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Aggression Dynamics and Hormone Fluctuations in Black and White Ruffed Lemurs (Varecia variegata)

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Abstract:

Black and white ruffed lemurs (*Varecia variegata*) are critically endangered primates from the Northeastern rainforests of Madagascar. This species shows clear female dominance in both feeding and social contexts. In captivity, this dominance hierarchy can lead to heightened aggression between females during the breeding season, which can result in animal separation or group reconfiguration. The objectives of this study are to determine the scope of this species-specific management strategy throughout AZA-accredited zoos, determine the influence of two types of feeding enrichment on aggression levels, and determine the effect of aggression on stress levels by measuring fecal glucocorticoids (fGC). Understanding aggression and its potential effects on stress levels can help animal managers keep multi-female groups together long-term, which promotes natural grouping configuration and social interactions. The multi-institution survey had a 90.9% response rate, with 70% of zoos reporting heightened aggression in their groups. A total of 52 hours of behavioral data were recorded by live observation and video at the Saint Louis Zoo and fecal samples were collected from all females daily. Study groups included a multi-female family group (3 females and 3 males) and an unrelated group consisting of 1 female and 2 males. Aggression rates were higher when food was presented in more complex enrichment feeders (males and females: $n=802$ interactions, $p<0.10$, exact sign test; females only: $n=604$ interactions, $p<0.05$, Wilcoxon signed-rank test), which is contrary to a previous study for this species. Furthermore, the breeding female living in the multi-female group shows significantly higher fGC than the female housed with only males (*$n=179$, $p<0.001$, exact sign test). Potential management strategies and future research can be suggested from these results.

Introduction:

Black and white ruffed lemurs (*Varecia variegata*) are critically endangered primates from the Northeastern rainforests of Madagascar. This species shows clear female dominance during both feeding and social contexts (Kaufman 1991, Raps & White 1995, Overdorff et al. 2005). There is an age-reversed dominance hierarchy in which daughters typically outrank their mothers by the age of 3 (White et al. 1992, White 2009). This social system has created significant challenges for managing captive family groups because the dominant individual is often aggressive towards subordinates. While agonistic interactions can be important for maintaining the dominance hierarchy and group cohesion, these interactions can occasionally escalate and result in injury, sometimes leading animal managers to temporarily separate individuals, or in some cases, reconfigure the social groups entirely (Porton, pers. comm. 2014).

Black and white ruffed lemurs are strictly seasonal breeders, with the breeding season occurring between May and July in Madagascar; however, because their breeding season is primarily dependent on photoperiod, the breeding season occurs October through April in the Northern hemisphere (Brockman, et al. 1987). The female's vulva is impenetrable, except during a short window of estrus, which can occur up to three times per breeding season (Brockman, et al. 1987). During estrus, the female's vulva swells and opens, followed by a color change from light pink to deep pink when it opens fully for a period lasting from 24 hours up to 5 days (Boskoff 1977, Brockman, et al. 1987, Foerg 1982). This external presentation of estrus allows animal managers to easily monitor and record reproductive status in captive females throughout the breeding season.

From 2014 through 2015, the Saint Louis Zoo housed 2 groups of *V. variegata*. The first was a family group consisting of a breeding pair and their 2 sons and 2 daughters. The age-reversed dominance hierarchy indicates that the older of the 2 daughters was the dominant

individual for that group because the younger daughter was still a juvenile at 1.5 years of age during the main study season (2014-2015). The second group contained 1 breeding female and 2 males, both of whom were genetically appropriate matches for the female. These groups provided a suitable study system - a multi-female group and a control single-female group - for preliminary observations on the influence of housing multiple females together.

In addition to understanding the behavioral aspects of intragroup aggression, it is necessary to begin understanding the underlying physiological states that can be influenced by behavior and social setting; specifically, the release of glucocorticoids (GCs). When a mammal undergoes stress, the anterior pituitary gland secretes adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to release GCs. When released, GCs promote the synthesis of glucose, resulting in the creation of the necessary energy to cope with the acute stress (Hadley 2000). Over long periods of time, the prolonged release of GCs can have negative health effects, ultimately leading to a shorter lifespan (Sapolski et al. 2000, Pride 2005). For this study, monitoring fecal glucocorticoids (fGC) was a straightforward and noninvasive way to measure the physiological stress response. By measuring daily fGC, it was possible to determine whether individuals had a prolonged, or chronic, release of glucocorticoids, which have been associated with higher mortality rates in a related species (*Lemur catta*, Pride 2005). Furthermore, a study on wild *L. catta* shows higher fGC levels in dominant individuals (i.e. the individuals who initiate more aggressive interactions) (Cavigelli et al. 2003).

One environmental factor that could have an effect on group behavior and stress is enrichment. Enrichment is anything added to an animals' environment that can encourage natural behaviors; for example, adding trees to encourage climbing or adding a mixture of wood shavings and seeds to encourage natural foraging. Captive animal managers have used feeding enrichment devices in a variety of ways to influence animals' behavior, including approaches to minimizing

aggression (Boccia & Hijazi 1998, Zimmerman & Feistner 1996). A previous study examining the effects of food presentation using enrichment showed decreased levels of both intra- and inter-specific aggression between *V. variegata* and *L. catta* in mixed species exhibits when food was presented in hanging enrichment devices rather than scattered on the ground (Zimmerman & Feistner 1996). However, a study examining intrasex aggression for *V. variegata* is absent from the literature. These observations would be particularly valuable during the breeding season when female-female aggression is at its highest.

These results can have both short-term and long-term impacts on the management of this species. By determining the factors that potentially lead to stress, animal managers can develop strategies to either keep groups together, or separate when necessary. This study seeks to clarify the potential causes of heightened aggression between females, which could lead to the establishment of a management plan to keep multi-female groups together long-term, when possible. By integrating the results from behavioral study and the endocrine data, it will be possible to determine a threshold of acceptable levels of aggression within a group that avoids widespread or chronic stress. Furthermore, collecting and analyzing survey data from several AZA-accredited institutions housing multi-female groups will lead to a better understanding of the scope of this management complication and the importance of developing mitigation strategies.

Because of their critically endangered status, this species is managed in captivity under an Association of Zoos and Aquariums (AZA) Species Survival Plan (SSP). Captive-bred individuals have been successfully released into the wild in the past (released from 1997-2001, Britt et al. 2004), and the captive population must be managed as if this is a possibility in the future. Therefore, maintaining natural behaviors, groupings, and social interactions is essential. In the wild, this species can be found in multi-female, multi-male groups of up to 30 individuals when resources are plentiful (Pereira et al. 1988). There is evidence of social learning in this species, as

well as direct mother-offspring exchange of information crucial to survival; therefore, maintaining natural behaviors will have lasting effects as these individuals reproduce in captivity (Stoinski et al. 2011). Management practices involving premature separation may result in decreased social development and stimulation for individuals involved and could ultimately decrease their candidacy for reintroduction programs in the future. However, if chronic stress due to social environment is decreasing the health and life expectancy for individuals involved, potential reintroduction candidacy may not be the top priority as there are no plans for reintroduction in the foreseeable future.

Predictions:

1) Aggression rates among group members will be lower when provided with more complex enrichment.
2) Group aggression and individual stress levels will be positively correlated.
3) Stress levels will be higher for the females living in the multi-female group; specifically, the dominant female will have the highest overall fGC levels.

Methods:

This multifaceted project incorporated survey data from other AZA-accredited zoos holding multi-female groups, as well as live behavioral observations at the Saint Louis Zoo, including video recordings, fecal collection, radioimmunoassay, and supplementary records kept by the animal keeper staff. Methods for each approach are described below.

Institution Surveys:

A survey was sent to 11 AZA accredited zoos that housed multi-female groups of *V. variegata* as of February 2015 using the online survey program "Surveymonkey". The questions were focused on intragroup aggression with additional questions for institutions that indicated female-female heightened aggression. The results of the survey provide a clearer picture of the breadth of this species-specific management strategy.

In order to measure the reliability of the responses, participants were asked to rank their ability to identify individual lemurs when they are stationary and when rapidly moving. Rankings were between 1 (Not at all confident) and 5 (Very confident). If an institution scored less than an average of 3 for both categories, their results were not used in further analyses. Specific survey questions are provided in Appendix I.

Behavioral Observations:

One of the primary objectives of the study was to document the differences in agonistic interactions throughout the breeding season when food was presented in two different feeding enrichment devices. One feeding device is considered easy for the animals to retrieve the food, and the second device is considered difficult. The "easy" devices are hanging bowls in which all diet items were presented in rubber bowls hanging from multiple plastic chains. These require little to no manipulation and time commitment (<10 seconds) from the animal to retrieve a diet item. The "difficult" devices are suet feeders, which are mesh boxes (8" x 4" x 7") traditionally used as bird feeders. They provided the animals with a challenge by requiring them to use their hands and teeth to manipulate and pull the diet items through the holes in the wire mesh. The suet feeders required a prolonged time commitment (>30 seconds) from the animal to retrieve a diet item. There were 6 enrichment devices offered each day – 1 per animal to eliminate competition over limited feeding

stations. The diet items were rotated based on availability, but no high quality items such as fruit or special treats were offered on observation days. Diet items consisted of a rotation of vegetables and starches, a rotation of leafy greens, and Mazuri brand primate chows.

Figure 1: Bowl ("Easy") Feeder in use (left; photo by Alicia Marty); Suet ("Difficult") Feeder in use (right; photo by Ethan Riepl)

Behavioral observations took place 4 days per week from 01 December 2014 through 28 February 2015 for the family group only. The team of 4 research assistants underwent a minimum of 3 training days with the primary author (AM) to ensure 90% or greater inter-observer reliability; additional validation was possible due to the video recordings (described below), which allowed us to confirm each individual behavior scored. On each observation day, the group was observed for 30 minutes coinciding with their AM feeding and again for 30 minutes during the PM feeding, based on the pilot season results described in appendix III. The group was shifted into the off-exhibit area prior to the beginning of each observation, food was placed on exhibit only in the specified feeding enrichment devices, which were always hung in the same locations in the habitat, and the

observation began the moment the shift door opened to give the animals access to the public exhibit. Of the 4 observation days per week, there were 2 days in which each enrichment type was used. This led to a final sample size of 52 observation periods for each enrichment device.

Observers were stationed on the public side of the exhibit and recorded all occurrences of ethogram behaviors (Appendix II), which included five event behaviors: chase, charge, cuff, displace, and a combination charge/cuff behavior (adapted from Pereira et al.1988; ethogram created by primary author AM). The information recorded for each behavior included: exact time to the second, location in the exhibit, the individual that initiated the agonism, the recipient of the agonism, and the specific behavior observed. In addition to the live observations, video recordings were taken from 4 different angles. By having both a live observer and the video recordings it was possible to accurately identify the individuals in real time, and watch the interactions repeatedly to gather detailed sequence and time information; many of the interactions occurred in rapid succession and were difficult to identify all details in real time.

Fecal Collection & Radioimmunoassay to measure hormone levels

Fecal samples were collected daily from all females in the family group as well as the female in the single-female group; this allowed us to compare stress levels for females in different social settings. Every morning, individuals were given 1.0mL of veterinarian approved non-toxic food coloring on a diet item. Each individual was given a unique color, allowing the animal keepers to collect daily fecal samples from each individual with 100% certainty. Keepers collected the fecal samples by entering the exhibit area while the animals were secured in an off-exhibit location. The samples were collected with a wooden tongue depressor and placed in a labeled plastic bag and immediately frozen.

Fecal samples (total n=360) were extracted, prepared, and analyzed using

radioimmunoassay following the methods described in Dumonceaux (2006); methods that have been validated for a variety of species. Approximately 0.5g of wet feces from each sample was used for the extraction process. Samples were extracted using a 1:1 solution of phosphate-buffered saline and Methanol. The samples were then shaken overnight and the remaining liquid was poured off the solid fecal material the next morning, which was then centrifuged and used for the radioimmunoassay. The solid fecal material that remained after being shaken overnight was dried and weighed; all fGC values reported in the results are based on the dried weight. All assays were conducted by the primary author (AM) at the Saint Louis Zoo's Endocrinology Lab using MP Biomedicals, LLC ImmuChem™ Double Antibody Corticosterone ^{125}I RIA Kit. The lower detection limit of the assay was 0.26 ng/ml and upper detection limit was 20 ng/ml. Assays were performed according to manufacturer's protocols with the exception that the matrices were equalized by adding standard diluent to the fecal extracts and fecal extraction buffer (containing 50% methanol) to the standards. Concentrations were determined as ng/ml, and then divided by the dry weight of extracted feces to give the results as ng/g feces. All samples were assayed in duplicate.

Supplementary Records:

The animal keepers monitored the reproductive status of all female lemurs every day throughout the breeding season by non-invasive visual examinations. Each female lemur was trained to climb on the mesh in the off-exhibit areas. When the lemurs were in the hanging position, keepers were able to view the vulva, as well as conduct a full-body scan for any injuries. Detailed records were kept regarding the coloration, presence of swelling, and status of their vaginal opening. These records were used to compare the behavior and endocrine data with the estrous stages of the study females.

Statistical Analyses:

All data comparisons were first measured for normality using the Shapiro-Wilk test. All non-normal data that could not be transformed to become normal and not skewed were analyzed using Mann-Whitney U-test, Wilcoxin signed rank test, or an exact sign test, depending on the presence of skew in the data. The transformation used, if needed, was the square root of the value + ½ for the aggression data, a standard transformation used for count data.

Results:

Survey Results:

The survey resulted in a 90.9% response rate; 10 of the 11 contacted institutions responded. All of the respondents reported an average score of 3 or higher for the individual identification questions, indicating that they assessed their responses as reliable. Of the 10, 7 zoos reported that their multi-female group experienced heightened aggression at some time of the year. The remaining results pertain to only the 7 institutions indicating heightened group aggression.

The heightened aggression was described in the survey as consistent occurrences of charging, chasing, hitting, or biting that do not appear to be related to play. Of the institutions that indicated that their group did experience heightened aggression, 85.7%, or 6 out 7, report that the agonism occurs specifically between females, and 83.3%, or 5 out of 6, of the female-female agonism occurs between mother and daughter. Furthermore, 57.1%, or 4 out 7, of the institutions report that the interactions have resulted in injury requiring veterinary attention, although only rarely. Only 2 institutions indicated a specific time of year that heightened aggression occurred – November through February; the remaining 5 institutions indicated that they were unsure or there was no specific time of year.

Behavior Results:

A total of 52 hours of observations were conducted, consisting of 104 30-minute sessions. The most frequently occurring behavior was "charge/cuff," while "charge" (with no physical contact resulting) occurred the least; all behaviors occurred the most between females (Figure 2, Table 1).

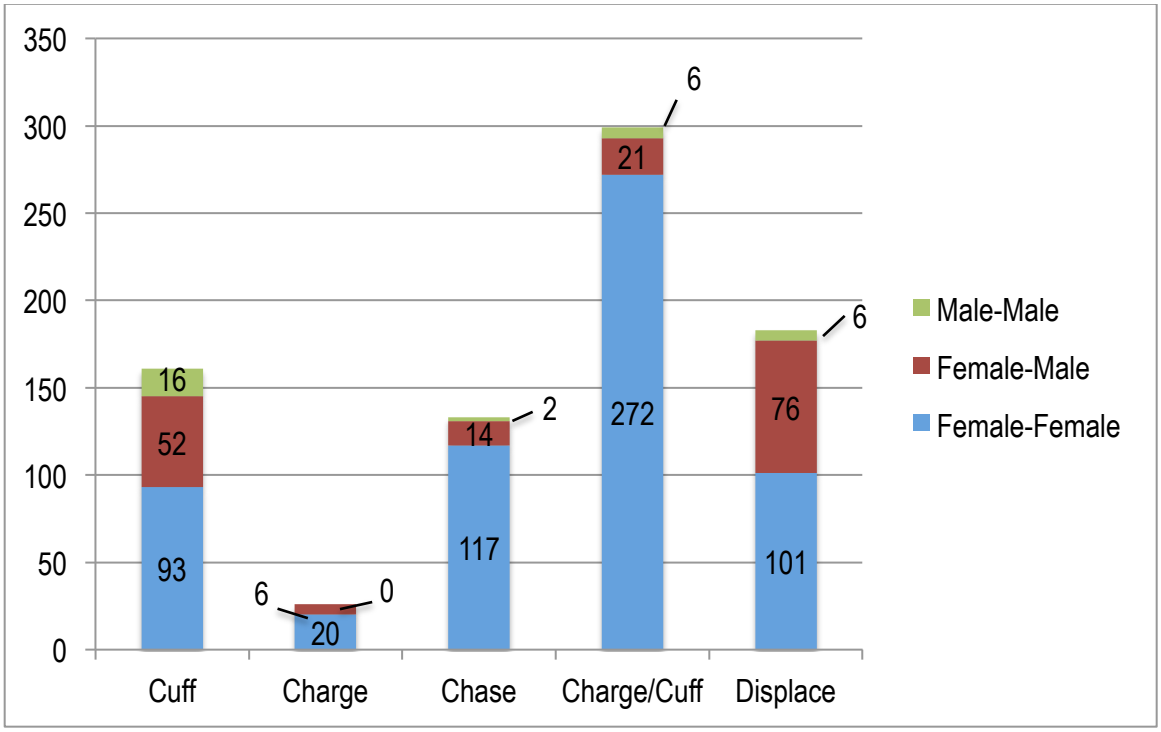

Figure 2: Number of occurrences of each behavior, divided into: female-female interactions (blue), female-male interactions (red), and male-male interactions (green).

	Cuff	Charge	Chase	Charge/Cuff	Displace	Totals
Female-Female	93	20	117	272	102	604
Female-Male	52	6	14	21	75	168
Male-Male	16	0	2	6	6	30

Table 1: Number of occurrences of each behavior, divided into: female-female interactions, female-male interactions, and male-male interactions.

There were a total of 802 agonistic interactions initiated between group members, 604 between females only. Higher agonism occurred during suet feeder observation days than bowl feeder observation days; this relationship is present when all agonistic interactions are included, both between females and female-male (n=802 interactions, $p<0.10$, exact sign test, table 2) and when only female-female interactions are considered (n=604 interactions, $p<0.05$, Wilcoxon signed-rank test, transformed using "square root of the value + 1/2," table 2).

	# Observation Sessions	a) Total # of Aggressive Interactions (Group)	a) Average # of Aggressive Interactions (Group)	b) Total # of Aggressive Interactions (Females only)	b) Average # of Aggressive Interactions (Females only)
Suet Feeders	52	480	9.23*	344	6.61**
Hanging Bowls	52	322	6.19	260	5.00

Table 2: Number of aggressive interactions per observation session for either a) all group members or b) females only. Differences between enrichment types were nearly statistically significant for all group members (*n=802 interactions, $p<0.10$, exact sign test) and female interactions only (**n=604 interactions, $p<0.05$, Wilcoxon signed-rank test, transformed using "square root of the value + 1/2,").

As expected, the dominant daughter initiated the majority of agonistic interactions: 680 of 802 interactions, or 84.7%. The dominant female also received the second least number of agonistic interactions, with only the geriatric adult male receiving less. Figure 3 depicts the

distribution of agonistic interactions across all group members. Of the 680 agonistic interactions initiated by the dominant female, 559 or 82.2% were directed towards other females (table 3; $p<0.000$; exact sign test). The juvenile daughter was the second highest aggressor, with 72 interactions. All of these interactions were directed towards the dominant daughter or the males; she never showed any aggressive behavior towards her mother during the observations.

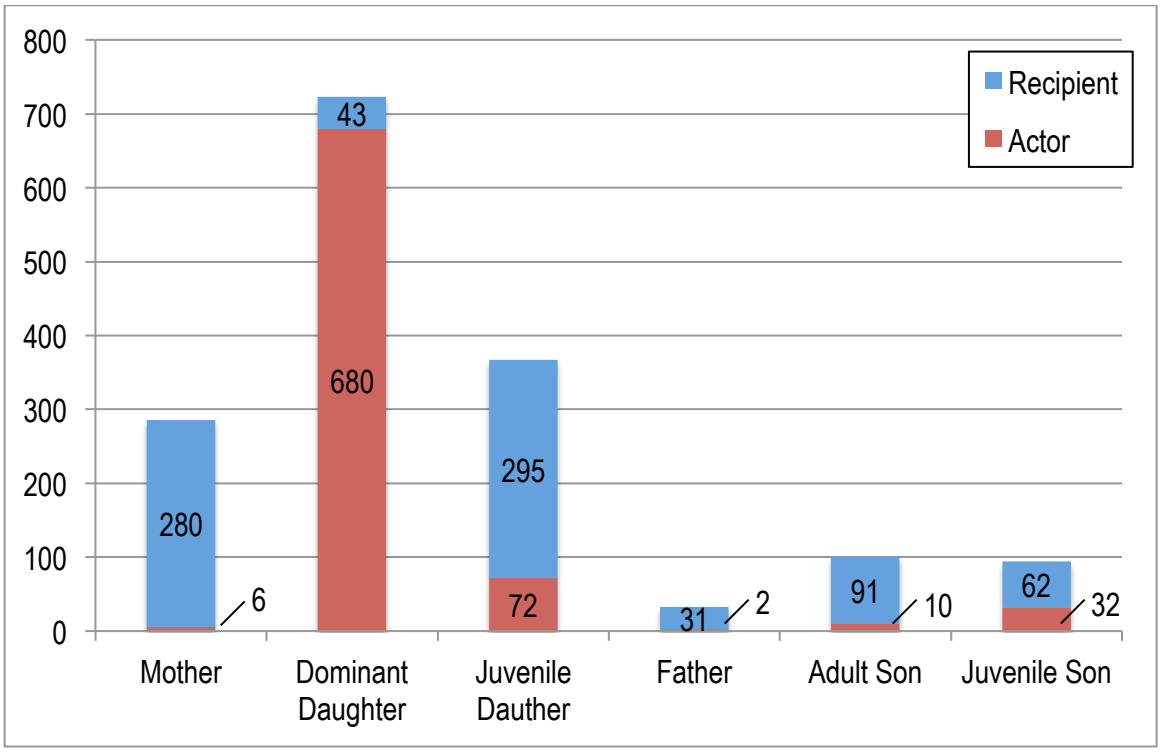

Figure 3: Number of agonistic interactions received (blue) and initiated (red) by each group member.

	Adult Female	Younger Daughter	Adult Male	Older Son	Younger Son	Total
Number of Interactions	273	286	21	64	36	680
% of Interactions	40.1%	42.1%	3.1%	9.4%	5.3%	

Table 3: Distribution of agonistic interactions initiated by the older daughter (the dominant individual) among other group members. The number of agonistic interaction directed at females versus males is highly significant (n=680; p<0.001; exact sign test).

Endocrinology Results:

The females living in the multi-family group had higher and more variable fGC levels than the female living in the single-female group (Figure 4). Furthermore, the breeding female living in the multi-female group had significantly higher fGC levels than all other females, including the female living in the single-female group (n=179 , p<0.001, exact sign test, table 4). It was clear within the multi-female family group, although not officially recorded, that the breeding female appeared more "stressed" than the other group members. She would typically not interact with enrichment very often, she appeared to be vigilant at all times, and she rarely left the lowest level of the habitat.

Female	n	Range (ng/g dry fecal)	Mean ± SE (ng/g dry fecal)
Breeding Female (Multi-Female Group)	89	5.71 - 784.25	52.78 ± 9.66 *
Dominant Daughter	90	4.97 - 526.48	31.99 ± 5.98
Juvenile Daughter	90	6.32 - 292.81	29.14 ± 4.21
Breeding Female (Single-Female Group)	90	8.04 - 73.95	17.79 ± 1.48 *

Table 4: Fecal glucocorticoid ranges and means ± standard error for all females. The breeding female in the multi-female group had a significantly higher average fGC than the female in the single-female group (*n=179, p<0.001, exact sign test).

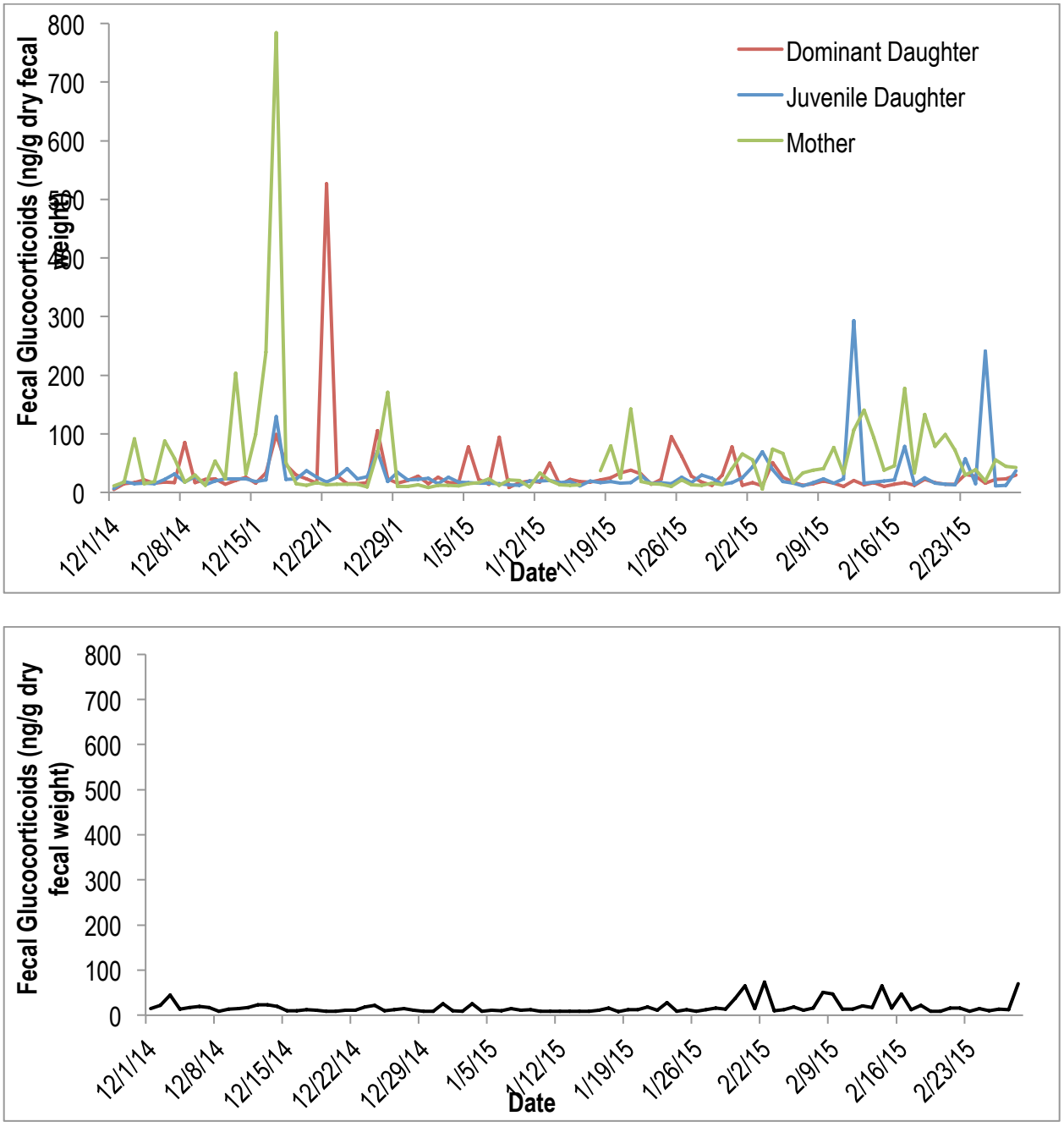

Figure 4: Top - Fecal glucocorticoid levels for the multi-female family group: Mother (green), dominant daughter (red), juvenile daughter (blue). Bottom – Fecal glucocorticoid levels for the breeding female in the single-female group.

Behavior, specifically the number of agonistic interactions, and fGC levels were tested for correlations; there was no positive or negative relationship found. Behavior of the group and behavior of only the females were compared to: individual female fGC levels, average fGC levels for all 3 females, fGC levels on the same day as the behavior occurred and the day after the behavior occurred. We tested for correlations between behavior and fGCs on the day after the behavior occurred because we were using fecal GCs rather than GCs in blood. Fecal GCs are a composite representation of glucocorticoids released over a period of time, whereas blood-bound GCs are a fairly immediate assessment of stress at that time; therefore, there is a lag time between the stressful event and the rise in fGC to consider when interpreting these values of up to 48 hours (Peel, et al. 2005; Whitten, et al. 1998).

Supplementary Records Results:

Reproductive status of all females was monitored through brief visual examinations daily. Throughout the study season, none of the females in the multi-female group showed external evidence of estrus; their vulvas remained almost entirely black in color and imperforate. However, this was to be expected for the 2 daughters, who were both given depot medroxyprogesterone acetate (Depo-Provera, Upjohn Pharmacia, Kalamazoo, MI; the contraceptive product recommended for lemurs by the AZA Wildlife Contraception Center) via intramuscular injections every 42 days throughout the breeding season. However, for the breeding female in the multi-female group who was not contracepted, this resulted in the second breeding season in a row that she did not show any signs of cycling.

The breeding female in the single-female group did show external evidence of a reproductive cycle between 6 February 2015 and 18 February 2015, during which time her average fGC increased from 16.21 ng/g (mean value across all non-estrous days) to 27.18 ng/g (mean

value between 6 Feb 2015 – 18 Feb 2015) (n=90, $p<0.01$, Mann-Whitney U-test). During this time, the female's vulva opened, breeding behavior was observed multiple times, and her vulva closed completely; however, she did not give birth to any offspring as a result.

Discussion:

Hypothesis #1: Aggression rates among group members will be lower when provided with more complex enrichment.

This study indicates that *more* complex, or difficult, enrichment resulted in higher aggression among group members – contrary to the original hypothesis that providing more complex enrichment will decrease group aggression. These results are also contradictory to another study for this species (Zimmerman & Feistner 1996). The results do fit with anecdotal information from the animal keepers, who noticed this trend in previous years and tried to provide minimal enrichment during the breeding season. Potential management implications can be made from this study, particularly regarding food presentation. If the heightened aggression occurs primarily during the breeding season, food could be presented in an easy to retrieve manner. By providing opportunities to retrieve food and vacate a station quickly, the subordinate lemurs may be able to avoid agonistic interactions with the dominant individual that are related to the enrichment stations. However, it is crucial that sensory or environmental enrichment still be provided to maximize mental and physical stimulation; for example, various scents sprayed in their habitat, cloth sheets hanging from branches, or additional substrates or climbing surfaces. This enrichment and food presentation strategy can likely be extrapolated throughout the year if agonism does not appear to be restricted to the breeding season alone.

Another approach could be to provide multiple types of complex enrichment to extend the

time directed at enrichment rather than agonistic interactions. Additionally, by providing more than 6 enrichment devices (more than 1 per animal) it could be possible to decrease the perception of competition over food; a 1:1 ratio of enrichment device to animal may not be the ideal ratio for minimizing aggression during feeding times. The lemurs could have perceived a competitive and tense environment based on the time commitment involved in retrieving the food at potentially limited feeding stations. Additionally, while high-quality food items, such as bananas or grapes, were provided in the enrichment devices during the study, the lemurs have received those diet items in those feeders in the past. They may have a high level of tension immediately after gaining access to the display with the enrichment devices because they are anticipating high quality food items that may be worth fighting over.

Hypothesis #2: Group aggression and individual fGC levels will be positively correlated.

The results from this study did not indicate a statistical relationship between the number of agonistic interactions and the fGC levels for the females in the multi-female group. On 17 December 2014, a rise in fGC is visible for all three females in the multi-female group. Although behavioral observations were not conducted on that day specifically, the animal keepers made note of particularly high aggression among the females that resulted in minor injuries for both of the daughters. The breeding female was noted to have avoided the interactions, although her fGC rise is substantially higher than her daughters (784.25 ng/g versus 99.28ng/g for the dominant daughter and 129.832 ng/g for the juvenile daughter). However, the heightened aggression and the fGC rise for the females occurred on the same day, which does not show the expected lag time between stressful events and visible GC rises in the feces. Another potential explanation is that the heightened fGC was the precursor for the agonism, rather than the opposite. There is another instance where all three females show a rise in fGC simultaneously, on 28 December 2014.

Behavioral observations did take place on this particular day and the number of agonistic interactions was less than the average of 6.13 interactions per session on bowl feeder days; there were 2 agonistic interactions during the AM observation and 4 agonistic interactions during the PM observation. The 2 days prior also had less than average agonistic events recorded during the observation periods. Although our behavioral observations spanned 3 months, they only represent 1 hour total on 4 days per week, meaning that these results are only representative of a very small window of time. Conducting a video study that records behavior for a larger portion of time may provide different insight into group behavior.

The main restriction on interpreting the behavior and endocrine data is that the fecal samples were not collected at a standard time each day. Ruffed lemurs have a rapid gut-transit system and they can defecate as often as every 3 hours. Therefore, if fecal samples were collected in the morning on one particular day and the afternoon on the next day, these results could show the fGC resulting from either the previous day's behavior or that same morning's behavior. A future study for this species should aim to collect fecal samples around the same time of day, or each lemur's first daily defecation, for example.

Hypothesis #3: fGC levels will be higher for the females living in the multi-female group; specifically, the dominant female will have the highest overall fGC levels.

Overall higher and more variable fGC levels for the females in the multi-female group was expected based on a more complex social environment (Gould et al. 2005) – the family group had 6 members compared to 3 in the single-female group. The breeding female in the multi-female group had the highest overall average fGC of all females and based on the age-reversed dominance hierarchy and our behavioral observations, it is clear that the breeding female is

subordinate to her oldest daughter.

A previous study on a related species led us to predict that the dominant individual would have the highest fGC; however, that was not the case in this situation in which the subordinate adult breeding female had the highest mean fGC of all females in the study. Additionally, the breeding female was the recipient of 40.1%, or 273 out of 680 agonistic interactions initiated by the dominant daughter. She did not receive the highest number of interactions, the juvenile daughter received slightly more at 286 out of 680 or 42.1%. This indicates that while the breeding female did not receive the highest number of agonistic interactions, the combination of her timid and cautious behavior and the highest mean fGCs shows that she is potentially impacted negatively by the agonism. The fact that the breeding female has not shown external evidence of an estrous cycle for the past 2 seasons also indicates that she may be impacted physiologically by the targeted agonism. The next steps for this study would be to determine the costs and benefits of group separation compared to keeping the females together, despite the resulting effects of the agonism.

This research can serve as the basis for many future studies. Primarily, studies involving a larger number of social groups could strengthen the validity of our conclusions, which in turn would assist our understanding of best practices for managing this species. *V. variegata* is difficult to study in the wild due to their habitat in the thick rainforest canopy; therefore, by generating a greater understanding of captive populations, it may be possible to make basic predictions regarding their wild counterparts. There are currently more than 200 individuals at over 70 institutions in North America, which creates a great potential for a large-scale project.

One aspect of this study that could be examined further is the influence of contraceptives on behavior. The two daughters in the multi-female family group were both contracepted with Depo-Provera throughout this study, making the specific influence of contraception impossible to

delineate. A previous study on a related species showed that Depo-Provera can have androgenizing effects on females because the females of a sexually dichromatic species experienced a transition to male coloration while contracepted (Asa et al. 2007); this effect could potentially impact the level of aggression exhibited by females contracepted with Depo-Provera. By observing multiple groups, with and without the use of contraceptives, it could be determined whether or not contraceptives can influence female behavior and glucocorticoid release. An alternative management strategy to the use of contraceptives is to briefly separate the females and males during the females' short window of estrous, as copulation is not possible at any other time.

Additionally, many research opportunities arise with a greater sample size; for example, the influence of group size or configuration, habitat size and setting (indoors versus outdoors), diet items, and management strategies (e.g. overnight separation). This potential research can have important implications for the captive population of *V. variegata*. Previous success with reintroductions and an increasing urgency for conservation action makes it more important than ever to ensure that captive populations exhibit natural behaviors, are housed in natural groupings when possible, and are breeding based on genetic recommendations.

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Appendix I:

Survey questions:

1. What is the name of your institution?
   _____

2. Are there any discrepancies between the taxon report included in the email and your current population of Black and White Ruffed Lemurs?
   _____

3. In your opinion, does each individual have unique natural markings that aid in identification and distinction from other group members?
   Yes
   No

4. Does your institution use any non-natural identification markers? For example: colored collars, tags, hair dye, shaved areas, etc.
   Yes
   No

5. On a scale of 1-5, how confident are the staff members in telling individuals apart when the animals are stationary?
   1 Not confident at all
   2
   3
   4
   5 Very confident

6. On a scale of 1-5, how confident are the staff members in telling individuals apart when the animals are running or interacting?
   1 Not confident at all
   2
   3
   4
   5 Very confident

7. Have you ever noticed heightened agonistic behaviors between individuals? Examples of agonistic behaviors: charging, chasing, hitting, or biting that do not appear to be related to play. (Some interactions are normal and healthy in order to maintain the groups' hierarchy.)

Yes
No

If yes is clicked, the following questions will appear:

a. Between which individuals have you seen agonistic behaviors (charging, chasing, hitting, biting)? Select all that apply.
   Brother & Sister
   Sisters
   Brothers
   Mother & Daughter
   Mother & Son
   Father & Daughter
   Father & Son
   Unrelated Male & Female
   Unrelated Males
   Unrelated Females

b. If 'Mother & Daughter' is selected, this question will appear: Does one individual initiate the agonistic interactions more than the other (which individual chases, hits, or bites the other)?
   Mother initiates the agonistic interactions more often
   Daughter initiates the agonistic interactions more often
   Both initiate an equal amount of interactions

c. If 'Sisters' is selected, this question will appear: Does one individual initiate the agonistic interactions more than the other (which individual chases, hits, or bites the other)?
   Older sister initiates the agonistic interactions more often
   Younger sister initiates the agonistic interactions more often
   The sisters are the same age
   Both initiate an equal amount of interactions

d. Is there a particular time of year that the group shows agonistic interactions more frequently?
   November – February
   March – June
   July – November
   N/A
   Other _____

e. Have the interactions ever resulted in an injury requiring veterinary involvement (For example: wounds requiring sutures)?
   Yes
   No
   If yes: How often?
      Rarely: Less than twice a year
      Often: 3-5 times per year

Frequently: 6-10 times per year
Monthly or Weekly

f. Have animal managers ever had to separate individuals for an extended period of time (More than 1 day) because of excessive agonistic interactions?
   Yes, temporarily
   Yes, permanently
   No

8. In a typical day, are the group members ever separated from each other (unable to have physical contact)? For example: during training sessions, habitat cleaning, feeding, etc.
   Yes
      Less than 10 minutes
      10 – 30 minutes
      30 minutes – 1 hour
      1-2 hours
      2+ hours
      Overnight
   No

9. Do staff members regularly check for reproductive status in the females during the breeding season (vulva checks)?
   Yes
   No

   a. If yes: Are records kept regarding vulva changes for each female?
      Yes
      No

   b. If no: Would you be interested in receiving training to monitor these changes?
      Yes
      No

10. Would you be interested in discussing any of these topics further?
    a. If yes: Please provide your email address: _____

Additional comments?

_____

OPTIONAL:

What is your position (curator, full-time keeper, part-time keeper, other)?
How long have you worked with this species?

Appendix II:

Ethogram:

Cuff: Actor strikes with hand(s) at recipient from a tripedal or bipedal stance

Charge: Actor rapidly approaches recipient, but no physical contact made

Chase: Actor sprints in pursuit of recipient

Charge/Cuff: Actor rapidly approaches or pounces on recipient and cuffs

Displace: Actor approaches within 1 body of length of recipient; recipient rapidly abandons location within 5 seconds

Appendix III:

Pilot Season:

The pilot season took place from 01 December 2013 through 28 February 2014. The main objectives of the pilot season were to determine differences in aggression levels during feeding observation sessions and non-feeding observation sessions, the time frame of activity after being fed, and a general understanding of the female-female relationships.

A total of 90 hours of behavioral data were collected on the multi-female family group of *V. variegata* at the Saint Louis Zoo (group described in the introduction). Observations were 90 minutes long and occurred either: during their AM feeding or during the early afternoon before their PM feeding. The results showed significantly higher instances of aggression during the feeding times versus the non-feeding times ($p<0.0001$, $n=30$, t-test). When examining when the aggression occurred throughout the 90 minute feeding observation, 52.8% of aggressive interactions occurred during the first 30 minutes, 14.2% during the middle 30 minutes, and 32.9% during the last 30 minutes. Because the two peaks of activity were separated by a period of low activity, the second

peak of aggression may not still be related to the feeding event. The behavioral observations for the main study season were shortened to 30 minutes only, to capture the peak of aggression that coincided with the feeding event.

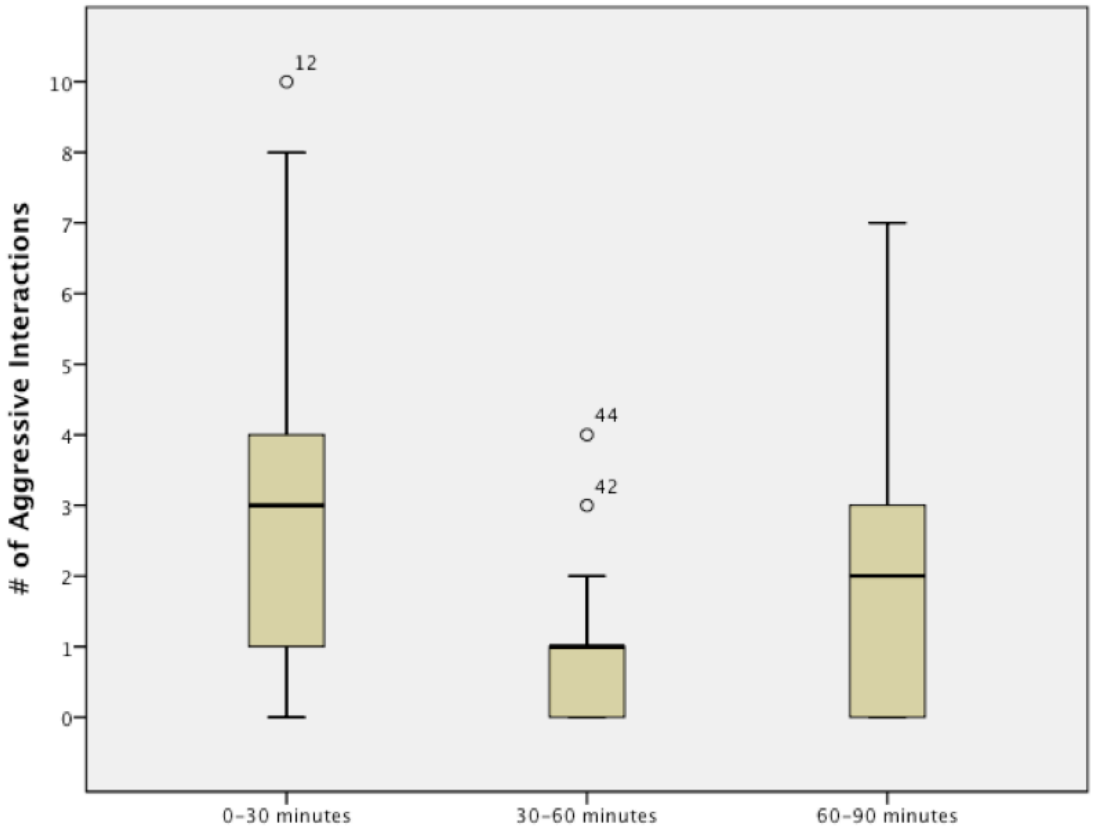

Figure 1: The number of aggressive interactions during 30-minute intervals of the 90 minute observation period for the feeding observations.

During the observations, the dominant daughter initiated 100% of the female-female aggressive interactions and all were directed towards the subordinate mother. The younger daughter was under 1 year of age and was not sexually mature; therefore, she was not involved in the aggressive interactions. However, she was included in the main study season because she

was approaching sexual maturity at that time and actively participated in the aggressive interactions with her sister and the males in the group.

The pilot season also served as a trial run to determine the appropriate number of fecal samples to collect from each lemur in order to have a clear representation of their fGC fluctuations throughout the breeding season. Samples were collected from the two focal females (the dominant daughter and subordinate mother) 3 times per week from December 2013 through February 2014. Additionally, when the samples were being processed and analyzed, there was an unrelated female *V. variegata* going through quarantine in the zoo's veterinary hospital. Quarantine typically lasts 30 days and takes place when any new animal is entering the zoo collection. Prior to being introduced to the zoo population, an animal undergoes a veterinary exam and monitoring for any health concerns. Quarantine keepers collected fecal samples from the quarantine female daily and we were able to compare her fGC levels to the "stressful" events that she experienced during her time in quarantine. We can see that she underwent a veterinary exam and 2 habitat changes during her quarantine stay. A visible rise in fGC occurs the day immediately following the stressful event and the fGC levels return to near-baseline the second day. This indicated that there is a short window of time to capture the fGC rise with fecal sample collection.

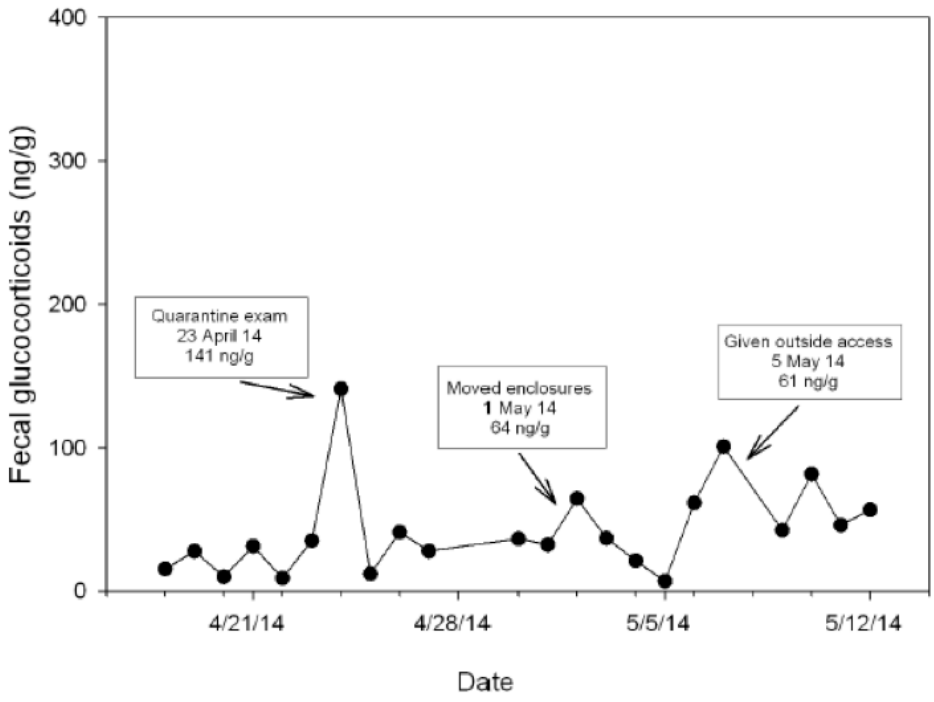

Figure 1: fGC results for the female in quarantine

These results showed that by measuring fecal samples three times per week, we were likely missing fGC changes because they occurred within a 2-day span. There were also a few instances during the pilot season where the study lemurs needed veterinary exams and there were no visible fGC rises because of the fecal collection schedule. This is the reason that we collected daily samples from the females throughout the main study season.

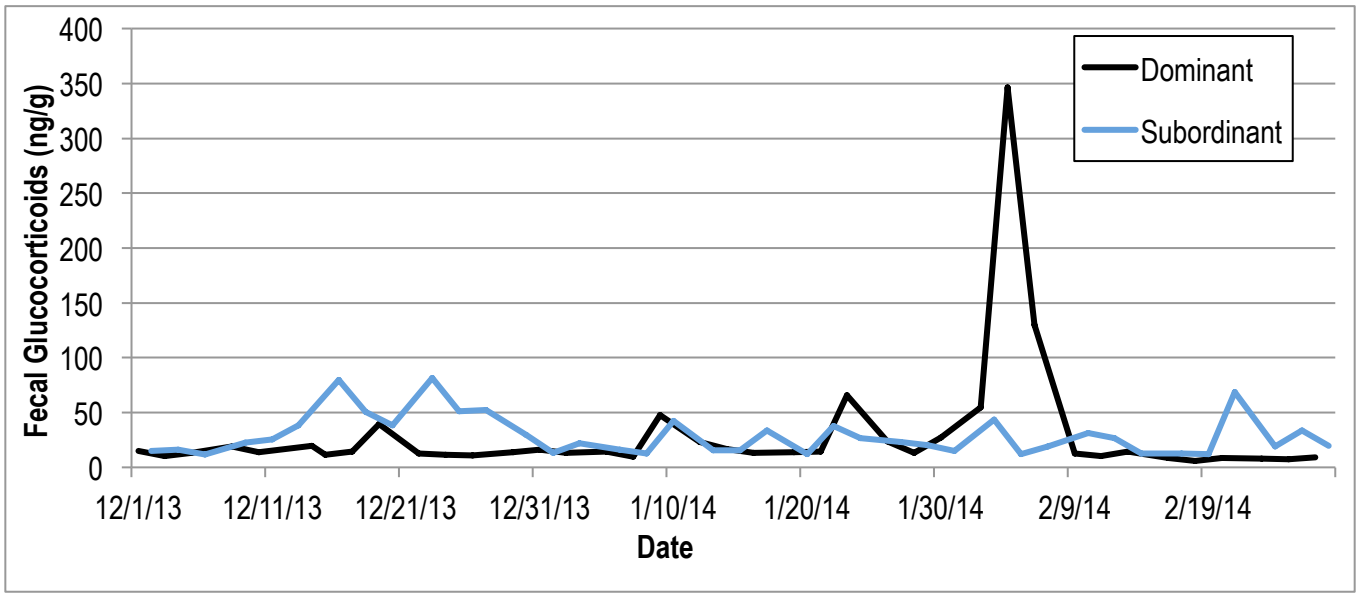

Figure 2: Fecal glucocorticoids (fGC) for the dominate and subordinate females during the pilot season. The dominant individual (shown in black) fluctuated between 5.85 ng/g and 346.39 ng/g with a mean value of 28.73 ± 8.85 ng/g. The subordinate female (shown in light blue) fluctuated between 11.74 ng/g and 81.22 ng/g with a mean value was 28.93 ± 2.95 ng/g.

Results of the radioimmunoassays for the dominant and subordinate females during the pilot season are shown in Figure 2. The dominant individual was generally stable except for a large peak on 2/4/2014 in which the fecal glucocorticoids increased over 10x higher than the mean value. Keeper notes for the day prior (2/3/2014) indicate that this individual was exhibiting excessive tail-carrying behavior throughout the day. This behavior, described as “tail manipulation” in Brockman et al. (1987) is believed to occur one week prior to parturition; however, the individual was not believed to be pregnant at the time and was in fact, receiving regular Depo-Provera contraception injections throughout the breeding season. The subordinate individual had several small increases, some of which coincide with small rises in the dominant individual’s fGC. As

previously stated, stressful events, including aggression, may not cause sustained increases in fGC; therefore, the need for daily samples to increase the resolution was crucial for understanding the relationship between aggressive behavior and stress.