Measuring Glucocorticoid Metabolite Levels, Behavioral Profiles, and Weight Gain in Two Hand-reared, Captive Clouded Leopards (*Neofelis nebulosa*): A Preliminary Assessment

by

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Abstract

Clouded leopards (*Neofelis nebulosa*), arguably the most distinct Pantherinae, or "big cat," species, are currently threatened by population decline *in situ* and *ex situ*. What little is known about this cat's behavior comes from captive studies. Modern research is focused on defining optimal rearing and mate-pairing protocols to mitigate severe mate incompatibility in this species. Evaluating the welfare of captive clouded leopards commonly entails the non-invasive measurements of behavioral ratings from the animals' keepers and glucocorticoid ("stress" hormone) metabolite concentrations in feces (fGMs). I assessed the welfare of two juvenile females born and hand-reared at Nashville Zoo at Grassmere (NZAG) by examining corticosterone (type of glucocorticoid) metabolite concentrations, weight gain, and behavioral ratings. A three-month period of fecal collections revealed baseline concentrations of 273.5 [$\pm$ 0.4 SD] and 293.5 [$\pm$ 0.3 SD] nanograms of corticosterone metabolites per gram of fecal mass for these cubs, Sip Saam and Natida, respectively. Behavioral ratings did not differ notably; each received a mean score of approximately 4.5 out of 5. Growth rate (weight gain/day) was similar between individuals ($y = 0.0452x$, $y = 0.0462x$, $r^2 = 0.99$). Baseline fGM levels were higher than most data reported for adult clouded leopards. This study reports the first investigation of a relationship between weight gain and fGM concentration in juvenile clouded leopards, of which I found no correlation. My data will be presented to the Association of Zoos and Aquariums' Clouded Leopard Species Survival Plan® to assist with future pairing recommendations. These data can also be used in future studies with these two individuals to investigate the consistency of fGM levels throughout their lifespan.

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1. Introduction

1.1 Natural History of the Clouded Leopard

The clouded leopard (*Neofelis nebulosa* Griffith 1821) is a member of the Pantherinae subfamily, which also includes jaguars (*Panthera onca* Linnaeus 1758), lions (*Panthera leo* Linnaeus 1758), leopards (*Panthera pardus* Linnaeus 1758), tigers (*Panthera tigris* Linnaeus 1758), snow leopards (*Panthera uncia* Schreber 1775), and Sunda clouded leopards (*Neofelis diardi* G. Cuvier 1823). Although *N. nebulosa* and *N. diardi* were historically considered a single species, recent evidence from morphological variation, mitochondrial DNA, and microsatellite loci analysis prompted the division of the taxon into the mainland (*N. nebulosa*) and island species (*N. diardi*) that are now currently recognized as the clouded leopard and Sunda clouded leopard, respectively (Buckley-Beason *et al*., 2006; Kitchener, Beaumont, & Richardson, 2006; Wilting *et al*., 2007; Grassman *et al*., 2015). Captive husbandry of clouded leopards began long before its reclassification, potentially resulting in hybrid captive populations. Nonetheless, captive populations are commonly referred to as *N. nebulosa*. The native range of *N. nebulosa* extends across the Himalayan foothills of Bhutan, India, and Nepal throughout the countries of Cambodia, Laos, Malaysia, Myanmar, Thailand, Vietnam, and as far northeast as the Chinese provinces of Shaanxi and Anhui (Grassman *et al*., 2015; Figure 1).

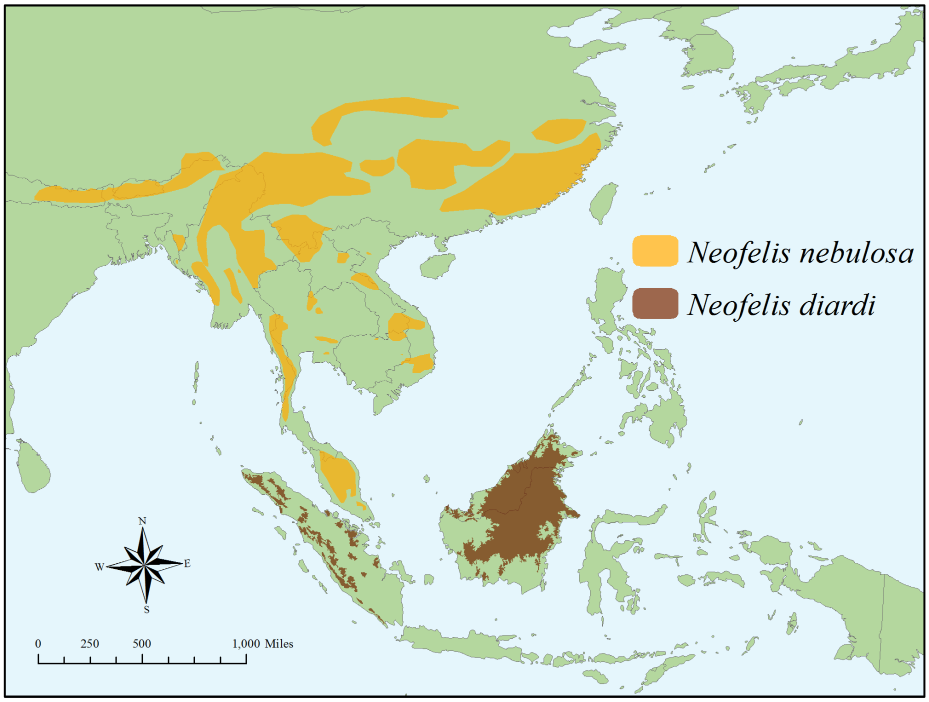

Figure 1: A range map of the clouded leopard (*Neofelis nebulosa*) and the Sunda clouded leopard (*Neofelis diardi*) illustrates the vicariance within the *Neofelis* genus between Southeastern Asia and Indonesia; created by Logan Whiles with credit to ESRI and IUCN for basemap and spatial data.

The clouded leopard is adorned in a coat of characteristic cloud-like markings that are typically colored in a gradient transition from black to tawny. The base color of the coat ranges from ochre to a grayish tan and is dotted with thick, black spots. The back of the ears and the fur along the spine are nearly completely black (Fletchall, 2000; Kitchener *et al*., 2006; Sunquist & Sunquist, 2002; Figure 2). Significant morphological features include an elongated tail (Sunquist & Sunquist, 2002) and the largest skull-to-canine ratio of all extant (living) felids, a convergent evolutionary trait often compared to the extinct saber-toothed cat species (Fletchall, 2000; Christiansen, 2008). They are known to be well suited for an arboreal lifestyle. Considered a nocturnal species, their activity patterns spike during crepuscular (dawn and dusk) hours (Austin *et al*., 2007;

Grassman *et al*., 2015; Sunquist & Sunquist, 2002). Despite their relationship to some of the world's largest felids, clouded leopards only reach weights of 25 to 50 pounds. This species is sexually dimorphic in size, with males growing nearly twice as large as females (Fletchall, 2000; Mackinnon 2008; Sunquist & Sunquist, 2002). Their breeding behavior and social organization has not been extensively documented *in-situ* (Fletchall, 2000; Grassman *et al*., 2005; Sunquist & Sunquist, 2002).

Figure 2: The sire of the two juvenile clouded leopards (*Neofelis nebulosa*) studied. Note the characteristic morphology and coloration of this individual, within his exhibit at Nashville Zoo at Grassmere. Photographed by Logan Whiles.

Recent studies estimate that populations are somewhat stable in only 12% of the clouded leopard's range, and less than 10,000 individuals live in the wild (Grassman *et al*., 2015). Deforestation and illegal wildlife trade are the primary threats to clouded leopard protection (Grassman *et al*., 2015; D'Cruze & Macdonald, 2015). Many sections of their native range have undergone some of the world's most extreme rates of deforestation from 1990 to 2007 (FAO, 2007; Grassman *et al*., 2015). Clouded leopards and their body parts are often exploited on the black market (Nijman & Shepherd, 2015). Nijman & Shepherd (2015) examined illegal wildlife trade and exploitation in Southeast Asia, focusing on two notorious markets. The clouded leopard was the most exploited species reported, with 482 observations recorded in 24 surveys over a period of 16 years. Because of declining numbers caused by numerous direct threats to their survival, the species is currently classified as "vulnerable" by the International Union of Conservation of Nature's (IUCN) Red List of Threatened Species, as it has been since records began in 1986 (Grassman *et al*., 2015). Clouded leopards are afforded maximum protection under the Convention on International Trade in Endangered Species of Wild Fauna and Flora, in Appendix I (CITES, 2015). However, this classification is stated with the caveat that more comprehensive data on wild populations are needed (Grassman *et al*., 2015).

1.2 Captive History of the Clouded Leopard

Zoological Institutions within the Association of Zoos and Aquariums (AZA) have housed clouded leopards for more than 100 years. Although the practice of supplementing the viability of the breeding program with wild-caught individuals was abandoned during the mid-1970s, the captive population is not self-sustaining (Breitbeil & Sullivan, 2014). A decrease of captive births during the late-1980s spurred innovative research and management strategies to overcome the ever-present challenges of pairing mates and rearing cubs in captivity (Breitbeil & Sullivan 2014; DeCaluwe *et al*., 2013; Wielebnowski *et al*., 2002). Extreme cases of aggression and spontaneous estrus cycles (*i.e.*, when a female is physiologically receptive to mating) have resulted in mostly failed reproductive encounters (Mackinnon, 2008). From 1988 to 2013, 25 attempted mate-pairings resulted in fatal attacks, which can be partially associated with heightened aggression of males paired after a year of age (DeCaluwe *et al*., 2013; Mackinnon, 2008). From 1975 to 1999, less than 25% of the captive population successfully bred, and the cub mortality rate rose above 40% (Wielebnowski *et al*., 2002). The cub mortality rate of NZAG is 15% (Karen Rice, personal communication). Many institutions have recently adopted the practice of raising clouded leopard cubs by hand and introducing them to potential mates early in life (Breitbeil & Sullivan, 2014; Mackinnon, 2008). Hand-rearing clouded leopards has become the "norm" in captivity because of the high risk of maternal maltreatment and infant mortality (Najera *et al*., 2015). The refinement and widespread implementation of these methods have shown improvements to the captive reproductive rate; during 2013 the captive population increased by 6%. However, the overall decline that began three decades ago has only been slowed in recent years, rather than reversed (Breitbeil & Sullivan, 2014). As of 2015, NZAG has successfully reared 22 cubs to adulthood, with at least 13 of the captive-reared cubs becoming successful breeders as adults (Karen Rice, personal communication). Of 87 individuals currently maintained in captivity, 51 are suitable for captive breeding. The genetic diversity of captive clouded leopards is currently below 90% of the founding population, and is projected to drop to

61.5% in 100 years, necessitating improvements in genetic integrity for the healthy survival of this species (Breitbeil & Sullivan, 2014).

Clouded leopards are known to show a variety of behavioral and physiological problems in captivity, as they are prone to anxiety and highly sensitive to environmental changes (DeCaluwe *et al*., 2013). Stereotypic behaviors in captivity (Stanton *et al*., 2015), especially self-biting on the tail and body as well as excessive pacing are likely observed more often in this species than any other member of the Pantherinae subfamily (Mackinnon, 2008; Wielebnowski *et al*., 2002). Clouded leopards are also thought to require unique exhibit design, as studies have shown stress levels to be negatively correlated with variables such as exhibit height and number of hiding and climbing structures (Shepherdson *et al*., 2004; Wielebnowski *et al*., 2002). Despite advances in improving captive welfare, defining the typical temperament and environmental preferences of the species as a whole has proven difficult. This has resulted in a variety of efforts to evaluate the well being of clouded leopards in captivity (Iseman, 2005; DeCaluwe *et al*., 2013; Whitham & Wielebnowski, 2009; Wielebnowski *et al*., 2002), the most significant of which are discussed in the following section.

1.3 Measuring Welfare in Captivity

In the past few decades, zookeepers' behavioral ratings of the animals in their care have been deemed consistent and reliable in numerous studies (DeCaluwe *et al*., 2013; Whitham & Wielebnowski, 2009). For many years, the AZA has developed Animal Care Manuals for its captive species. Now, recent research has helped develop the implementation of keeper ratings further by creating ethograms, score sheets, behavioral reaction tests, and welfare monitoring programs for each individual animal (DeCaluwe *et al*., 2013; Stanton *et al*., 2015; Whitham & Wielebnowski, 2009).

Monitoring the concentrations and fluctuations of adrenocortical hormones is a common practice in the science of animal welfare. Glucocorticoids, such as corticosterone, are known to have a vital role in the cascade of effects that result from activation of the hypothalamic-pituitary-adrenal (HPA) axis (Young *et al*., 2004). Exogenous and endogenous stimuli *e.g.*, loud noises and disease, induce the HPA axis to secrete several hormones that initiate a stress-induced response (Sapolsky *et al*., 2000; Young *et al*., 2004). The metabolites from these hormones can be readily measured and are known to be useful indicators of animal welfare because consistently high levels can correlate with poor behavioral and physiological states (Young *et al*., 2004). However, these negative effects on behavior and physiology are found with consistently extreme levels of "stress" hormones; the natural functioning of an organism's coping mechanism alone is insufficient evidence of poor welfare and/or physiology (Sapolsky *et al*., 2000; Mackinnon, 2008; Young *et al*., 2004).

Glucocorticoids, at modulated and basal levels, regulate some of the body's basic metabolic components, such as salt and glucose concentrations (Busch & Hayward, 2009; Figure 3). At low levels, glucocorticoids bind to mineralocorticoid receptors (MR) that have a higher affinity (attraction to the glucocorticoid) than glucocorticoid receptors (GR) (Busch & Hayward, 2009). When circulating levels of glucocorticoids are high enough to saturate the MRs, they can bind to the GRs with enough frequency to illicit what is considered a stress response (Busch & Hayward, 2009; Figure 3). However, the nature of this response is also affected by individuals' life histories, characteristics, and perceptions (Busch & Hayward, 2009). Because glucocorticoids and the physiological integration of external stimuli do not have a simple or singular manifestation, defining "stress" can be as difficult as it is necessary. Busch and Hayward (2009) describe this phenomenon as "an individual's perception that they must focus energy on coping with a short-term threat to survival and curtail long-term investments in functions such as courtship, territorial defense, reproduction, growth, and/or immune defense." I adopt Busch and Hayward's (2009) definition for purposes of this study. Furthermore, "chronic stress" refers to the duration of glucocorticoid overload for extended periods of time, at which point the hormone becomes detrimental for an organism's fitness (Busch & Hayward, 2009). Repeated exposure to stress can result in either acclimation (lessened responsiveness) or sensitization (increased responsiveness) to glucocorticoids, and this possibility must be considered when assessing the fitness or welfare of an individual (Busch & Hayward, 2009).

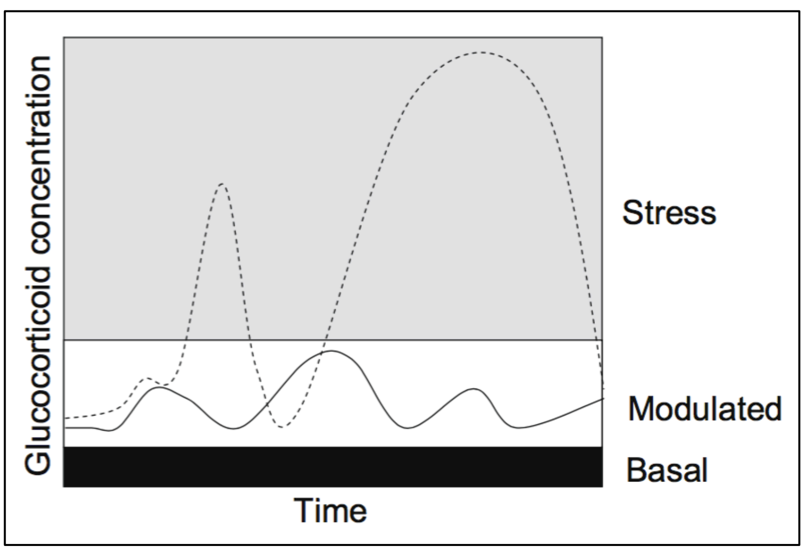

Figure 3: Visualization of glucocorticoid fluctuation as described by Busch & Hayward (2009). The solid line represents concentrations during typical, energetic functions and the dotted line represents concentrations during a perceived stressful event.

Adrenocortical hormone concentrations can be measured by drawing blood from the study animal and assaying serum composition. Such invasive techniques affect circulating levels of the hormones being studied, which can add a confounding variable to an experiment (Möstl & Palme, 2002; Young *et al*., 2004). Alternatively, innovations in non-invasive techniques allow researchers to utilize fecal material as a more practical method to measure hormone metabolites. Feces can be gathered without excessively alerting the study animal to a researcher's presence and are often gathered and disposed of as part of typical captive husbandry routines (Busch & Hayward, 2009; Möstl & Palme, 2002; Young *et al*., 2004). In addition to its non-invasive benefits, monitoring glucocorticoid metabolites in feces may be more representative of the organism's overall well being. Following the immediate onset of a stressor, glucocorticoids can take minutes to secrete and an hour to exert most of their actions upon the pathway. Physiologists therefore consider the circulation of these hormones to be slow and comparatively long lasting (Sapolsky *et al*., 2000). The measurement of serum hormones can be too instantaneous to detect representative baseline concentrations, whereas feces contain an accumulation of glucocorticoid metabolites over time and are more applicable to longitudinal studies. (Young *et al*., 2004).

The literature surrounding adrenocortical hormone levels in carnivores regularly addresses the reliability and efficiency of different assay methods. Radioimmunoassay (RIA) of corticosterone (a type of glucocorticoid) metabolites extracted from dried fecal samples (fGM) is generally considered the most effective technique. This assay is especially preferred when monitoring glucocorticoid concentrations in clouded leopards (Iseman, 2005; Young *et al*., 2004). Development of a validated enzyme-linked immunoassay (EIA) using cortisol (a type of glucocorticoid) has been attempted and successfully implemented more often in recent years. Although an EIA is preferred for some species, such as black footed ferret (*Mustela nigripes* Audobon & Bachman 1851) and domestic cat (*Felis catus* Linnaeus 1758), a corticosterone RIA is more reliable for clouded leopards (Iseman, 2005; Young *et al*., 2004).

1.4 Project Objectives

The main purposes of this welfare assessment were to conduct a pilot study for using glucocorticoid assay with juvenile clouded leopards and to gain hands-on experience in captive wildlife welfare monitoring. Because of a small sample size (n = 2), no statistical analyses were used to draw inferences based on previous studies. However, these data can be used in future studies within the field and will be presented to the AZA's Clouded Leopard Species Survival Plan (SSP®). General comparisons to relevant literature are presented in the discussion.

2. Animals, Materials, and Methods

2.1 Study Animals and Captive Environment

I studied two juvenile, female clouded leopards (Figure 4) that were hand-reared and housed together at NZAG. This study focused on a time period when each cub was 3 – 6 months of age. Their AZA Studbook identification numbers are 4723 and 4728. Sip Saam (#4723) was born on 13 March 2015 and Natida (#4728) was born on 18 March 2015. Each cub was hand-reared from birth (day 0). They are offspring of different dams and the same sire; these three adults are currently housed together on exhibit at NZAG. During gestation, keepers restricted tactile contact between the male and females. The sire was completely removed to separate, off-exhibit housing at NZAG during the final weeks of the dams' pregnancy in an effort to minimize stress during this critical period for successful parturition (Fletchall, 2000).

Animal keepers separated each cub from their dam immediately after birth (< 6 h) and commenced 24-hour care of the individuals. Prior to weaning, keepers bottle-fed Zoological Milk Matrix 33/40© (PetAg, Hampshire, IL) to the cubs approximately six times per day. Keepers offered approximately 20% of each cub's body weight in formula per day. Keepers used Toronto Feline Diet© (Milliken Meat Products Ltd., Ontario, CAN) during and after weaning them off of their liquid-based diet. During the developmental and study period, four primary keepers cared for the cubs for four or more days per week, and 4 other keepers cared for the cubs less than four days per week.

Figure 4: The investigator with Natida (#4728; left) and Sip Saam (#4723; right) at approximately seven weeks of age. Photographed by Andy Heidt

On 15 June 2016, immediately prior to the fecal collection period, the cubs were moved from keeper care to permanent housing. The indoor component of this enclosure was approximately 10 ft high by 12 ft wide by10 ft deep with several artificial ledges and climbing structures. Separated from the inside by a keeper-operated shift door, the outdoor component of this enclosure was approximately 15 ft high by 20 ft wide by 15 ft deep with many horizontal and vertical climbing structures, as well as an elevated ledge and nest box. The entire building containing this enclosure also contained an identical enclosure that housed one adult, female clouded leopard.

For the duration of the fecal collection period, the cubs had visual, olfactory, and auditory interaction with the neighboring adult; however, the cubs did not have tactile contact with this conspecific. Initially, the cubs performed curious and affiliative behaviors in the direction of the adult, which hissed back at them but elicited no reaction from the cubs. Beyond this, no aggressive or stressed behaviors were noted, and the animals soon became accustomed to one another's presence (Karen Rice, personal communication). In addition to potentially influencing glucocorticoid levels, these interactions also played an important role in the keeper ratings of the study animals' behavioral profiles.

Nearly one month after the fecal collection period, the study individuals were introduced to another juvenile clouded leopard for the purposes of potential mating. Jack (studbook identification #4936), a slightly younger individual, weighed approximately 3.5 pounds less than the study individuals (*i.e.* Jack was approximately 88% of the study individuals' body size) during the introduction period. The introduction was relatively typical for successful mate pairing occurrences with this species. The protocol, in general, involved an initial period of visual, olfactory, and auditory contact, then progressed to one-on-one, tactile interactions between the animals. After a week of gradually longer-lasting interaction periods and simultaneous interaction with both females, keepers determined the introduction period to be complete and Jack remained in constant tactile contact with the study individuals (Karen Rice, personal communication). No breeding behaviors were noted and no individual in the trio was thought to be sexually mature at the time of this study. Although feces were not collected during the introduction period, these interactions played an important role in the keeper ratings of the study animals' behavioral profiles.

2.2 Sample Collection and Processing

The study animals' keepers collected fecal samples opportunistically during their typical, morning husbandry routine from 19 June 2015 through 19 September 2015. Keepers collected feces immediately after defecation with marked, plastic bags and stored the samples in an ultra-low freezer at -80°C. Keepers collected 38 samples from Sip Saam, with one sample obtained approximately every 2.4 d. Keepers collected 33 samples from Natida, with one sample obtained approximately every 2.8 d.

After the collection period, I transported all samples from NZAG in Styrofoam© containers of dry ice to an ultra-low freezer at -80°C at Middle Tennessee State University. The samples were on dry ice for less than 1 h.

To dry samples I removed them from the ultra low freezer, placed them in a boat made of aluminum foil, and placed them in a drying oven at 60°C for 48 h. Dried samples were stored and pulverized in a fume hood with air flow set at approximately 100 ft/min. Prior to pulverization, I removed obvious debris (leaf litter, soil, and cardboard that was used for behavioral enrichment and incidentally ingested) from feces. To pulverize a sample, I initially struck it with a rubber mallet to break up the fecal mass. I then poured the fecal material into a hand-operated coffee grinder (Chef's Star®) and ground the feces into a fine powder that collected into a glass jar. I disassembled, washed, and dried all equipment after each sample was processed. Approximately 2 g of fecal powder from each sample was stored in BD Falcon™ 15 ml polypropylene, conical tubes (Corning Inc., Tewksbury, MA). I wrapped all tubes (n = 71) in bubble wrap and shipped them in single package overnight by FedEx to the Endocrinology Lab at the Smithsonian Conservation Biology Institute (SCBI).

2.3 Steroid Extraction and Corticosterone RIA

Drs. Janine Brown, Natalia Prado-Oviedo, and their team at the SCBI Endocrinology Lab extracted steroid hormones from each sample and assayed the extractions for corticosterone metabolite levels using an RIA, in accordance with methodology originally developed in Brown *et al*. (1994). The lab used a double-antibody I^{125} RIA for corticosterone. The poly-clonal antiserum cross-reacts with corticosterone 100%, desoxycorticosterone 0.34%, testosterone 0.1%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5a-dihydrotestosterone 0.01%, and <0.01% with all other steroids tested (MP Biomedical LLC, Santa Ana, CA).

Briefly, approximately 0.2 g of each homogenized, dried, and crushed sample was transferred to a 16x125 mm glass extraction tube. Corticosterone-H^3 (0.1 ml) was added to each tube as a means of evaluating the steroid extraction efficiency. Ethanol (5.0 ml of 90%) was added to each tube and vortexed until all fecal powder was suspended in the solution. Tubes were securely capped and placed on a multi-pulse vortexer (Glas-Col, Terre Haute, IN) for 30 minutes set at a motor speed of 60 rpm. Tubes were then centrifuged for 20 min at 2,000 rpm. The supernatant was poured into labeled 16x125 mm glass extraction tubes (referred to as “duplicate tubes” below) and set aside. Ethanol (5.0 ml of 90%) was added to each of the tubes containing the fecal pellet, vortexed again for 30 s and then centrifuged again for 15 min at 2,000 rpm. The resulting supernatants were poured into their respective duplicate tubes containing the first supernatant. These duplicate tubes were then air-dried underneath a fume hood until the supernatant evaporated. The sides of the tubes were rinsed with 1 ml of methanol and sonicated for 15 min to reconstitute the extract. These tubes were then air-dried in a fume hood until the methanol evaporated. Dilution buffer (1 ml; 0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.14 M NaCl; pH 7.0) was used to rinse the sides of the tubes. The tubes were then briefly vortexed and sonicated for 15 min to reconstitute the extract in the new solution. This solution (15 µl) was added to scintillation vials along with 3 ml of scintillation fluid (Ultima Gold, Perkin-Elmer, Waltham, MA). These scintillation vials were then securely capped, manually shaken and incubated at room temperature for 1 h. Extraction efficiencies were then counted on a beta counter (Beckman Coulter, Inc., Brea, CA) with two blank scintillation tubes containing 3 ml of scintillation fluid and two total count scintillation tubes containing 100 of corticosterone-H^3 each.

The RIA protocol is supplied within the instruction manual for the Corticosterone-I^{125} RIA Kits (MP Biomedical LLC, Santa Ana, CA). Briefly, samples were brought to room temperature and diluted to 1:100 by adding 15.2 µl of neat sample to 1.5 ml of dilution buffer. Steroid diluent (0.15 ml of phosphosaline gelatin buffer) was added to the non-specific binding (NSB) tubes 1 and 2 and 0.05 ml of steroid diluent was added to the max binding (zero) tubes 3 and 4. Corticosterone calibrators (0.05 ml) were added to duplicate tubes (5-16). Calibrator values ranged from12.5 ng/ml to 1,000 ng/ml. Corticosterone controls were reconstituted with 2.0 ml of distilled water and incubated at room temperature for 30 m before use. Reconstituted corticosterone controls (0.05 ml) were added to tubes 17-18 and 19-20. Control values were 320 ng/ml and 160 ng/ml. The extracted sample (0.05 ml) was added to labeled tubes in duplicate. Corticosterone-I^{125} (0.1 ml) was added to all tubes and anti-rabbit corticosterone antiserum was added to all tubes except for totals and NSB tubes. All tubes were vortexed and incubated at approximately 23°C for 2 h. After incubation, 0.25 ml of precipitant solution was added to all tubes and thoroughly vortexed. All tubes were then centrifuged for 15 min at approximately 2,400 rpm. The supernatant was decanted and inverted on a paper towel for 30 seconds to remove all remaining liquid. Finally, a gamma counter (ISOdata 20/20; GMI Inc., Ramsey, MN) was used to quantify the hormone content of the samples.

The distribution of fGM data recorded for each individual was tested for normality on a TI-84 Plus© (Texas Instruments, Dallas, TX) using MATH200A© (Basic Statistics Utilities V7.2, Stan Brown). Neither distribution was normal (Sip Saam: $r = 0.8870$, critical value = 0.9702; Natida: $r = 0.8336$, critical value = 0.9666) so each data point was transformed with the formula $\log_{10}(X_i+1)$ to fall within a normal distribution, where X_i is equal to an individual data point. The results were transformed back using the same formula and solving for X_i to report fGM data. Therefore, the means and standard deviations reported are geometric, rather than arithmetic. A baseline fGM concentration was calculated by excluding any values that exceeded the mean by 1.5 standard deviations, then calculating a new mean with the remaining values and reiterating this process until all values were within 1.5 standard deviations of the final mean (Wielebnowski *et al*., 2002). The mean of all excluded values is reported as the peak mean. The overall mean, range, and coefficient of variation were also calculated.

I compared glucocorticoid metabolite levels in reference to behavioral scores and weight gain data to highlight variation in individual animals or correlations between behavior and other parameters (*e.g.* cortisol metabolite levels and overall behavioral score or *e.g.* inconsistency in growth rate and consistent stereotypic pacing).

2.4 Questionnaires

2.4.1 Rearing Protocol Survey

I used a survey to investigate the variability in clouded leopard rearing methods among zoological institutions (Appendix 1). This was primarily developed through direct correspondence with Karen Rice, Heather Robertson, and Margarita Woc Culburn. I used the responses to compare the protocols and success of captive husbandry of clouded leopards at NZAG to other institutions. I also used these responses to distinguish the recent efforts to develop a "cooperative-rearing" protocol that is distinct from "hand-rearing" or "parent-rearing." The survey was hosted by SurveyMonkey® (SurveyMonkey, Palo Alto, CA) and sent to recipients via email. Recipients are the listed clouded leopard SSP® correspondents for each AZA institution involved within the last 15 years.

2.4.2 Behavioral Survey

I used a behavioral survey to quantify the temperament, physical condition, and overall welfare of the study animals (Appendix 2). A behavioral score that does not include physical condition was calculated for each individual by averaging its score for each parameter. The questions and scoring system were inspired and adapted from previous behavioral studies (Whitham & Wielebnowski 2013) and direct correspondence with Karen Rice, Heather Robertson, Jessica Whitham, and Nadja Wielebnowski, Margarita Woc Culburn. Scores from 1 – 5 were available for selection with a score of 1 representing an extremely negative manifestation of the parameter (*e.g.* reacts to environmental variation or enrichment in a manner typical of unhealthy or unsuccessful clouded leopards) and a score of 5 representing an extremely positive manifestation of the parameter (*e.g.* reacts to environmental variation or enrichment in a manner typical of healthy or successful clouded leopards). Near the end of the entire study period, I hand-delivered the survey to all NZAG animal care staff responsible for the care of the study animals. One response was not included in the calculations as it exhibited very weak inter-keeper and intra-keeper agreement.

2.5 Body Mass Dynamics and Physical Condition

The animal care staff at NZAG weighed the clouded leopard cubs each week, and they provided me with 50 weight data points from Sip Saam from 0 – 301 days of age, and 41 weight data points from Natida taken from 0 – 296 days of age. This time period encompassed the 3-month fecal collection period. NZAG personnel also provided me with additional comments on growth rates, body conformation, and body condition scores (a universal rating of physical condition used by zoological institutions; a rating of 2.5 is "moderate" for adults, and scores range from 1 or "emaciated" to 5 or "obese") for each animal. I plotted weight data points on a line graph, with slopes and regression calculated for each individual. I used weight data and slopes to compare the two study animals' growth rate and to previous work on body mass dynamics (Najera *et al*., 2015).

2.6 Ethical Approval

Because of the non-invasive nature of this study, this project did not need to undergo formal review by the Institutional Animal Care and Use Committee (Appendix 3).

3. Results

3.1 Corticosterone RIA Data

Sip Saam's fGM baseline concentration was 273.5 ng/g [± 0.4 SD]. Her peak mean concentration was 615.9 ng/g [± 0.4 SD] and her overall mean concentration was 310.9 ng/g [± 0.5]. Coefficient of variation was 0.1906 and sample concentrations ranged from 117.7 ng/g to 1083.6 ng/g (Table 1).

Natida's fGM baseline concentration was 293.5 ng/g [± 0.3 SD]. Her peak mean concentration was 601.5 ng/g [± 0.5 SD] and her overall mean concentration was 328.3 ng/g [± 0.1]. Coefficient of variation was 0.1644 and sample concentrations ranged from 141.0 ng/g to 1,139.3 ng/g (Table 1).

A graphical representation of longitudinal fGM concentrations for each cub is supplied in Appendix 4. This data shows the fluctuation and progression of fGM levels during an approximate 3-month period. The fGM profile of each cub, as well as the baseline concentration of each cub, is displayed simultaneously. A table of individual sample collection dates and corresponding fGM concentrations for each cub is supplied in Appendix 5.

Table 1: fGM (corticosterone) values in ng/g for Sip Saam (#4723) and Natida (#4728).

	Baseline	Peak Mean	Overall Mean	CV (%)
Sip Saam	273.5 ± 0.4	615.9 ± 0.4	310.9 ± 0.5	0.1906
Natida	293.5 ± 0.3	601.5 ± 0.5	328.3 ± 0.1	0.1644

3.2 Questionnaire Data

3.2.1 Rearing Protocol Survey

These results were not statistically analyzed, as the questionnaire provided the option for a written response and many breeding experiences proved difficult to quantify and standardize because of variability across institutions. Most AZA institutions involved with clouded leopard husbandry did not respond. In total, 11 responses were received; these respondents make up the majority of AZA institutions with individuals that are currently recommended for breeding. Of the 8 received responses that are applicable to the following statements, the majority of institutions (n = 5) permanently separated cubs from the dam within 6 hours of birth. Half of the institutions (n = 4) employ 5 or more keepers to regularly interact and care for the cubs. Most institutions surveyed hand-rear their clouded leopards (n = 6), reporting mostly positive results. One response indicated a "cooperative-rearing" strategy that was passed on to the Clouded Leopard SSP® Studbook Keeper for purposes of better defining rearing protocols. Although hand-rearing was the most common protocol surveyed, one response reported a mother-rearing protocol that has also been successful.

3.2.2 Behavioral Survey

Sip Saam's average overall behavioral score was 4.4 [$\pm$ 0.5]. Her overall scores from 7 keepers ranged from 3.6 to 5. Her average physical condition score was 4.8.

Natida's average overall behavioral score was 4.6 [$\pm$ 0.5]. Her overall scores from 7 keepers ranged from 3.6 to 5. Her average physical condition score was 4.6.

The average rating for each behavioral category, as well as an average overall behavioral score, for each cub is displayed in Appendix 6. Ratings from 7 keepers were used.

3.3 Body Mass Dynamics and Physical Data

Primary keepers supplied 41 weight data points for Sip Saam taken from 24 March 2015 to 8 January 2016. During this time period she gained 12.79 kg, averaging a gain of 46.2 g/day. In the behavioral survey, keepers gave Sip Saam an average physical condition rating of 4.8. Primary keepers gave Sip Saam a body condition score of approximately 3.25 by the end of the fecal collection period and considered Sip Saam to have a more stocky build than Natida (Karen Rice, personal communication).

Primary keepers supplied 50 weight data points for Natida taken from 13 March 2015 to 8 January 2016. During this time she gained 12.61 kg, averaging a gain of 45.2 g/day. In the behavioral survey, keepers gave Natida an average physical condition rating of 4.6. Primary keepers gave Natida a body condition score of approximately 3.25 by the end of the fecal collection period and considered Natida to have a more slender build than Sip Saam (Karen Rice, personal communication).

The weight gain for each cub is displayed in Appendix 7 and Appendix 8. Individual weight data points are shown alongside a linear trend line representing average growth rate during a time period of approximately 300 days. It should be noted that the fecal collection period corresponds with 98 – 190 days of age for Sip Saam (#4723) and 93 – 185 days of age for Natida (#4723).

4. Discussion

The study of wildlife welfare is a complex and difficult task, both *in situ* (in a natural habitat) and *ex situ* (in captivity). The primary purpose of keeping wildlife in captivity is to maintain a genetic resource representative of wild populations, while reliably studying aspects of ecology that may represent populations and ecosystems *in situ*. However, captive animals of the same species may differ in age, sex, life history, and their responses to husbandry (Fanson & Wielebnowski, 2013). Therefore, drawing consistent inferences about a species from captive research requires experimental design that considers the inevitable variation across institutions and among individuals. Clouded leopard ecology has been studied only *ex situ*. This research has validated the use of behavioral observations and hormone assays for evaluating the welfare of this species, knowledge that is crucial for maintaining a healthy and self-sustaining captive population.

I used fecal corticosterone (glucocorticoid) metabolite (fGM) concentrations, weight gain, and keeper ratings of animal behavior to investigate the welfare of two juvenile, female, hand-reared clouded leopards at Nashville Zoo at Grassmere (NZAG). My project primarily functioned as a pilot study to aid investigations of the validity of fGM monitoring for juvenile clouded leopards. To my knowledge, Mackinnon (2008) was the first to use this method with clouded leopards less than one year of age. However, her study was focused on potential relationships between mate-pairing success and factors such as fGM concentrations. Therefore, her fGM data were reported with the confounding variable of potentially stressful conspecific introductions during the duration of her study, and cannot be used as "control" data for comparative purposes. Likewise, the presence of the adult conspecific during the duration of my study could be considered a confounding variable, but keeper observations do not suggest that this factor had an impact on the cubs' stress or welfare.

Various measurements of fGM concentrations are typically used to quantify aspects of clouded leopard welfare. The most notable indices are "baseline," "peak mean," and "overall mean" (calculations are described in Chapter 2.3; Busch & Hayward, 2009; DeCaluwe *et al*., 2013; Wielebnowski, 2002). Specifically, the coefficient of variation of fGM data from the sample mean can be a more accurate indicator for the reproductive success of a female clouded leopard (Mackinnon, 2008). Lidgard *et al* (2008) also found this relationship in male grey seals (*Halichoerus grypus* Fabricius 1791). Wielebnowski *et al*. (2002) report baseline glucocorticoid concentration in clouded leopards is higher in females (121.7 ng/g [± 20.3]) than in males (59.8 ng/g [± 10.0]). A relationship between sex and fGM levels has also been found in the Sumatran tiger (Parnell *et al*., 2014; *Panthera tigris* ssp. *sumatrae* Pocock1929). Overall mean concentrations negatively correlate with availability of climbing and hiding structures, as well as time spent with primary keepers (Shepherdson *et al*., 2004; Wielebnowski *et al*., 2002). Overall mean concentrations in clouded leopards positively correlate with exposure to potential predators, habitual self-injuring behavior, quantity of keepers, and failed mating attempts in males as well as keeper ratings of "time sleeping," "tense," "hiding," and "pacing" (Mackinnon, 2008; Wielebnowski *et al*., 2002). Mackinnon (2008) did not find a correlation between keeper ratings of "pacing" and fGM concentrations, and suggests that this stereotypic behavior could sometimes be habituated previous to hormone measurements and remain regardless of stress. Mean concentrations do not correlate with overall enclosure size, age of the individual (although no juveniles were studied), and rearing history (Wielebnowski *et al*., 2002). These findings indicate that inferences from fGM concentrations can vary across studies or differ in relation to seemingly subtle discrepancy between similar parameters, such as evaluations of enclosure size. Furthermore, Fanson & Wielebnowski (2013) found that 70% of fGM concentration peaks did not correlate with a recorded stressful event. This counter-intuitive finding can simply reflect a gap in the perception of stressful events between humans (*Homo sapiens* Linnaeus 1758) and non-human animals, or suggest an incomplete understanding of the function of "stress" hormones.

Sip Saam's and Natida's fGM levels are generally higher than previously reported data for adults of this species. Wielebnowski *et al*. (2002) report mean baseline fGM concentrations of 72 adult individuals, which is the largest sample size of any study relevant to my project. Female mean baseline concentration was 121.7 ng/g [$\pm$ 20.3 SEM] and peak mean concentration was 332.7 ng/g [$\pm$ 54.8 SEM]. The relatively high mean fGM levels of the study individuals (Sip Saam's baseline = 273.5 ng/g $\pm$ 0.4 SD, peak mean = 615.9 ng/g $\pm$ 0.4 SD; Natida's baseline = 293.5 ng/g $\pm$ 0.3 SD, peak mean = 601.5 ng/g $\pm$ 0.5 SD) could be attributed to a variety of factors. Busch & Hayward (2009) emphasize the importance of scrutiny when drawing inferences about wildlife welfare from glucocorticoid concentrations, as this hormone also plays a critical role in energy regulation. Physiological and behavioral observations, specifically the cubs' recorded interactions with keepers and conspecifics, do not reflect those of clouded leopards known to be under consistently high levels of stress. Therefore, I postulate that Sip

Saam's and Natida's unusually high glucocorticoid metabolite levels are associated with energy regulation during this developmental stage, rather than stress.

On the other hand, these cubs were often playful and interactive with one another, as is common for feline species in the early stages of life (personal observations; Crowell-Davis *et al*., 2003). Intensely playful behaviors are expected for juvenile clouded leopards (Fletchall, 2000), and are likely beneficial for socialization. Mackinnon (2008) reported that the observed frequency of possible play-fighting behaviors positively correlates with mate-pairing success, though little is known about the specifics of play behaviors in this species. Sip Saam's and Natida's high frequency of playful stalking, pouncing, and chasing behaviors could be associated with the high baseline fGM levels recorded in this study. Further research should be done on glucocorticoid concentrations in juvenile clouded leopards to investigate the effects of development and socialization.

This study is the first report of glucocorticoid metabolite concentrations alongside weight gain during the juvenile period of this species. There is no apparent relationship between fGM concentrations and growth rate. Najera *et al*. (2015) reports an average growth rate of 30.0 g/day [± 1.2 g SD] for healthy, female clouded leopards during a period of 0 – 90 days of age. Najera *et al*. (2015) also reported an average birth weight of 232.6 g [± 33.0 SD] and an average weight of 2,931.4 g [± 436.5 SD] at 90 days of age for healthy, female cubs. Sip Saam's and Natida's birth weights are typical in comparison to data reported for this species (220.0 and 250.0 g, respectively). Conversely, the cubs' pre-weaned growth rate (Sip Saam = 42.9 g/day during 0 – 89 days of age; Natida = 46.3 g/day during 0 – 92 days of age) and weight after 90 days of age were high in comparison (Sip Saam = 4,218.4 g at 89 days of age; Natida = 4,580.0 g at 92 days of age). These results suggest that the study individuals are uncharacteristically large individuals, or NZAG's hand-rearing protocol is more nutritionally robust than in the institutions recorded by Najera *et al*. (2015). Another juvenile clouded leopard, named Jack (#4936), was transferred from another institution to NZAG and introduced to the study individuals near the end of this study. A partial weight record for Jack was supplied in addition to weight data for Sip Saam and Natida during 19 September 2015 and 30 October 2015 (Appendix 9) During this time period, Jack's growth rate was three times that of the other two cubs, and he nearly reached their body weight within about 0.5 kg. Najera *et al*. (2015) reported that males grow at a rate of approximately 1.15 times that of females during 0 – 90 days of age, but body weight at 90 days of age does not differ between sexes in this species. Jack's body condition score also improved at a comparatively rapid rate after being transferred to NZAG. His physical improvement supports my suggestion the high body weight and growth rate exhibited by the individuals in this study are possibly attributed to NZAG's rearing protocol.

Developing the behavioral survey used in this study was difficult and limited to scarce behavioral research for this species. While relevant studies are reputable and extensive, clouded leopard behaviorists state the need for a more complete understanding of this little-known species (DeCaluwe *et al*., 2013; Fazio, 2010; Mackinnon, 2008). Because a trained clouded leopard behaviorist was not directly involved with this study, I decided to generalize the behavioral survey (in comparison to previous, more intensive studies) in attempt to create a simple questionnaire that still covered the most essential welfare indicators of this species (Appendix 2). With the help Jessica Whitham and Nadja Wielebnowski, in addition to experienced NZAG staff, I produced a survey with five general categories applicable to clouded leopard behavior. The results of this survey are considered highly positive ratings for this species (Appendix 6). I would recommend that future research neglect the use of a 1 – 5 scale in favor of a scale that parallels that of Body Condition Scores (*i.e.* a median value is perfect, while the highest and lowest values represent opposing extremes), which the keepers in this study were more accustomed to using. I would also recommend that more time be devoted to understanding individual animals' behavioral states. Some related studies utilize behavioral observation periods that are brief in comparison to an individual animal's entire day or life (Fazio, 2010; Mackinnon, 2008). Other relevant papers use single behavioral surveys or request that keepers record potentially stressful events (Fanson & Wielebnowski, 2013; Wielebnowski *et al*., 2013). While keeper ratings have been validated, using continuously recorded video to record an animal's daily behaviors without the presence of a human keeper could potentially reveal more accurate behavioral profiles. Video recording could reduce the need for additional researchers devoted solely to behavioral observations. This could also alleviate dependence on the accuracy and memory of keepers who often care for many other animals and may only have the opportunity to periodically observe study individuals.

Finally, it should be restated that the study individuals are half-sisters raised in nearly identical conditions. Glucocorticoid metabolite values, behavioral profiles, and growth rates appear similar between these individuals (with the possible exception of fGM values, but these statistics could not be reliably analyzed). The results from the three main components of this study suggest the intuitive assumption that similarity in environment, rearing protocol, and genetics can result in similar data for separate individuals. It is unclear which, if any, had the greatest effect on the data. Nonetheless, considering and understanding the potential dichotomy between "nature and nurture" in this species could have important implications for the current captive breeding program. Institutional transfers are necessary for maintaining genetic integrity within the Clouded Leopard SSP®, and there is still great interest in defining optimal rearing protocols for this species (Breitbeil & Sullivan, 2014). Previous research has not focused on the consistency or "inheritability" of welfare parameters in related individuals, but such knowledge could have implications for mate-pairing recommendations.

In conclusion, the results from this pilot study suggest that further research is needed on 1) defining expected glucocorticoid metabolite levels for juvenile clouded leopards, 2) the effects of NZAG's rearing protocol on juvenile clouded leopard weight gain in comparison to other zoological institutions, and 3) the similarity in glucocorticoid metabolite levels, weight gain, and behavioral profiles in siblings compared to unrelated individuals exposed to the same husbandry practices.

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Appendices

Appendix 1: Historical Survey (pp. 33 – 37).

Non-invasive Study of Neofelis nebulosa Rearing, Physiology, and Behavior

Rearing Information

This information will be used to define and account for the institutional variation of rearing protocols.

1. Approximately how many hours following birth is the dam in tactile contact with her cubs?

- ◯ Less than 6 hours
- ◯ 6 - 12 hours
- ◯ 12 - 24 hours
- ◯ 24 - 48 hours
- ◯ 2 days - 1 week
- ◯ More than 1 week
- ◯ The dam is not separated from her cubs
- ◯ Other (please specify)

2. Approximately how many hours following birth is the dam in olfactory, visual, or auditory contact with her cubs?

- ◯ Less than 6 hours
- ◯ 6 - 12 hours
- ◯ 12 - 24 hours
- ◯ 24 - 48 hours
- ◯ 2 days - 1 week
- ◯ More than 1 week
- ◯ The dam is not separated from her cubs
- ◯ Other (please specify)

1

3. Are the cubs temporarily separated from the dam for any reason (for medical exams, etc.)?

- Yes
- No
- Other (please specify)

4. At what approximate hour following birth is the dam reintroduced to her cubs? I.e., "at approximately 48 hours the cubs are returned to their dam [after medical examination, etc.]."

- Less than 6 hours
- 6 - 12 hours
- 12 - 24 hours
- 24 - 48 hours
- 2 days - 1 week
- More than 1 week
- The dam is not reintroduced to her cubs
- Other (please specify)

5. How many times are the cubs separated from their dam during the first six months following birth? I.e, "every morning the cubs are separated for (x) hours for medical examination/every night the cubs are separated for (x) hours for safety/etc.

6. How many zookeepers are in regular (approximately every day), tactile (free) contact with the cubs during the first six months following birth?

- ◯ 0
- ◯ 1
- ◯ 2
- ◯ 3
- ◯ 4
- ◯ 5 or more keepers
- ◯ Other (please specify)

7. How many zookeepers are in regular olfactory, visual, or auditory (protected) contact with the cubs during the first six months following birth?

- ◯ 0
- ◯ 1
- ◯ 2
- ◯ 3
- ◯ 4
- ◯ 5 or more keepers
- ◯ Other (please specify)

8. How many cubs were born in each litter this spring? I.e., litter 1: 0.1; litter 2: 1.2; etc.

9. When was each litter delivered? I.e., Litter 1: March 1st, 2015; Litter 2: April 2, 2015; etc.

10. How many cubs from each litter delivered this spring were lost during the first six months following birth, and on what date? If possible, a brief description of each suspected cause is requested.

11. How many physical, behavioral, or procedural complications were observed with the cubs during the first six months following birth? If possible, a brief description of each complication is requested.

Non-invasive Study of Neofelis nebulosa Rearing, Physiology, and Behavior

Historical Information

This information will be used to distinguish the protocols and results of each institutions' breeding events. Cooperative-rearing can define a mother-reared infancy with a frequent, tactile presence of one or more zookeepers throughout the stages of cub development. Hand-rearing can be used to define the sole presence of a human keeper(s) throughout the stages of cub development.

We request this institution's records on weight gain for each of its 1) mother-reared 2) hand-reared, and 3) cooperatively-reared litters.

12. How many individuals have been 1) mother-reared, 2) hand-reared, or 3) cooperatively-reared at this institution?

13. What is the survival rate of 1) mother-reared, 2) hand-reared, or 3) cooperatively-reared infancies at this institution?

14. How many of this institution's individuals of a 1) mother-reared, 2) hand-reared, or 3) cooperatively-reared infancy have been successful breeders?

15. How many of this institution's individuals of a 1) mother-reared, 2) hand-reared, or 3) cooperatively-reared infancy have been excluded from breeding recommendations due to genetic overrepresentation, show/exhibit training, sexual immaturity, etc. (not for health or behavioral reasons)?

16. Have any of this institution's individuals of a 1) mother-reared, 2) hand-reared, or 3) cooperatively-reared infancy demonstrated behavioral or physiological complications upon exhibit or mate introduction? If possible, a brief description of each complication is requested.

Appendix 2: Behavioral Survey (pp. 38 - 41).

<u>Clouded Leopard Cub Behavioral Survey</u>

Rate behaviors on a scale of 1 (unhealthy/poor/atypical behavior) to 5 (healthy/excellent/typical behavior). Use NA for any question that cannot be confidently answered.

Q1: **Activity**
Rating of 1: Obvious and consistent lethargy or hyperactivity.
Rating of 5: Displays good behavioral diversity and spends time resting as well as interacting with the environment.
A: Sip Saam: ____, Natida: ____.

Q2: **Physical Condition**
Rating of 1: Obvious signs of injury, lack of muscle tone, common ocular, nasal, or oral discharge.
Rating of 5: Perfect body score, maintains good posture, and rarely displays illness or discomfort.
A: Sip Saam: ____, Natida: ____.

Q3: **Environmental Stimulation**
Do not include behaviors associated with conspecifics, keepers, or other animals in this rating.
Rating of 1: Overly reactive and/or behavior is negatively altered from enrichment and novel stimuli for sustained periods of time, or is completely unaware of enrichment and novel stimuli.
Rating of 5: Curiously investigates and/or recognizes enrichment and novel stimuli in a positive way, remains aware of the status of her enclosure.
A: Sip Saam: ____, Natida: ____.

Q4: **Conspecific Stimulation**
Rating of 1: Overly reactive and/or behavior is negatively altered for sustained periods of time from novel conspecific presence and displays serious aggression towards conspecifics and attempts to injure them, or is completely unaware of conspecific presence.

Rating of 5: Initially aware of conspecific, establishes comfort towards conspecific after a reasonable period of time, vocally and visually communicates with conspecific, plays or grooms conspecific, and comfortably coexists and eats with conspecifics present.
A: Sip Saam: ____, Natida: ____.

Q5: **Keeper Stimulation**
Rating of 1: Overly reactive and/or behavior is negatively altered for sustained periods of time from novel keeper presence and displays serious aggression towards keepers and attempts to injure them, or is completely unaware of keeper presence.
Rating of 5: Initially aware of keeper, recognizes keeper after a reasonable period of time, vocally and visually acknowledges keeper, displays natural behaviors and comfortably coexists and eats with keepers present.
A: Sip Saam: ____, Natida: ____.

Q6: **Stress**
Rating of 1: Hides as often as possible, consistently displays fearful or tense behaviors, posture, extreme stereotypic behaviors, self-injurious behaviors, and frenetic behaviors.
Rating of 5: Appears to have a healthy level of stress, *i.e.*, is not consistently lethargic but does display a consistent level of comfort and general awareness in her typical environment.
A: Sip Saam: ____, Natida: ____.

Background Information
Q: How many days per week does your routine include observing and caring for Sip Saam and Natida?
A:

Q for Karen Rice: On what date were Sip Saam and Natida moved to Rajani's neighboring stall?
A:

Q for Karen Rice: Was introduction protocol followed when introduced to Rajani?
A:

Q for Karen Rice: On what date were Sip Saam and Natida introduced to Jack?
A:

Q for Karen Rice: Was introduction protocol followed when introduced to Jack?
A:

Q for Karen Rice: Need comprehensive data for weight gain and any observations on physical condition.
A:

Q for Karen Rice: Need comprehensive data for diet and diet changes.
A:

Correspondence:
This survey was developed by Logan Whiles (Primary Investigator) as part of ongoing research with captive clouded leopards. Email: ljw3b@mtmail.mtsu.edu.

References and Acknowledgments:
This survey was adapted from various sources, with consideration towards proven reliability of certain questions and significant parameters. Primary sources are listed in alphabetical order:

Gartner, M.C., Powell, D.M., Weiss, A. 2014. Personality Structure in the Domestic Cat (Felis silvestris catus), Scottish Wildcat (Felis silvestris grampia), Clouded Leopard (Neofelis nebulosa), Snow Leopard (Panthera uncia), and African Lion (Panthera leo): A Comparative Study. Journal of Comparative Psychology 128:4, 414-426.

Fletchall N. 2000. Clouded leopard (Neofelis nebulosa) husbandry guidelines. Grand Rapids, Michigan: John Ball Zoological Garden, 32–40.

Stanton, L., 2013. A Standardized Ethogram for Felids. Manchester Metropolitan University.

Whitham, J.C., Wielebnowski, N., 2009. Animal-Based Welfare Monitoring: Using Keeper Ratings as an Assessment Tool. Zoo Biology 28, 545-560.

Wielebnowski, N. C., Fletchall, N., Carlstead, K., Busso, J. M., Brown, J. L., 2002. Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. Zoo Biology 21, 77–98.

Whitham, J.C., Wielebnowski, N.C., 2015. (personal communication).

Appendix 3: Correspondence with Dr. Gore Ervin of MTSU concerning IACUC approval requirement. Hosted by MTMail.

Fwd: IACUC

Brian Miller <Brian.Miller@mtsu.edu>

Thu 1/28/2016 12:11 PM

To: Logan J Whiles <ljw3b@mtmail.mtsu.edu>;

Begin forwarded message:

From: Max Ervin <Max.Ervin@mtsu.edu>
Subject: RE: IACUC
Date: January 21, 2015 at 2:46:18 PM CST
To: Brian Miller <Brian.Miller@mtsu.edu>

Hi Brian,

In this instance IACUC review and approval is not required.

Hope this helps,

Gore

-----Original Message-----
From: Brian Miller
Sent: Wednesday, January 21, 2015 9:03 AM
To: Max Ervin
Subject: IACUC

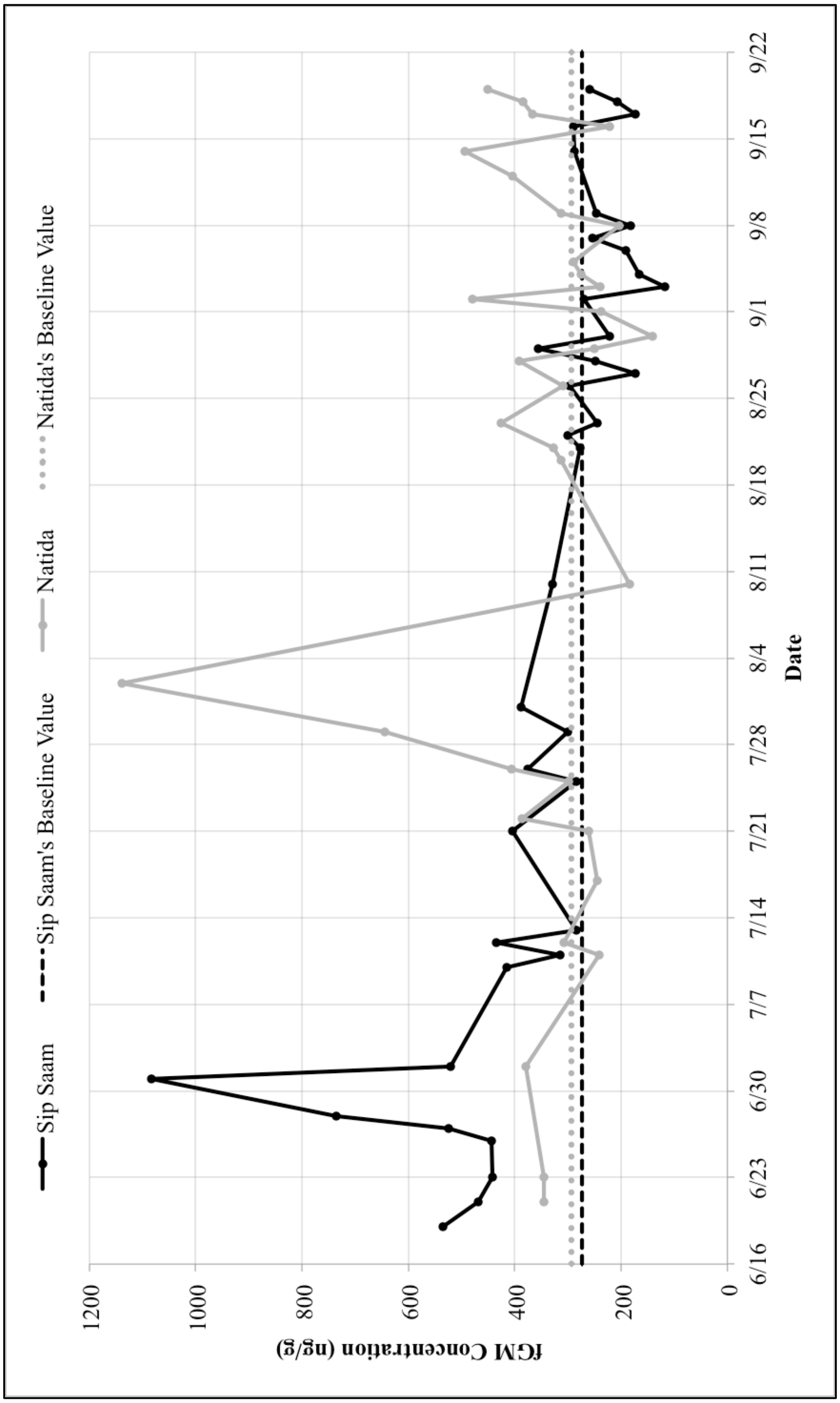
Hi Gore,

An honors student, Logan Whiles, is gathering feces from Clouded leopards to analyze hormones. He is not working with the cats, but having keepers collect and freeze the samples. Does he need to fill out an IACUC form?

Thanks in advance,

Brian

Appendix 4: Graphical representation of fGM concentrations for Sip Saam (#4723) and Natida (#4728) during a period of approximately three months. Baseline concentration for each cub is also displayed.

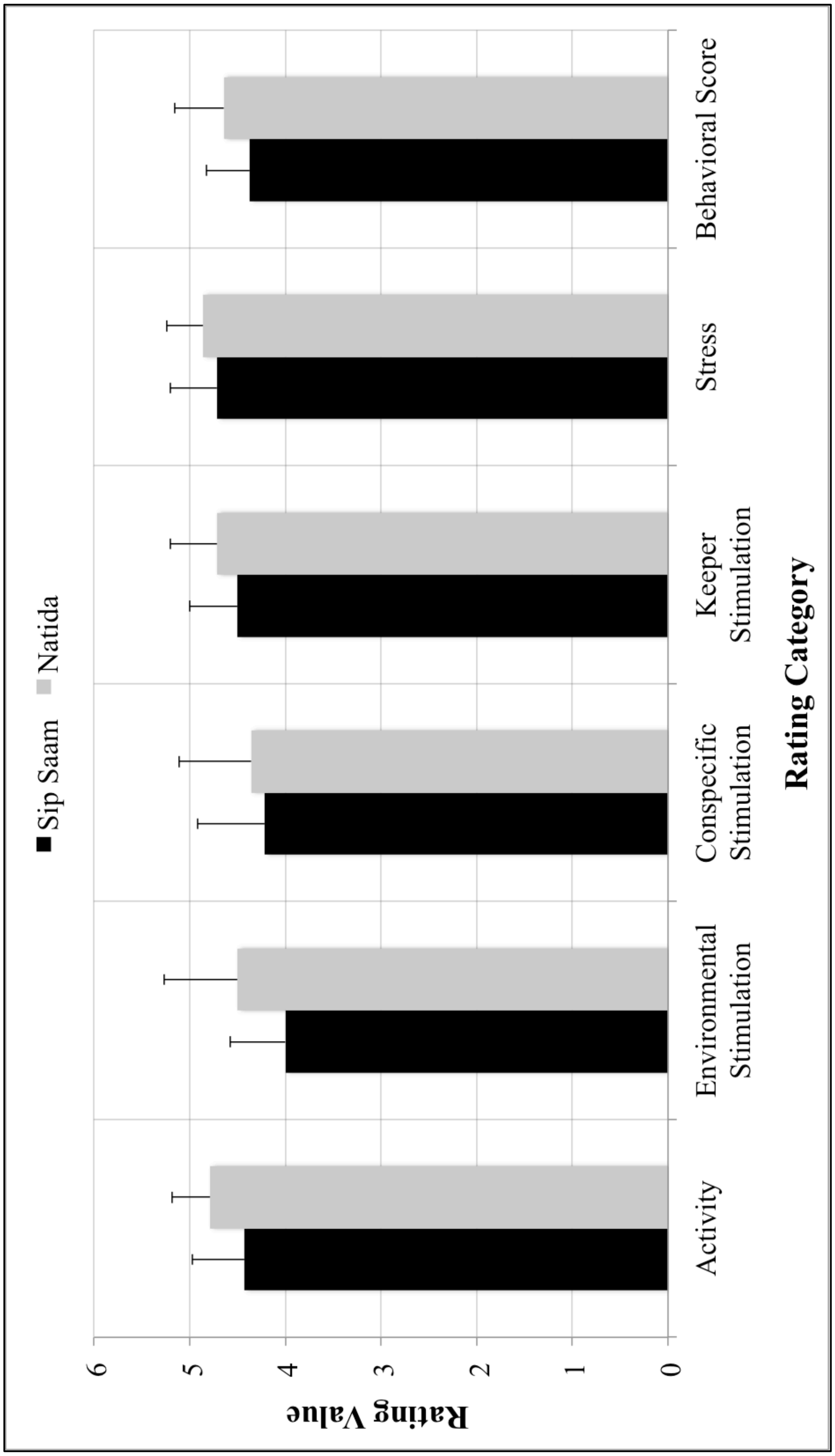


Appendix 5: Tables displaying individual sample collection dates and corresponding fGM concentrations (ng/g) for Sip Saam (#4723) and Natida (#4728).

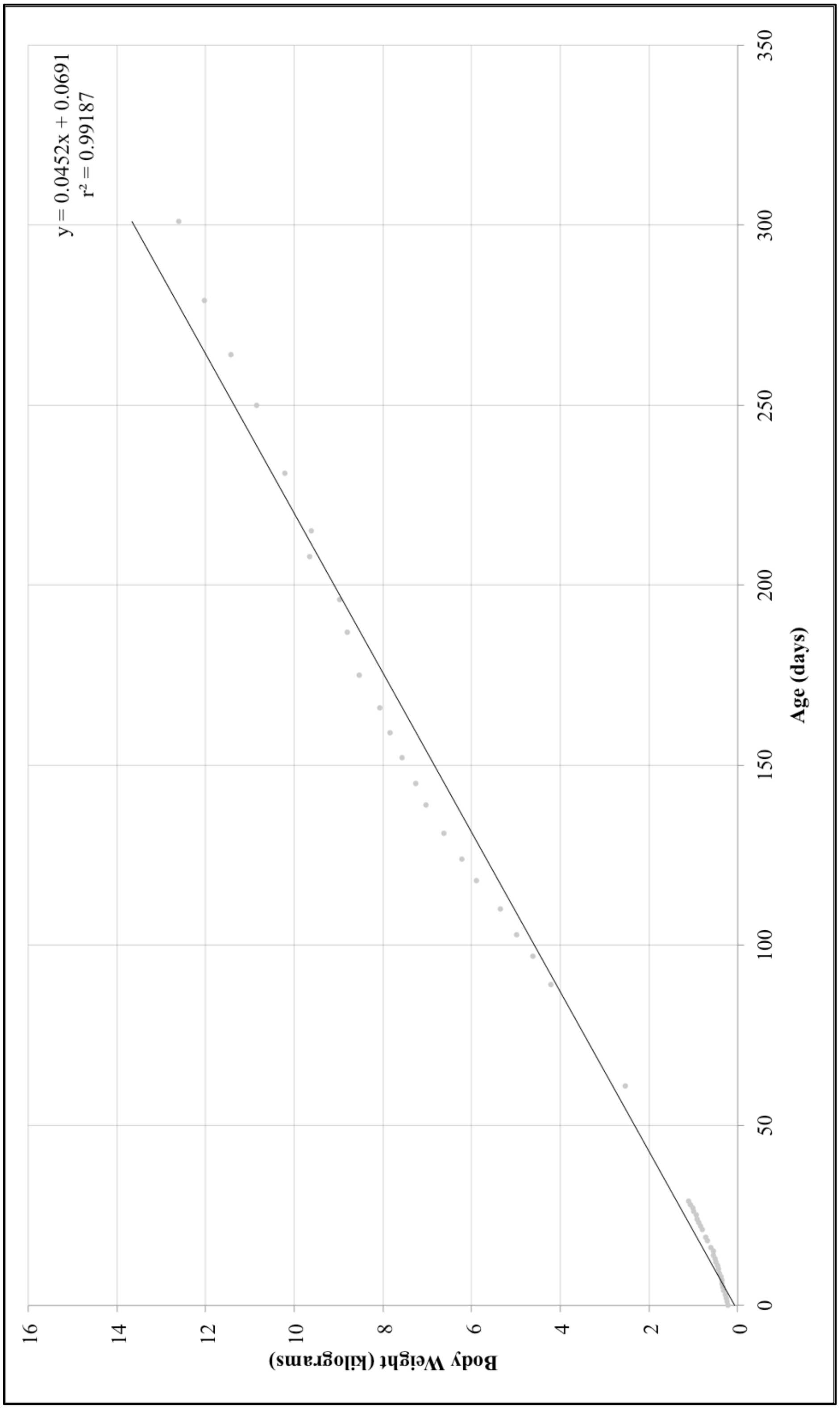
Sip Saam (#4723)	
19-Jun	535.5
21-Jun	468.7
23-Jun	441.9
26-Jun	443.2
27-Jun	523.8
28-Jun	735.9
1-Jul	1083.6
2-Jul	520.4
10-Jul	415.3
11-Jul	313.8
12-Jul	433.8
13-Jul	284.7
21-Jul	403.4
25-Jul	283.5
26-Jul	376.2
29-Jul	299.7
31-Jul	388.0
10-Aug	329.8
21-Aug	277.6
22-Aug	300.9
23-Aug	245.5
26-Aug	297.5
27-Aug	173.3
28-Aug	248.2
29-Aug	355.9
30-Aug	221.4
2-Sep	269.7
3-Sep	117.7
4-Sep	166.3
6-Sep	190.3
7-Sep	254.4
8-Sep	181.6
9-Sep	247.5
14-Sep	287.2
16-Sep	289.8
17-Sep	172.6
18-Sep	206.7
19-Sep	259.9

Natida (#4728)	
21-Jun	344.5
23-Jun	345.0
2-Jul	379.8
11-Jul	241.0
12-Jul	306.8
17-Jul	244.2
21-Jul	261.0
22-Jul	386.6
25-Jul	298.4
26-Jul	406.7
29-Jul	645.2
2-Aug	1139.2
10-Aug	183.7
20-Aug	313.2
21-Aug	327.8
23-Aug	426.2
26-Aug	309.7
28-Aug	392.3
29-Aug	249.7
30-Aug	141.0
1-Sep	236.8
2-Sep	480.4
3-Sep	239.6
4-Sep	275.3
5-Sep	289.0
8-Sep	203.0
9-Sep	312.5
12-Sep	404.8
14-Sep	494.6
16-Sep	220.9
17-Sep	366.5
18-Sep	384.9
19-Sep	450.6

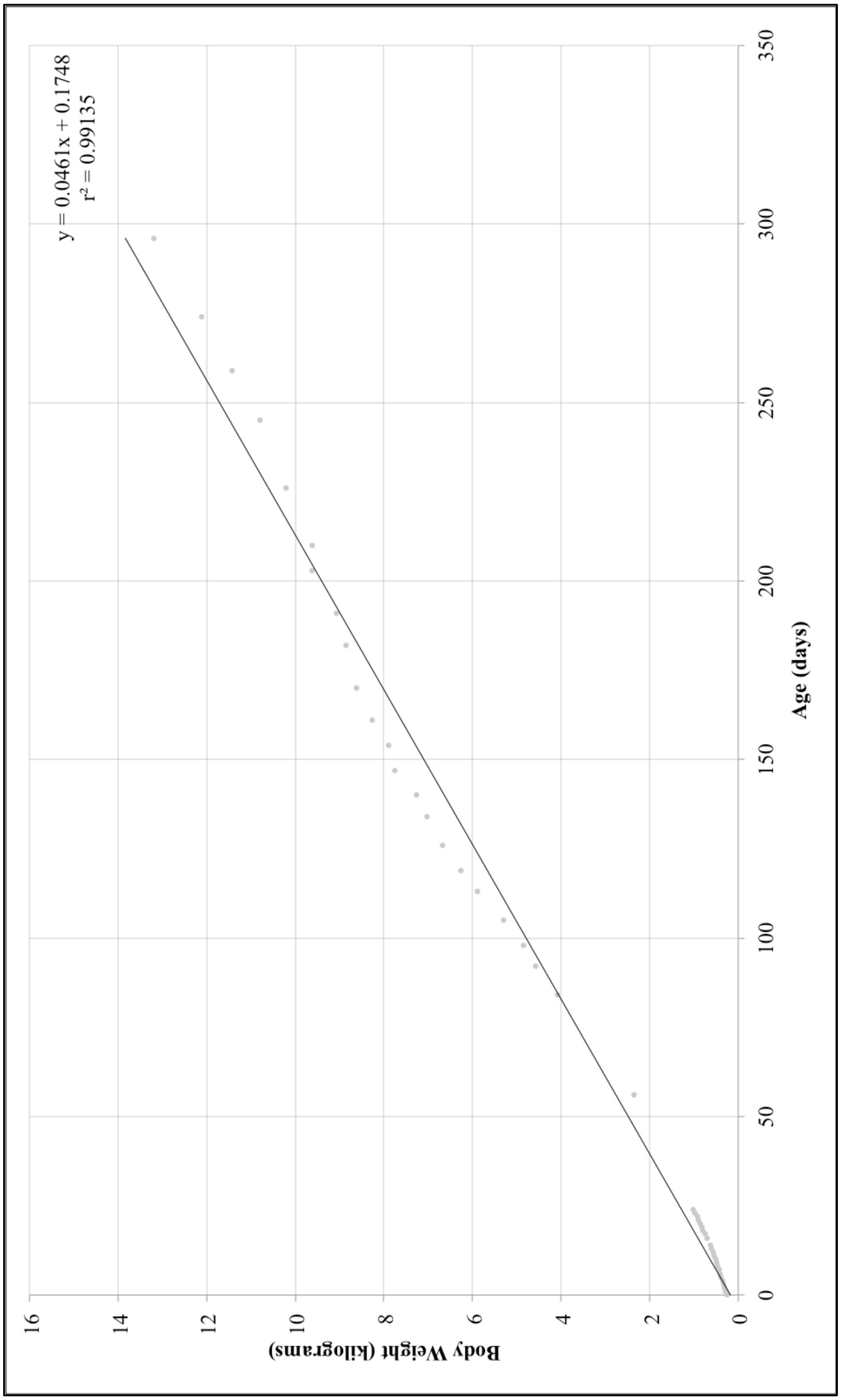
Appendix 6: Behavioral Ratings for Sip Saam (#4723) and Natida (#4728).



Appendix 7: Growth rate of Sip Saam (#4723). Individual weight data points are given with a linear trend line (y = 0.0452x; r2 = 0.99).



Appendix 8: Growth rate of Natida (#4723). Individual weight data points are given with a linear trend line (y = 0.0461; r2 = 0.99).



Appendix 9: Graphical representation of growth rate for the study individuals compared to Jack (#4729), a cub introduced to the study individuals' enclosure after the fecal sample collection period. Graph supplied by Karen Rice.