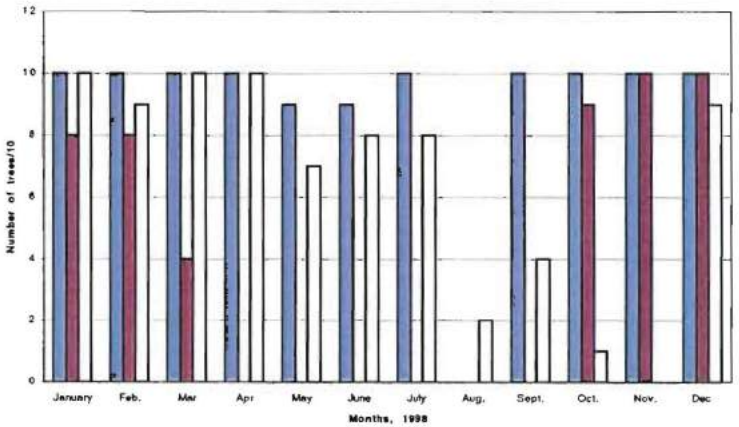

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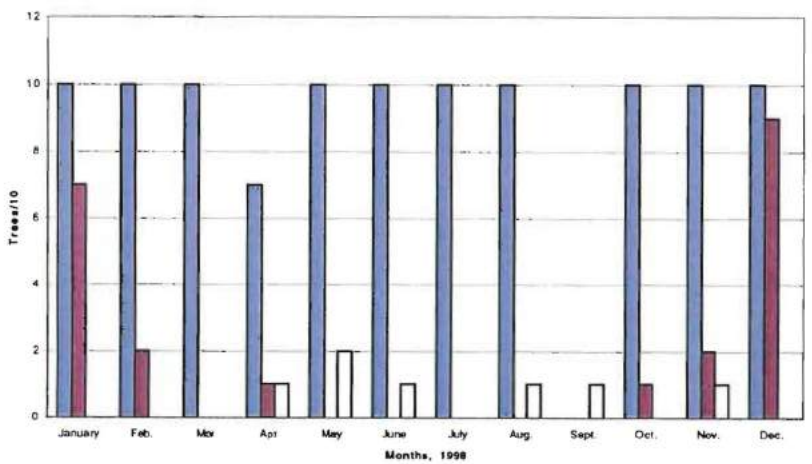

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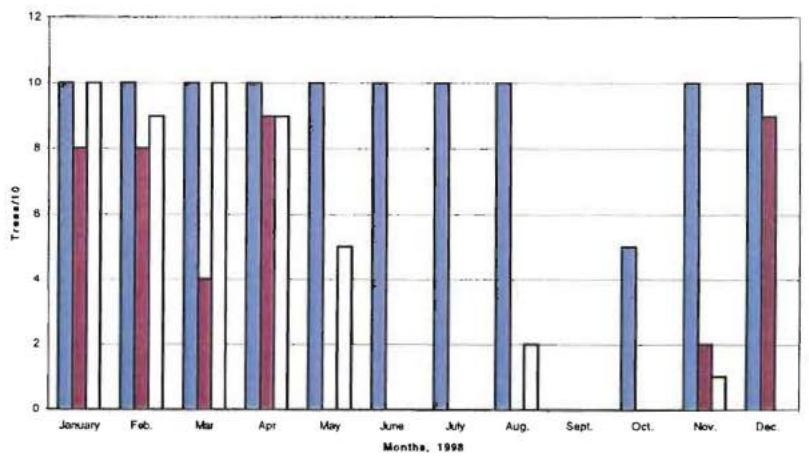

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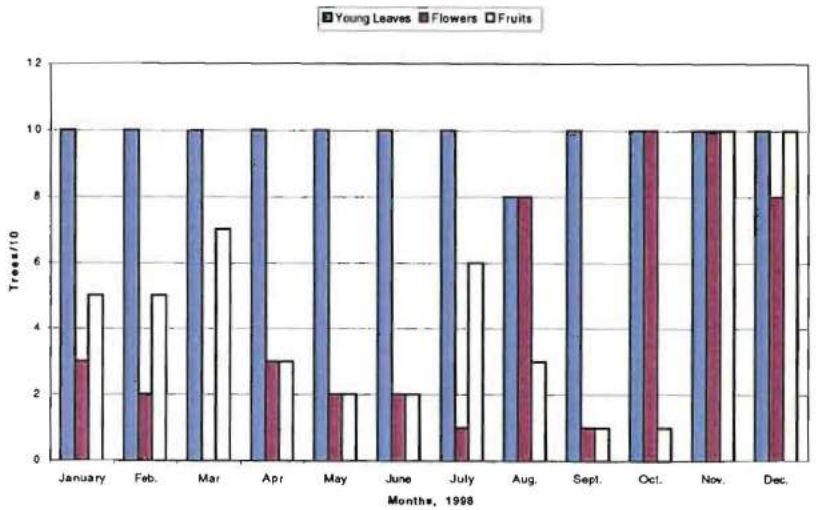

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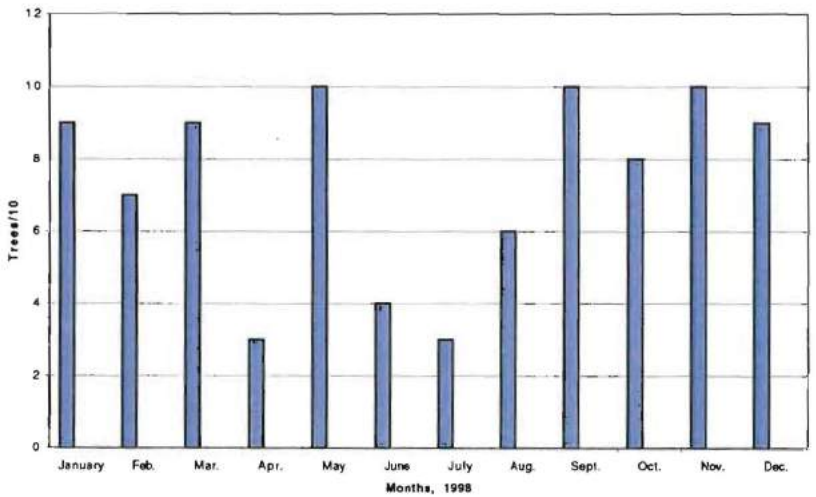
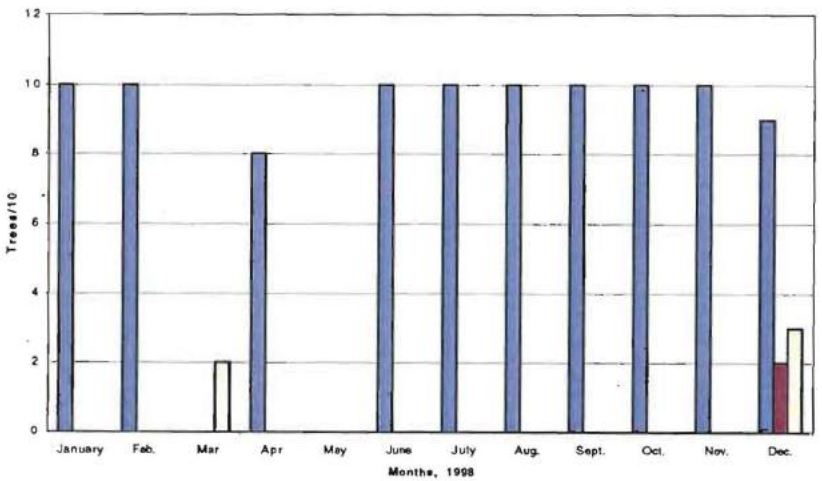
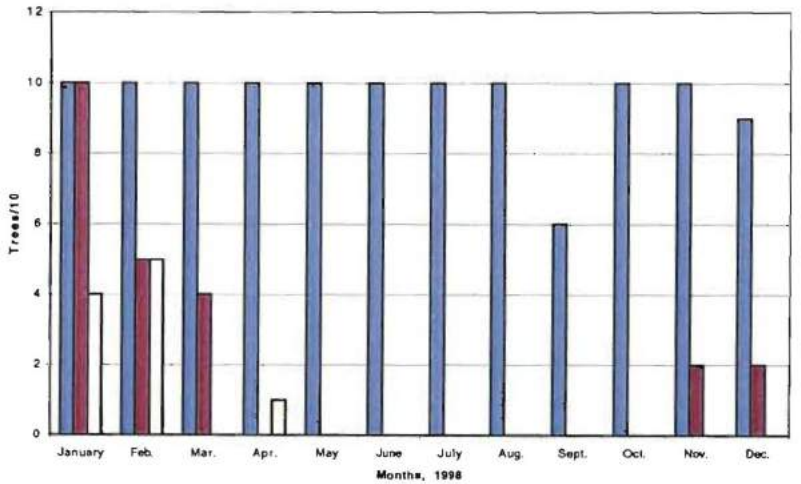


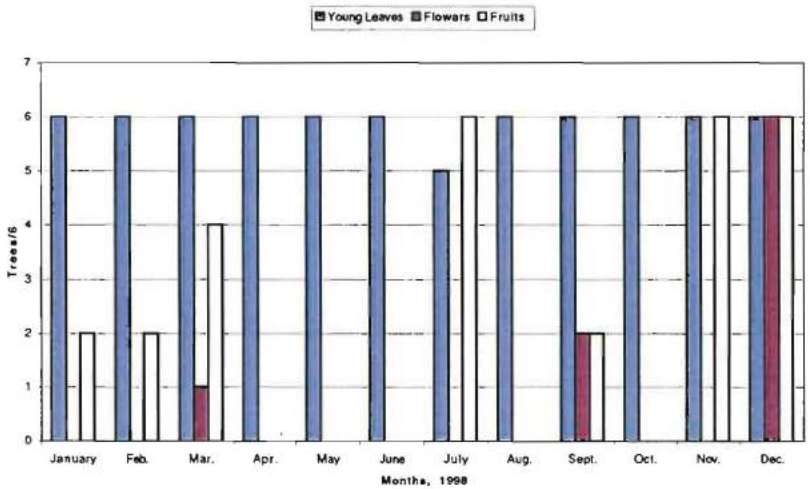
Lecaniodiscus Fraxinifolious



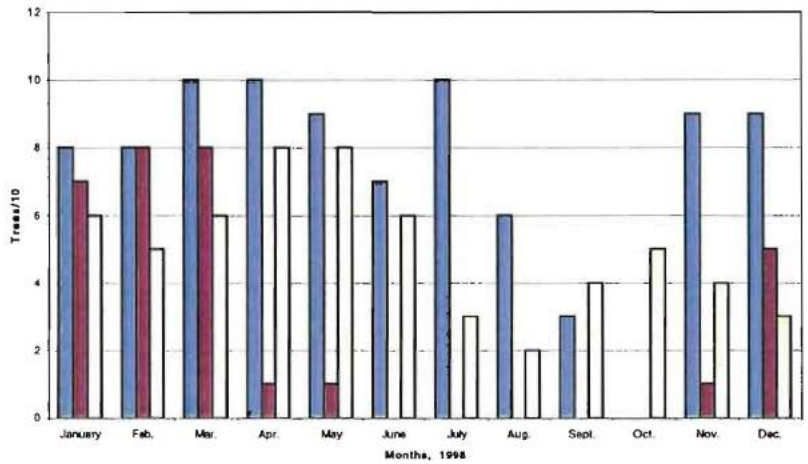
Lanea Welwitschi



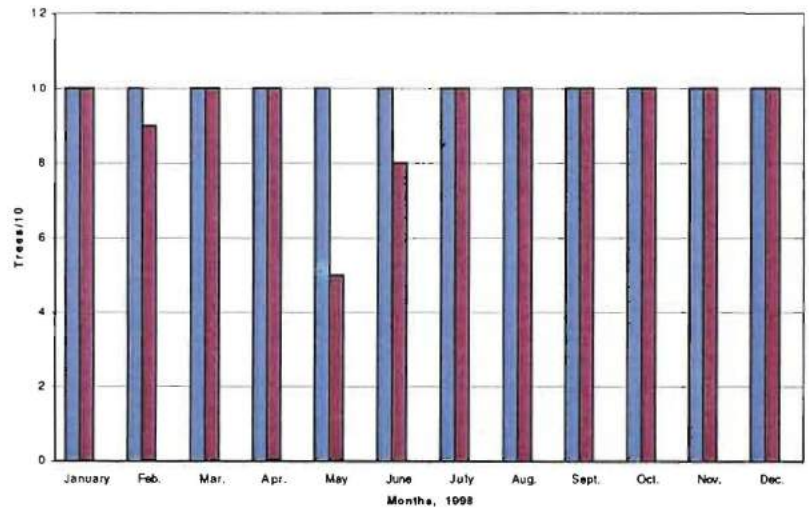
Ficus Lingua



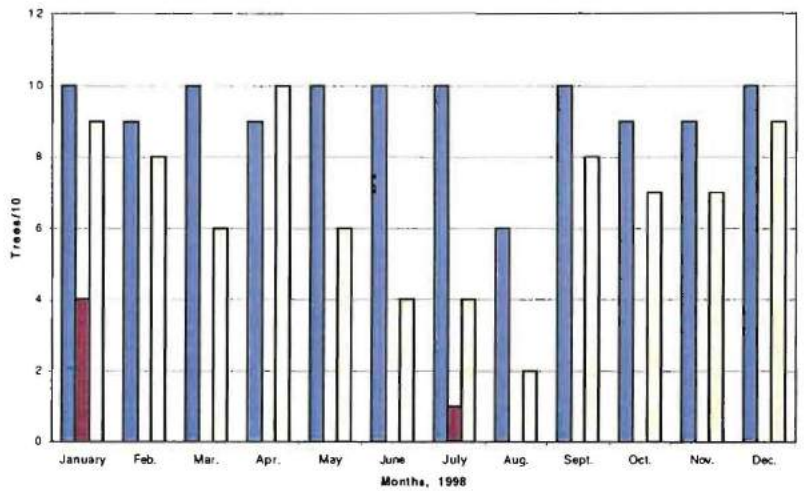
Adansonia Digitata (Baobab)



Bourgainvillea spectabilis



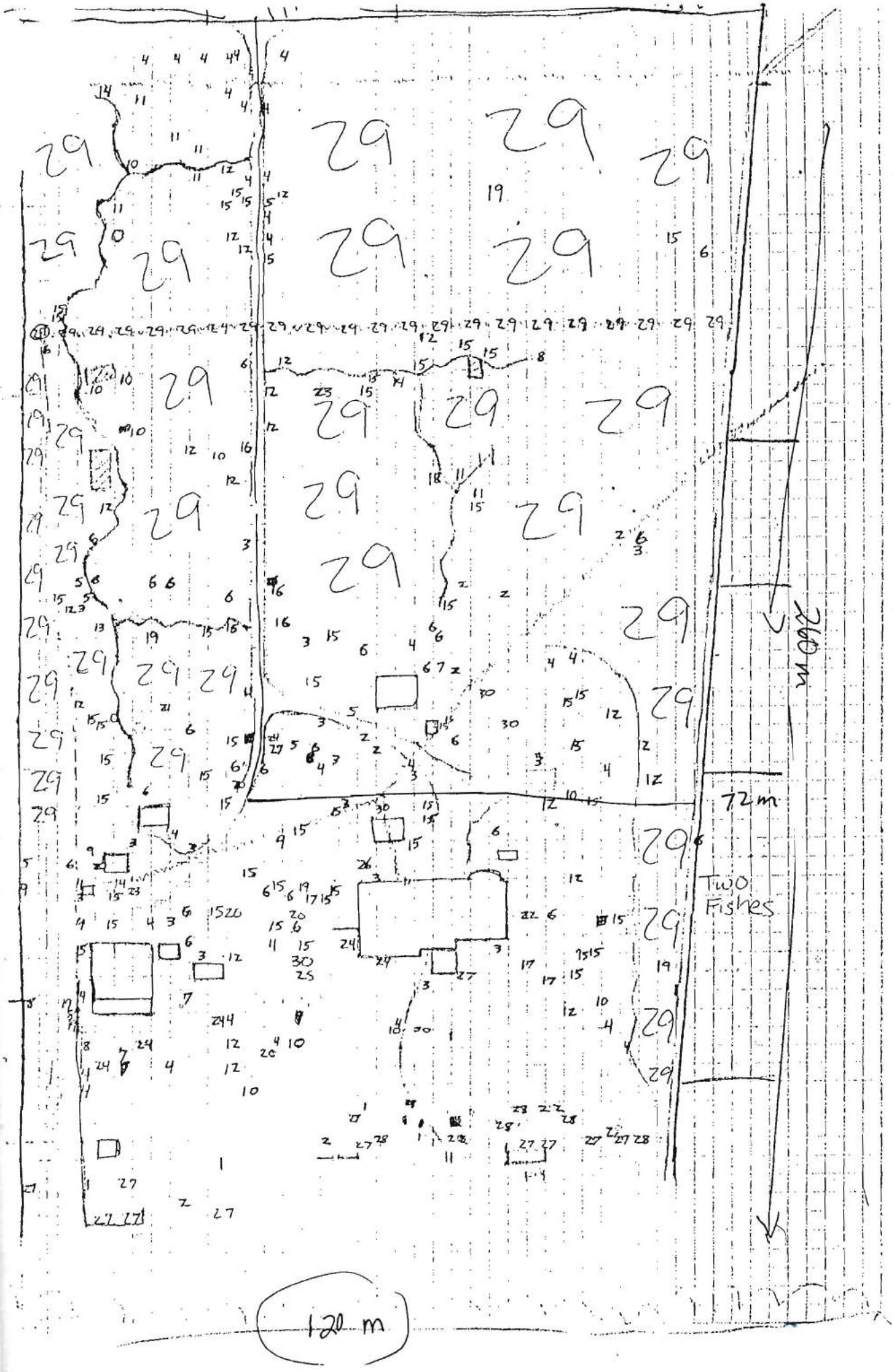
Ficus Sycamorous



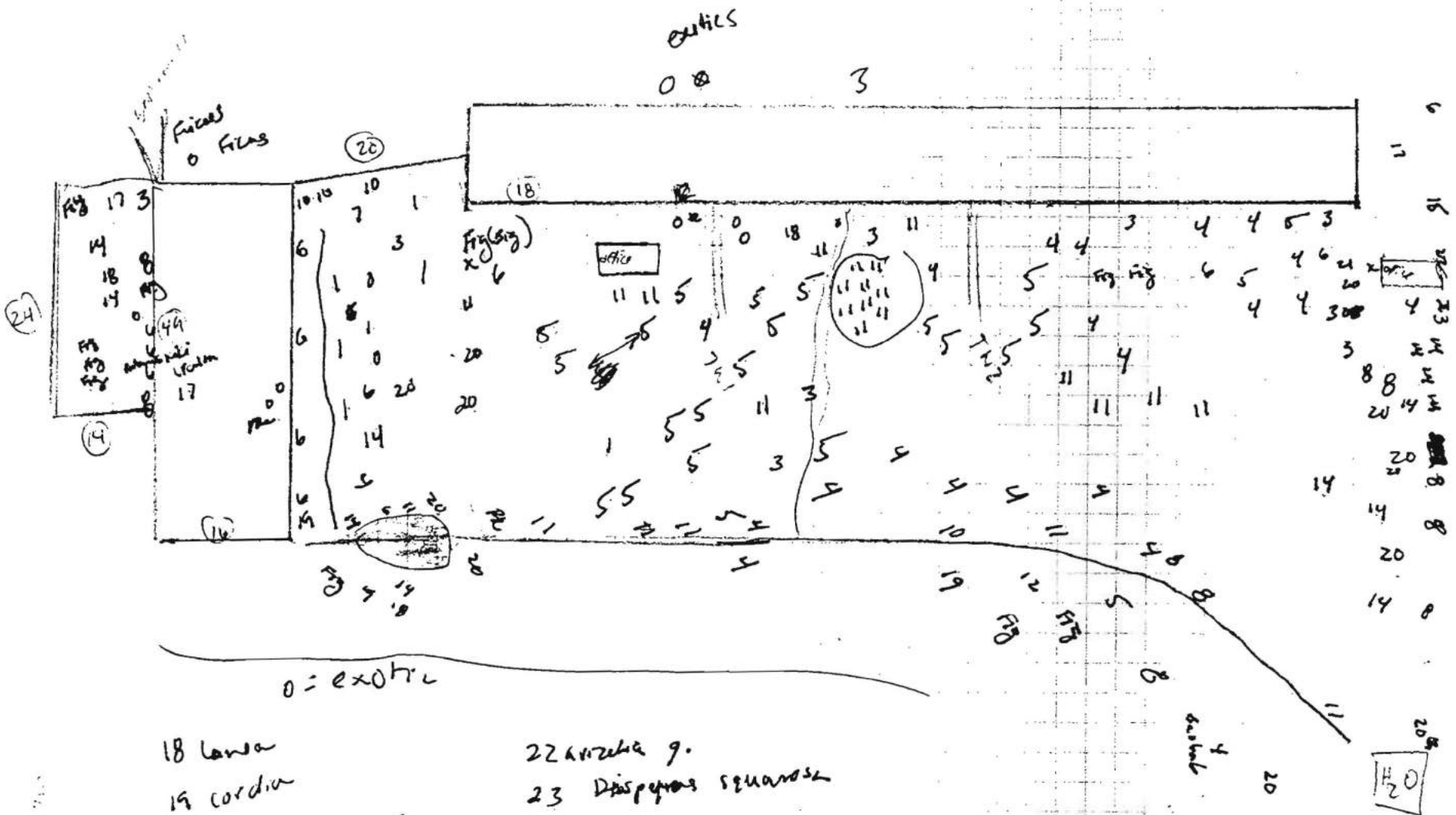
VEGETATION KEY, COLOBUS COTTAGE

1- Whispering palm, *Casuarina equisetifolia*
2- *Sideroxylan inerme*
3- *Diospyros squarrosa*
4- Flamboyant tree, *Delonix elata*
5- Neem tree, *Azadrachta indica*
6- *Lannea welwitschii*
7- *Ficus lingua*
8- *Ficus sur*
9- *Ficus sycamorus*
10- Prickly ash, *Zanthoxylum chalybeum*
11- *Suregada zabzibariensis*
12- *Grewia plagiophylla*
13- *Grewia vaughanii*
14- Baobab, *Adansonia digitata*
15- *Lecaniodiscus fraxinifolius*
16- *Leucaena latisiliqua*
17- *Haplocoelum inoploeum*
18- *Zizphus mucronata*
19- *Trichilia emetica*
20- *Pycnocoma littoralis*
21- *Majidea zanguebarica*
22- *Terminalia catappa*
23- *Combretum schumannii*
24- *Franch pane*
25- *Bougenvilla*
26- Mango, *Mangifera indica*
27- *Pandanus kirkii*
28- Coconut palm, *Cocos nucifera,*
29- Low vegetation
30- Exotic plant

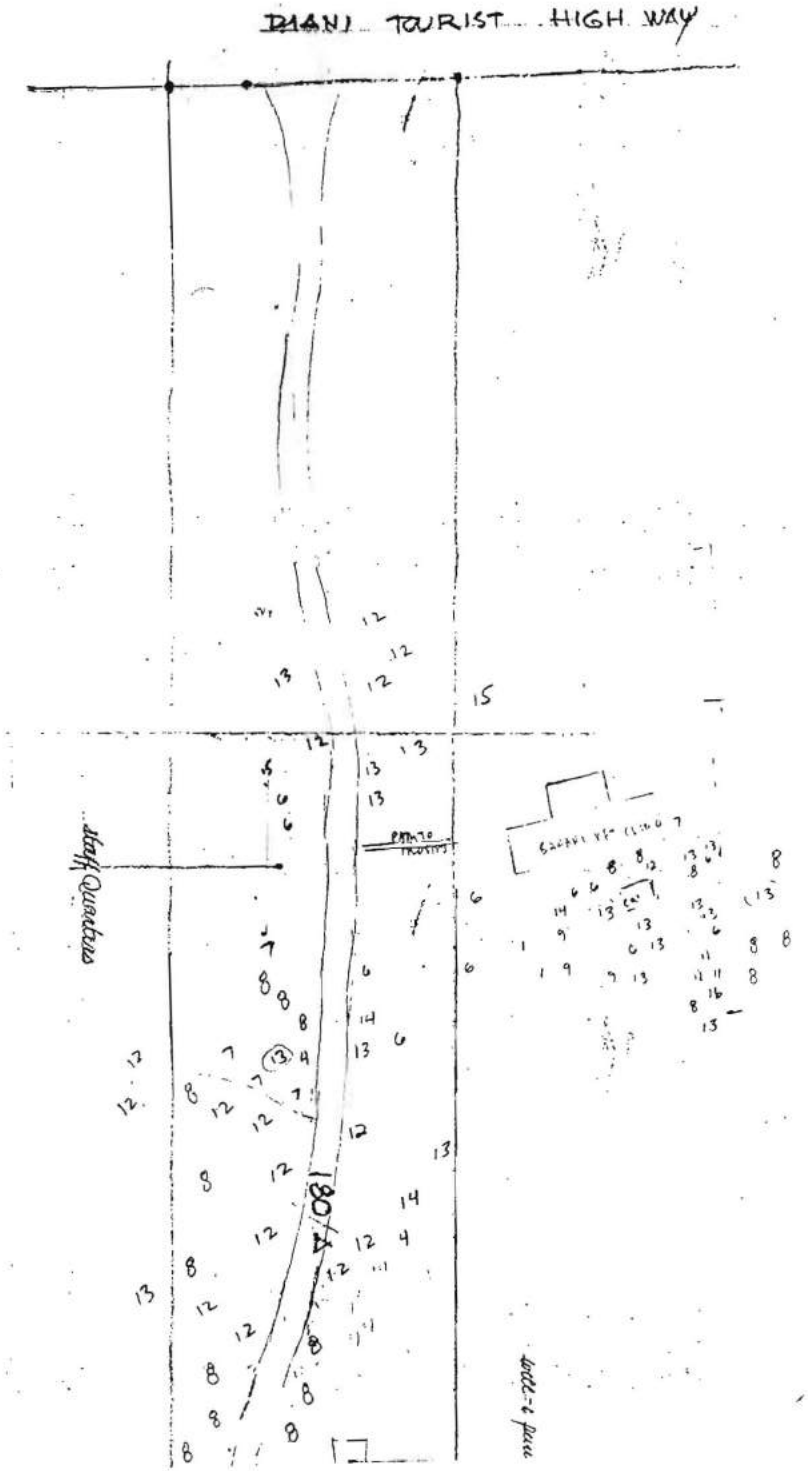
COLOBUS COTTAGE

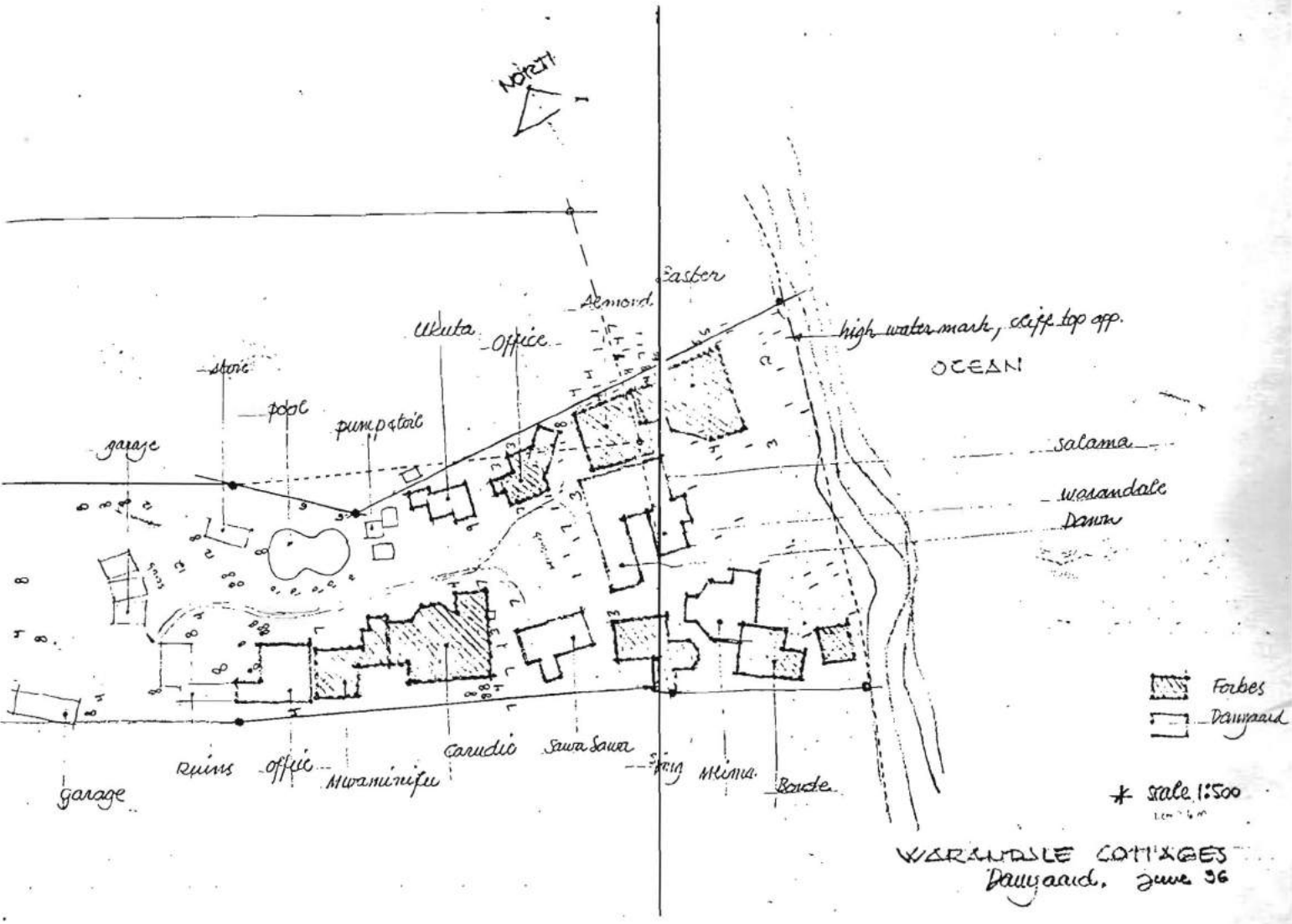


Trade winds



18 Lonas
19 cordia
20 ficus sycamorus
21 Trachilia emitica
22 arizelia 9.
23 Diospyros squarrosa


DIANI TOURIST HIGH WAY
Staff Quarters
Petrol station



WARANDALE COTTAGES
Dunyaad. June 96

Appendix C. Laboratory Analysis

The first laboratory procedure I used to prepare my fecal samples for radioimmunoassay (RIA) was modeled after a method designed by Samuel Wasser at University of Washington (Wasser *et al.*, 1991, 1994, 1996). With a mortar and pestle, 0.5 g of each fecal sample was ground into a fine powder. Large seeds, coral chips, and other inert materials were removed before weighing. Weighed samples were placed in 15 ml, 17 x 120 mm screw-cap vials (Sarstedt Inc., Newton, NJ) and boiled in 10 ml absolute ethanol for 20 minutes. Samples were then centrifuged for 10 minutes at 4,400 g's (20° C) and stored at –80° C until used for testing. For the first test, two tubes were thawed and centrifuged for 5 minutes at 4,400 g's. Then, the ethanol fraction was poured into a new centrifuge tube and the fecal pellet was re-extracted with another 5 ml of ethanol. Each tube was vortexed for a minute and then centrifuged for 5 minutes at 4,400 g's at room temperature. The second ethanol extract was then added to the first, and a variety of volumes of the combined extract were analyzed with a RIA kit according to the manufacturer's directions (Diagnostic Products Corporation, Los Angeles, CA; Coat-a-Count Cortisol). In the antibody-coated tubes, 50, 100, 200, and 500 µL aliquots were dried under a stream of nitrogen and reconstituted with 25 µL of human serum and 1 ml of I^{125}-labeled cortisol. These tubes were then incubated for 45 minutes at 37° C. After the incubation, tubes were emptied into a radioactive waste depository and allowed to drain upside down to dryness. Radioactive counts were done for one minute on a gamma counter (Beckman Coulter, Inc., Fullerton, CA). Data were not indicative

of the proportional changes in volume, likely because of the large amount of solid impurities remaining in the bottom of the tube. It is possible that these impurities prevented cortisol from binding to the antibody sites on the walls of the tube. Also, drying ethanol under nitrogen is takes quite a long time (25 minutes for 500 µl).

In an attempt to remove some of the solid impurities from the sample, I used a 0.2 µm syringe filter (Whatman Inc., Clifton, NJ) to strain the ethanol extract before evaporating the samples in the coated tubes. Filtering helped to remove some of the largest particulate matter, but the tubes used for RIA still had impurities that did not permit accurate replicate tests (60% variation in replicates).

The next series of methods that I tried involved extracting the ethanol sample with dichloromethane (CH_2Cl_2). The extra extraction would hopefully remove solid particulate or other polar-solvable compounds and allow the drying stage of RIA sample preparation to proceed more quickly. Simply adding CH_2Cl_2 to the ethanol extract however would require a physical separation. Snap freezing the tubes in a liquid nitrogen and toluene slurry (-100°C) was considered, but was too labor intensive for the large number of samples required and I could not rule out the possibility of some cortisol remaining in the ethanol fraction. Instead, the ethanol extracts were evaporated to dryness in a rotovap (BRAND!) and reconstituted in CH_2Cl_2. Replicates from testing after this additional extraction had approximately 50% variation. In a final modification of this technique aimed at removing more of the impurities which were causing problems in the RIA tubes, I evaporated the ethanol in each sample, reconstituted in water, and did a competitive extraction with an equal volume of

CH_2Cl_2. Replicates using this technique were much better (10-40% variation in replicate and spiked tests). On the basis of this success, I tried 48 samples with this technique. However, the end result was still too varied to be acceptable (35-64% variation). Again, particulate and brown impurities seemed to play a major role in the sample variation.

Next, ethanol extracts from samples were filtered on silica gel columns (60-200 c.u. and 10-40 μ silica gels) before being dried. The results here were less than satisfying however, and samples had 35-50 % variation. The main problem, other than impurities, could be the difficulty of suspending cortisol in ethanol as a homogenous mixture.

In an attempt to correct for this lack of homogeneity, fresh samples (0.25 g) were ground and extracted with 2 ml of methanol/acetone mixture (4:1). The extraction was filtered through a C18 Sep Pak Plus syringe filter (Waters Corp., MA). Columns were first washed with 5 ml water and then activated with 1 ml of methanol. Then, the sample was loaded onto the column, and the cortisol was eluted with 2 ml of methanol. Sample replicates were much more accurate with this technique (less than 19% variation) and colored impurities were not present after reverse column chromatography. This laboratory success prompted the purchase of 100 C18 Sep Pak VAC tubes and the procedure as described in the methods section of the Cortisol chapter.

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