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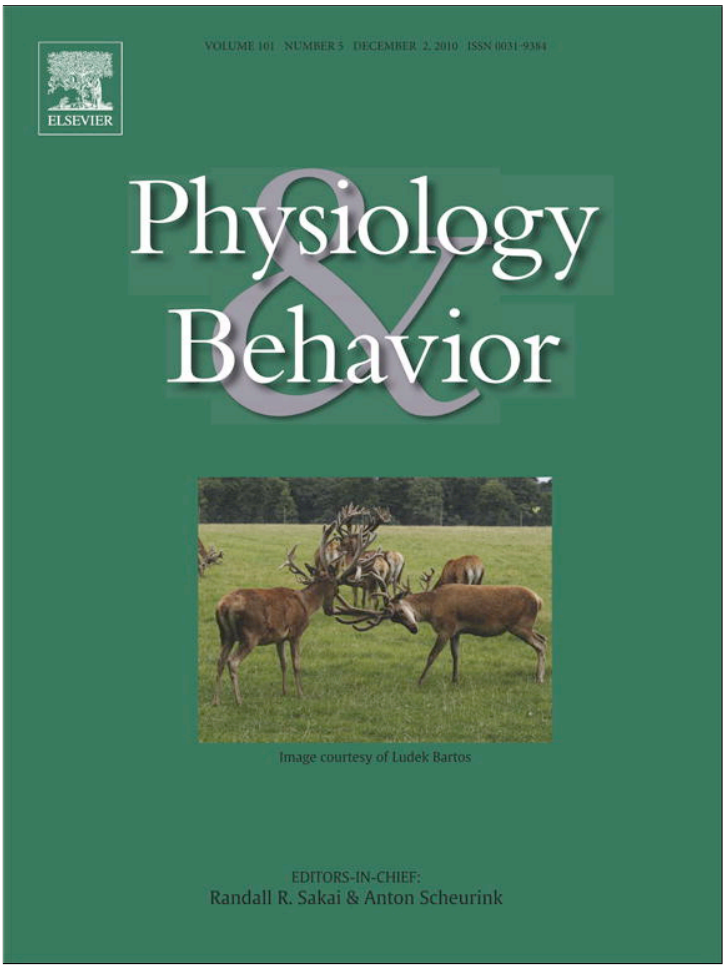

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Measuring salivary analytes from free-ranging monkeys

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ABSTRACT

Studies of large free-ranging mammals have been revolutionized by non-invasive methods for assessing physiology, which usually involve the measurement of fecal or urinary biomarkers. However, such techniques are limited by numerous factors. To expand the range of physiological variables measurable non-invasively from free-ranging primates, we developed techniques for sampling monkey saliva by offering monkeys ropes with oral swabs sewn on the ends. We evaluated different attractants for encouraging individuals to offer samples, and proportions of individuals in different age/sex categories willing to give samples. We tested the saliva samples we obtained in three commercially available assays: cortisol, salivary alpha amylase, and secretory immunoglobulin A. We show that habituated free-ranging rhesus macaques will give saliva samples voluntarily without training, with 100% of infants, and over 50% of adults willing to chew on collection devices. Our field methods are robust even for analytes that show poor recovery from cotton, and/or that have concentrations dependent on salivary flow rate. We validated the cortisol and SAA assays for use in rhesus macaques by showing aspects of analytical validation, such as that samples dilute linearly and in parallel to assay standards. We also found that values measured correlated with biologically meaningful characteristics of sampled individuals (age and dominance rank). The SIgA assay tested did not react to samples. Given the wide range of analytes measurable in saliva but not in feces or urine, our methods considerably improve our ability to study physiological aspects of the behavior and ecology of free-ranging primates, and are also potentially adaptable to other mammalian taxa.

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1. Introduction

The past few decades have seen great advances in the non-invasive assessment of animal physiological condition. These techniques have proved particularly valuable in studies of the behavior, ecology and evolution of larger-bodied free-ranging mammals, which unlike smaller mammals, birds, reptiles, and other groups, cannot easily be trapped and sampled in the field for practical and ethical reasons. In particular, methods for measuring the metabolic products of steroid hormones in feces or urine have become widespread, and have allowed investigation of a variety of ecological and evolutionary questions that depend on such physiological measures for analysis. Examples include: studies of the effects of seasonal changes in socioecology on variation in elephant (*Loxondonta africana*) androgen and glucocorticoid levels [1]; relationships between reproductive hormones and behavior in Mohor gazelle (*Gazella dama mhorr*) [2]; and the functional significance of primate male and female sexual signals [e.g. male mandrills *Mandrillus sphinx*, 3; female baboons *Papio hamadryas anubis*, 4,5; female chimpanzees *Pan troglodytes verus*, 6,7].

Studies of free-ranging non-human primates are among the biggest beneficiaries of such methodological developments. Early progress in measuring endocrine status came in the form of non-invasive techniques for the measurement of female primate reproductive hormones [e.g., 8], while more recent developments have added the ability to measure adrenal [e.g.,9], and testicular androgen [e.g., 10] function. More recently still, several studies have incorporated measurements of concentrations of urinary c-peptide of insulin, a small polypeptide cleaved from proinsulin when it is converted to insulin, into studies of primate energetics, ecology and behavior [11–13].

Despite such clear progress in the non-invasive assessment of primate physiology, there are many potential methodological obstacles associated with the measurement of metabolic products in feces and/or urine. These include: 1) that only certain physiological markers are broken down and excreted via these pathways in analyzable forms; 2) that certain excreted metabolites may stem from analytes of different origin [e.g. androgens from testes and the adrenal system, 9] or can be very similar in structure due to similarities in metabolic pathways [e.g. metabolites from testosterone and cortisol, 14], hence making it impossible to know how variation in metabolite excretion reflects variation in one variable over another; 3) that unlike native hormones in circulation, which vary little (if at all) across mammals, excreted hormone metabolites are often

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species-specific, requiring laborious biological validations every time an assay is used on a new species [15,16]; 4) that it is difficult to know from fecal measures whether assay responses may in part just be measuring hormones in diet that pass straight through the gut without having biological impact on the animal [for example, when wild baboons consume phyto-hormone mimics, 17]; 5) that fecal concentrations of hormone metabolites are dependent on the fecal sample's fiber content [18,19], and so may vary seasonally with diet; and 6) that fecal measures represent integrated measures of endocrine variation over a few days, which may make it impossible to link short-term variation in behavior to fecal hormone metabolite output [as may be the case in 20].

In order to expand the availability of different substrates that can be collected non-invasively for the assessment of primate physiological condition, there has been recent interest in methods to obtain saliva [e.g. 21–23]. Salivary analyses have great potential in studies of primate behavior and ecology given the range of analytes measurable. These include measures of adrenal (e.g. cortisol) and testicular endocrine function (e.g. testosterone), and of female reproductive hormones (progestogens and estrogens). Although these steroid hormones can be measured through concentrations of fecal and urinary metabolites, nonetheless salivary measures are still of interest because saliva provides a sensitive reflection of acute (short-term) changes in blood hormone concentrations and thus allows a real-time assessment of endocrine status. Further, as saliva contains the native forms of these hormones, problems associated with species-specificity in hormone metabolism and excretion do not occur. Of even more interest, however, is the potential of salivary assays for measuring aspects of condition that are currently difficult or impossible to measure through other means. These include correlates of sympathetic axis function [e.g. salivary alpha amylase, SAA; 24], which unlike HPA axis function is poorly studied in free-ranging mammals due to measurement difficulties [25], measures of immune function [e.g. secretory immunoglobulin A, SIgA; 26], and other measures of health such as C-reactive protein [CRP, an acute phase protein secreted by the liver in response to inflammation that has been associated with cardiovascular and other disease in humans; 27]. The ability to measure such analytes could add a great deal to studies of certain ecological and evolutionary areas. Examples include comparative patterns of immune function [e.g., 28], tests of the immunohandicap hypothesis for primate color signals [e.g. mandrills, 29], and (as in humans) relationships between chronic stress and sympathetic axis function [30], immune function [e.g., 31] and disease [e.g., 32].

To date, methods for obtaining saliva have only been published for captive, rather than free-ranging, animals, who have been trained to give samples by chewing on cotton ropes [21,23], buds [22] or rolls [33]. Further, these samples were only assayed for steroid hormones [e.g. cortisol, 23; androgens, 34], which are relatively unproblematic analytes. Steroid hormones show typically consistent and high percentage recovery from cotton rope [though see 35], and do not depend on the salivary flow rate of the animal. In contrast, some of the most interesting salivary analytes have inconsistent recovery from cotton rope [36], and are also known to be dependent on the animal's salivary flow rate [e.g., SIgA, 37], requiring this to be measured so that assay values can be corrected appropriately.

Here, we present methods for collecting and analyzing saliva from free-ranging primates. We undertook a range of field testing on free-ranging rhesus macaques on Cayo Santiago, exploring the use of substrates for the animals to chew on to deliver saliva, materials to act as attractants, and methods for obtaining salivary flow rate measurements. We also explored how willing different individuals were to give samples freely, and without training, according to their age and sex class. We then tested saliva collected from a number of adult males in three different salivary assays (cortisol, SAA, and SIgA). We tested whether our saliva samples responded to the assays, and investigated whether our samples met analytical validation criteria. We also assessed the extent of diurnal variation in the tested analytes. Finally, we determined whether overall variation in the values produced varied with respect to biologically meaningful characteristics (age and dominance status) of the animals tested. Together these data represent the first systematic attempt to collect and measure saliva from free-ranging primates without capture.

2. Materials and methods

All research reported in this manuscript was approved by the University of Chicago's and the University of Puerto Rico's Institutional Animal Care and Use Committees, and adhered to the legal requirements of Puerto Rico and the United States.

2.1. Study site and population

This study took place on Cayo Santiago, a 15.2 ha island located 1 km off the coast of Puerto Rico. A rhesus macaque population was established on this island in 1938 from free-ranging individuals captured in India [38], with no new individuals introduced to the population since then except through natural births. The animals are provisioned daily with commercial monkey chow but are free-ranging, and consume other wild foods on the island. Females in this population currently undergo a 6-month birth season from Sept to Mar, followed by a 6-month breeding season that occurs from Mar to Aug [39]. Data presented in this study were collected between June 25th and August 6th 2009. During this time approximately 1000 animals lived on the island, in 6 naturally-formed social groups. Data were collected on individuals in Group R, which consisted of a mean ($\pm$SD) of 248.3 ± 4.2 (range = 245–253) individuals during the study period, with fluctuations in group size being accounted for by births, deaths, immigrations, and emigrations. All individuals in the population are tattooed and ear-notched, making it easy for observers to identify them.

2.2. Collecting saliva samples

We used standard white cotton rope approximately ¼ in.thick as the basis for our collection devices. However, as some of the analytes of interest show inconsistent recovery from cotton [36], we used fine cotton thread to hand-sew Salimetrics Oral Swabs (www.salimetrics.com) to the end of the rope. These swabs are made from a polymer, have verified recoveries for salivary analytes, and are known not to cause changes in sample pH. Unlike previous methods devised for obtaining saliva samples from captive primates using swabs or cotton rolls, where the roll is completely saturated in a sugar solution and then dried [33], we devised a method of making the swab attractive to the animals without adding any substance to the swab itself. This is primarily because we wanted to avoid adding any liquid to the swab that might not dry fully, as this would affect salivary volumes centrifuged from the swab, and hence our ability to assess salivary flow rate. (Unlike rope, oral swabs are difficult to dry after liquid has been added.) To do this, we wrapped the cotton rope around the swab numerous (>15) times, and then brushed the cotton rope (avoiding any contact with the swab itself) with a thick sugary mixture that we created by adding a very small amount of water to Tang drink crystals (Kraft Foods Inc.). We then allowed this to dry thoroughly so that there was no water content in the rope/swab collection device. When manufacturing other collection devices we tried adding several other substances to the rope, including honey and peanut butter. Finally, we tried all collection devices both with and without a grape added to the rope as an additional attractant. To do this we threaded the other end of the rope to the swab through a grape using a needle, and then moved the grape down to a position just above the swab.

After some initial pre-testing of individuals to test the relative efficacy of different attraction materials and designs, we then

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undertook systematic testing using our final collection device design. To test individuals for their willingness to give samples, we approached 28 different adult males, 26 adult females, and 21 juveniles and infants in the field who were not already eating, and wherever possible, were alone. Males were tested for willingness to give samples both during the peak of the mating season, and following the termination of this period. Though we tested individuals for willingness to give samples in these different age/sex categories, we only went on to collect and process samples from adult males, as part of our wider ongoing study on males taking place at that time. Testing and sample collection usually took place between 13:30 and 16:00. On one day, we also sampled two different adult males throughout the day to obtain samples that could be used to assess diurnal variation in analyte concentration.

To test or sample individuals, we pushed a collection device out to them, such that the swab rested against the ground a few feet away from us (Fig. 1). If animals looked interested, but appeared wary of the experimenter holding the rope, we then tied the rope to a nearby tree, so allowing the experimenter to step backwards away from the collection device. For all individuals we recorded whether they were willing to approach the collection device, take it in their mouth, and begin to chew. For those adult males from which we were interested in obtaining samples for testing, we then allowed the animal to continue chewing in order to deposit a sample. Using a stopwatch, we continuously recorded whether the swab part of the collection device was in the animal's mouth, stopping the timer when the swab was outside the mouth. When the animal abandoned the swab, or after 2 min of total chewing on the whole device, the experiment was terminated by the experimenter moving towards the collection device, at which point animals retreated. We then separated the cotton rope from the swab (wearing gloves), and placed the swab into a centrifuge tube with retainer. We also placed the cotton rope that had been in the animal's mouth into a separate centrifuge tube with retainer. Samples were then placed immediately into a cooler containing ice packs. When we returned from Cayo Santiago to mainland Puerto Rico (usually within 1–2 h of sample collection), we centrifuged each sample for 10 min. We then transferred the samples to 2 ml centrifuge tubes using 1 ml disposable Pasteur pipettes, and measured the volume of the saliva that had been deposited using gradations on the centrifuge tubes. Samples were then stored at −80 °C, until they were transported on ice to the Institute of Mind and Biology, University of Chicago, for analysis.

Fig. 1. A free-ranging rhesus macaque voluntarily approaches and chews on a collection device to deposit a saliva sample on Cayo Santiago, Puerto Rico.

2.3. Measurement of salivary analytes

We tested collected saliva samples in cortisol, SAA and SIgA Enzyme-Immuno-Assays (EIAs), all purchased from Salimetrics (www.salimetrics.com). Cortisol was measured from samples collected on cotton rope (since this analyte shows consistent recovery from cotton and is not flow rate dependent), whereas SAA and SIgA assays were tested on swab samples. For the cortisol and SAA assays, samples were diluted at the levels recommended for humans (cortisol, undiluted; SAA, 1:200). For the SIgA assay, we tried adding saliva concentrations at both a 1:5 dilution (as recommended) and undiluted, and also tried adding a range of undiluted salivary volumes to assay conjugate for incubation, from 10 µl (as recommended for humans) to 160 µl. We also tested a human saliva sample to confirm that the assay was measuring SIgA as it should (in humans), and tested for any effect of the Tang drink crystals on the assay by adding small amounts of this to replicates of controls. Previous work in rhesus macaques has also shown that the addition of drink crystals has no effect on salivary endocrine measures (e.g. cortisol values produced from plain rope and rope covered in drink crystals are strongly and highly significantly correlated) [21]. In the cortisol assay, we tested 28 samples from 5 males, in the SAA assay, 24 samples from 6 males, and in the SIgA assay, 22 samples from 4 males. We followed manufacturer instructions in assaying samples in the Cortisol and SIgA assays in duplicate, but in the SAA assay singularly. For the two assays that responded to our samples (SAA and Cortisol, see Results), measures of intra-assay variation are: SAA, high concentration = 2.5% (n = 10), low concentration = 7.2% (n = 10); Cortisol, high concentration = 3.4% (n = 18), low concentration = 3.7% (n = 14). As we measured all samples on just one plate, we did not record measures of inter-assay variation. Sensitivity of the cortisol assay, measured at 90% binding on our plate, was 0.037 µg/dL. Sensitivity of the kinetic SAA assay is determined by the change in absorbance between the 1 min and 3 min time points at which absorbance is measured; a change in optical density of less than 0.01 (at 405 nm) is below assay sensitivity. Samples spiked with known levels of each analyte show very high levels of recovery (cortisol—101%, SAA—101%). In addition, for each assay, we measured serial dilutions of several samples. For both assays we found that samples diluted linearly. For the cortisol assay, we also found that each sample tested diluted in parallel to the standard curve (t-tests on each of 3 samples from 3 different males, all $t<1.15$, all $p>0.3$). The kinetic SAA assay does not feature a standard curve and cannot therefore be assessed for parallelism.

2.4. Data treatment

For cortisol values measured from rope samples, we present values uncorrected for flow rate. For SAA values measured from swab samples, we present values both uncorrected and corrected for flow rate, since there is continued debate in the literature about whether this correction is necessary for this analyte, with the assay manufacturer recommending it (see http://www.salimetrics.com/assets/documents/news/2009/Flow_rate_correction_advisory,_sAA,_1-6-09.pdf), despite one study claiming that it is unnecessary [40]. The rationale for making such a correction is that as an animal produces more saliva, this dilutes the relative concentration of analytes in saliva, and leads them to appear relatively weak by comparison to a value from an animal currently producing less saliva. Of particular concern is that a stressor stimulating sympathetic axis function would decrease saliva flow rate through an inhibitory effect on parasympathetic function, which could lead to apparently higher concentrations of analytes in saliva without an actual increase in their secretion [40]. To adjust for variability in salivary flow rate, we used both the time the swab was in the mouth, and the final volume of saliva deposited. Multiplying the rate of saliva flow by the analyte concentration allows measured SAA amounts to be presented per unit time.

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Unlike in analysis of endocrine metabolites, which are known to be highly species-specific in structure, assays of salivary hormones are measuring analytes in their native forms. For the present analytes, it is known that cortisol is structurally identical across mammals (though some mammal species have corticosterone as the major glucocorticoid rather than cortisol in blood). Further, previous studies of cortisol in rhesus macaques have undertaken biological validations, demonstrating that plasma and salivary cortisol levels are highly correlated [41], that patterns of salivary cortisol variation broadly show the same patterns of life-history variation as plasma levels do [41], and that infant rhesus macaques have elevated levels of salivary cortisol following social bouts [42]. For SAA, studies of alpha amylase diversity between Old World primates also show very little inter-specific variation in this analyte [43]. Macaque and human SAA are known to be very similar structurally [44], while parotid alpha amylase is also known to be very similar in amino acid composition between the two species [45]. More importantly, antibodies raised against human alpha amylase show extremely high affinity for the alpha amylase of Old World monkeys, including rhesus macaques specifically [46]. In contrast to SAA and Cortisol, however, SIgA is known to be extremely hyper-variable in rhesus macaques (see Discussion) [47,48].

We tested to see how well the corrected and uncorrected sets of SAA values correlated using a Pearson's correlation. We then used Spearman's rank correlations to test whether values produced in our assays correlated with male age or rank (using both corrected and uncorrected values for SAA). These analyses are not intended to be new comprehensive biological validations, nor are they intended to provide a serious investigation of these variables in the study population—clearly much larger sample sizes are needed for this. Instead, we undertake these correlations merely to assess whether the values we obtained from our assays correlate with basic biological variation in the study animals, which may give us increased confidence that our assays are producing potentially reliable and interesting data. For cortisol, we might predict that lower ranked individuals would have higher levels, as seen in females in this population [49]. For SAA, this is to our knowledge the first investigation of this analyte with respect to variation in age and rank in a non-human primate; as such it is unclear whether we might predict any relationships at all. Male age was available from the Cayo Santiago database. Bivariate dominance interactions were used to establish dominance hierarchies for males in our study. During the period of saliva collection, dominance ranks were unstable [50], especially in the month of June. However samples were collected from the end of June to early August only, after the period of main rank instability, and our rank data from July and early August were sufficient to produce a statistically significant linear dominance hierarchy across this period [50], which was used in the present analyses. Two-tailed tests were used for correlations, with $p<0.05$ considered statistically significant; we present both asymptotic and exact probability values. Statistics were undertaken in SPSS 16.0.

3. Results

Though we tried honey and peanut butter as attractants on the rope, it became clear quickly that dried Tang drink crystals, being both high in sugar and highly colorful, were most attractive to the animals. We found that the addition of a grape to the device brought us to the attention of many monkeys, which made it difficult for us to select one target monkey for sampling. Further, the addition of a grape did not encourage individuals to chew on the swab; instead, monkeys stole the grape from the rope without paying attention to the swab. As such, our final collection device had rope brushed in Tang, but without a grape as an additional attractant. Using this device, we systematically explored willingness to give samples, and found that during the breeding season, around half of adult males and females, and all infants and juveniles, were willing to give samples by approaching and chewing on the collection devices (Table 1; Fig. 1). We successfully collected 26 swab samples from 6 adult males, and 41 rope samples from 6 adult males using these methods. Males chewed on swabs for an average of 42 s (±SEM 4.8, range 6–69) (time of rope in mouth was not measured, but this was usually longer). Salivary volumes deposited were: rope, mean = 454 μl ± SEM 29.4, range 100–900 μl; swabs, mean = 230 μl ± SEM 19.8, range 20–420 μl.

Table 1
Number of animals tested and willing to give samples according to age/sex category.

	Adult males		Adult females	Infants and juveniles
	During peak mating	After peak mating		
Number tested	28	28	26	21
Number willing to give samples	11	15	14	21
Proportion willing to give samples	39%	54%	54%	100%

No rhesus samples responded to SIgA assay at any concentration. Human saliva responded to the assay as anticipated, and the addition of Tang to assay controls had no effect on their concentration, demonstrating that it is the rhesus saliva itself that is unsuitable for the assay. Rhesus samples collected responded to the salivary cortisol assay, and the SAA assay, at the manufacturer recommended dilutions for humans. Values produced in the cortisol assay had a mean (across all samples, treating each sample separately) of 1.16 ± SEM 0.2 μg/dl. Some males were quite consistent in the values they produced (e.g. male 11Z, mean = 0.43 ± SEM 0.09 μg/dl, range = 0.15–0.74, n = 7), whereas other males were more variable (the maximum variation seen within any one male (male 54 V) was 0.26–3.0 μg/dl). Values produced in the SAA assay had a mean of 122.6 ± SEM 16.2 U/ml (uncorrected for flow rate) and 42.8 ± SEM 9.7 U/min (corrected for flow rate). Although consecutive samples for testing diurnal variability in cortisol and SAA were available from only 2 males, in the individuals tested there was diurnal variability in sample concentration for both of these analytes, with both being higher in the morning and afternoons than during the middle of the day, and values varying within an individual by over four-fold over the course of the day (Fig. 2). Salivary flow rate correction for SAA results produced values that correlated with uncorrected values (Pearson's correlation, $r = 0.60$, $n = 24$, $p = 0.002$), but with a great deal of variability in relative concentration (Fig. 3). Mean uncorrected male SAA levels ($r = -0.83$, $n = 6$, $p = 0.043$, exact $p = 0.058$), (but not corrected levels, $p > 0.1$), showed a relationship with age, with older males tending to have lower mean SAA levels. Neither corrected nor uncorrected SAA levels were correlated to male dominance rank (both $p > 0.1$). Male mean cortisol were related to male dominance rank ($r = 0.90$, $n = 5$, $p = 0.037$, exact $p = 0.083$), with lower ranked

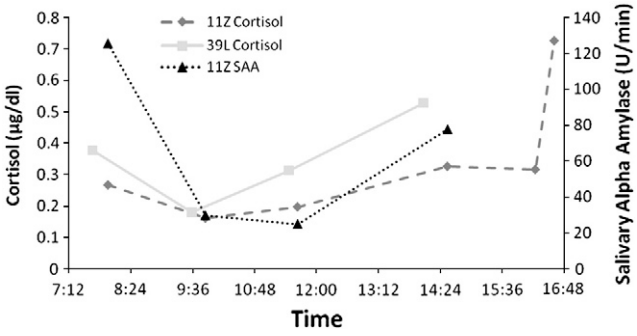

Fig. 2. Diurnal variation in salivary analyte concentration as measured on one day for salivary alpha amylase (one male, 11Z) and cortisol (two males, 11Z and 39 L).

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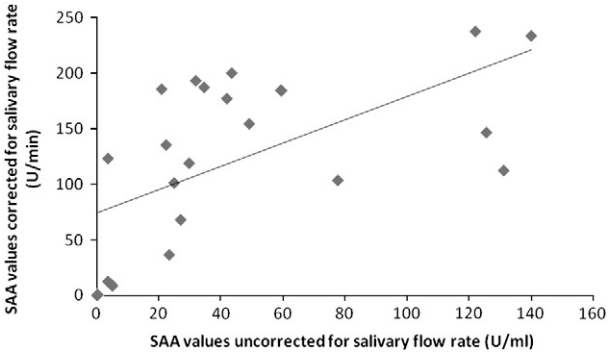

Fig. 3. Correlation between rhesus macaque salivary alpha amylase concentrations when considered with and without flow rate correction. Though values do correlate, consideration of flow rate does cause values to change considerably with respect to their relative concentrations, suggesting that the potential need to correct for flow rate needs to be considered carefully.

individuals tending to have higher mean cortisol levels. However mean cortisol levels were not correlated with age ($p>0.1$).

4. Discussion

Our results demonstrate that, for inquisitive and relatively terrestrial primate species such as macaques, non-invasive saliva collection can be undertaken safely and successfully from habituated free-ranging monkeys. Our study provides necessary information and methods for doing this, even when the analytes of interest are potentially flow-rate dependent, and show inconsistent recovery from cotton. When animals chewed on devices they typically pulled rope off the swab, and we observed them chewing on both the rope and the swab separately. From our observations, it appeared that saliva deposited on the swab was deposited directly on the swab, and that saliva deposited on the rope was deposited directly on the rope. However, it is worth bearing in mind that there are potential issues for saliva being deposited on the swab having passed through the rope if this becomes fully saturated, given that some analytes of interest may show poor recovery from cotton (hence demonstrating adherence to that material). We recommend testing of collection devices and careful observations of how saliva is being deposited.

We have demonstrated that saliva collected using our methods is not only viable for analysis in commercially available assays, but that samples meet typical assay validation criteria. Although it was not the aim of this manuscript to produce new biological validations, the values we produced did correlate with biologically meaningful characteristics of the animals from which they were collected. In our study, cortisol values tended to show higher levels in males of lower dominance rank, consistent with results for females in this population, where low ranking individuals tend to have higher levels of plasma cortisol [49], and show higher increases in cortisol when moving into more costly reproductive phases [51]. In addition, SAA values uncorrected for flow rate tended to show lower mean levels among older individuals, though it is unclear whether this might be predicted [see Materials and methods, and 53]. Our other results confirmed data from other studies suggesting that it is important to control for collection time [for example, for SAA, 52]. In our case, samples for both measurable analytes showed higher values in the early morning and in the afternoon than in the late-morning. It seems unlikely that this pattern is caused by sample degradation; if so we would expect the lowest values to come from the early morning, as these samples have the longest time before freezing in the field. We consider two possible non-mutually exclusive explanations. First, the pattern may be influenced by feeding, since the study animals are fed in feeding corrals first thing in the morning, and return to these corrals for additional feeding in the afternoon (although no new food is provided at this time). Second, the study animals are much more socially active in the early morning and afternoon than in the late morning and the middle of the day. As the animals encounter each other at higher rates and in higher densities in the feeding corrals, these factors necessarily occur together on Cayo Santiago, and are therefore extremely difficult to separate. We advise all researchers considering collecting similar data to standardize collection times as much as possible.

Although values of SAA corrected and uncorrected for salivary flow rate were highly correlated, the effect of controlling for flow rate caused values to change substantially relative to each other. Until flow rate issues surrounding this analyte are resolved, we recommend that authors measure this (as in the present study), and consider both sets of values in analyses. The failure of the SIgA assay to develop in response to rhesus samples was unfortunate. SIgA consists of two IgA monomers joined by a J-chain and an additional secretory component [53]. In turn, each IgA monomer is made up of 3 constant domains and a hyper-variable hinge region [53]. Although there has been relatively little polymorphic diversity identified in humans [who have two IgA loci, IGHA1 and IGHA2], rhesus macaques have been shown to have extremely high levels of allelic polymorphism, with each individual animal expressing two different forms of the single IGHA gene found in Old World monkeys, and a sample of just 4 rhesus macaques demonstrating 6 different allelic forms [47]. This sample also featured a high degree of variation in the hinge region (5 different hinge regions in the 8 immunoglobulins, each with a different number and composition of base pairs when compared with hinge regions present in hominoids) [47]. (Several authors have suggested that this hyper-variability probably reflects an evolutionary arms race between the body's defenses and fast-evolving bacteria [48].) In contrast, several studies have shown that ape species feature IgAs that are more similar to those found in humans [e.g. chimpanzees, 54]. As such, though the SIgA assay did not work for rhesus macaque saliva, this does not mean that studies of other non-human primates, especially apes, might not have more success.

There is a wide range of analytes measureable in saliva, representing aspects of physiology currently difficult to measure from other substrates, including numerous biomarkers of health and disease. This makes the techniques presented in this study extremely valuable for studies of free-ranging primates. For example, in the present study we successfully measured both cortisol and SAA levels; one potential topic that could utilize these assays further would be studies of chronic stress. In humans, chronic stress is thought to cause asynchrony between the HPA and sympathetic axes of the stress response causing increased variation in cortisol levels relative to levels of SAA [30], but to our knowledge this phenomenon has not been investigated in other mammalian species.

Although a percentage of adults were unwilling to give samples in the present study, the willingness of individuals to give samples could potentially be used as a criterion in focal selection at the commencement of studies if the study being undertaken required saliva sampling from all study animals. However, we caution against effectively selecting for specific qualities among study subjects, such as 'boldness', and then expecting the focal animals to be representative of the population. Even if it was only possible to obtain saliva samples from a subset of study animals, this may nonetheless be sufficient to provide novel and interesting data, at least for those individuals. Our data suggest that the natural curiosity of young primates is beneficial in this type of testing; all infants and juveniles were highly intrigued by the collection devices, approaching them and putting them in their mouths. Although we did not measure the salivary volumes produced during these interactions, many assays of interest require only very small volumes for measurement. For example, the SAA assay used in the present study requires just 10 μl of saliva; we are confident that such tiny saliva volumes will be deposited by even the smallest of individuals. As such, our methods would seem to have particular potential for studies of free-ranging

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infants that require non-invasive measures of physiological status. Given that urine and fecal samples are usually very difficult to collect from very young free-ranging animals, collection of saliva may indeed offer a more reliable alternative for generating physiological information from animals in this age class.

One important issue to bear in mind when considering using such techniques is disease transmission. Without due care, these techniques could induce this in two ways: between different study animals, and between study animals and human collectors. In order to prevent the former, it is important to observe all collection materials, and once one animal has had them in its mouth, remove them before another animal approaches. To prevent the second, gloves and other safety equipment should be used at all times, and researchers should ensure safe distances between themselves and study animals.

Given the constraints on obtaining samples using our methods, there are certainly some primate species for which methods for collecting saliva are likely to be very difficult to implement in free-ranging settings. These include the smaller, more skittish primates, and those that are usually found only high in the forest canopy. However, many primate groups, such as the Papionins, in which many species are relatively large bodied, bold, and terrestrial, seem to have promise for such collection techniques. Further, even for arboreal and/or shy species, ropes could be attached to branches and left with attractants to induce individuals to approach and give samples. In a similar way, such devices could be used to collect saliva from free-ranging mammals in other taxa. Salivary cortisol analyses have been undertaken on species as diverse as elephants [*Elephas maximus*; 55] and white-tailed deer [*Odocoileus virginianus*; 56], but these have almost always, if not exclusively, been undertaken on captive individuals. In some groups, such as ungulates, methods for attracting free-ranging individuals for measurement using provision of nutrition (salt licks) are already widespread [e.g. 57]. Addition of salivary chewing devices with appropriate attractants could enable measurement of critical new variables in such studies. As such, salivary collection and analysis methods such as those outlined in the present study improve not only our ability to study free-ranging primates, but have broad potential to increase the depth and scope of studies on the behavior, ecology and evolution of large-bodied free-ranging mammal populations.

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