Studying stress-associated non-invasive biomarkers in Japanese macaques

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Abstract

My aim for the present thesis was to explore and develop tools for understanding acute stress in a minimally disruptive way in Japanese macaques (*Macaca fuscata*). A main goal I have been focused on is using saliva to help increase the temporal resolution of studying the stress response of two primary stress pathways; the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis in this species. Studying sympathetic response in a non-invasive manner is particularly difficult due to a lack of methodological tools. However, saliva is a broadly useful medium because it contains proteins, steroids, and other meaningful molecules of interest to measure physiological responses. First, I developed a non-invasive method to collect saliva from unrestrained and awake single-housed Japanese macaques. This method allowed me to measure stress-related short-term changes of salivary alpha-amylase, a digestive enzyme that is also associated with autonomic nervous system activity. I showed that saliva collection is possible through cooperative training with Japanese macaques and that this can be used to study the stress response of an understudied system, the SAM axis. Later I led a study with the same captive group of monkeys to understand to what extent a housing relocation increased their stress response. There is little information on how stressful housing relocation is for Japanese macaques, though such information could help inform management practices. These monkeys were planned to move due to housing renovations and their stress response was opportunistically investigated. Fecal samples were collected to monitor activity of the steroid cortisol, a stress-associated hormone that indicates HPA axis activity. No statistical significance was found over the consecutive days of fecal cortisol monitoring; however, a minimal response was shown on the relocation day, which suggests that the relocation itself was mildly stressful. The findings were in line with the reported literature; i.e. that the non-application of anesthesia, short movement duration, and consistency in both environments were all likely contributing factors in stress reduction during housing relocation. In the final study, I expanded cooperative saliva collection to a wild living group of Japanese macaques to study short-term changes of stress-associated analytes. By utilizing attractants applied to cotton ropes, monkeys were habituated to sampling and characteristics such as their receptivity and chewing duration were recorded to modify and improve the method. Salivary alpha-amylase and salivary cortisol levels fluctuated in tandem with social behavior, in particular with levels decreasing following social grooming. The study of stress-related salivary analytes in *M. fuscata* was advanced by investigating the methodological feasibility in a wild living group of monkeys in relation to short-term changes of two complementary biomarkers that correspond to HPA and SAM axes, i.e. salivary cortisol and salivary alpha-amylase, respectively. The main findings of this thesis are that it is possible to collect saliva in a minimally disruptive way in both captive and free-range living Japanese macaques. Doing so allows us to simultaneously measure the two primary stress systems in an acute manner. The need for stress monitoring and its applicability from a management perspective was shown in a captive setting. Stress research can be further incorporated in this species in order to quantify and assess health.

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Abbreviations

ANOVA: analysis of variance

C: Celsius or centigrade

CV: coefficient of variation

GAS: general adaptation syndrome

h: hour(s)

HPA: hypothalamic-pituitary-adrenal

ID: identification

min: minute(s)

NHP: non-human primate

PB: peanut butter

PRT: positive-reinforcement training

SAM: sympathetic-adrenal-medullary

sAA: salivary alpha-amylase enzyme

sC: salivary cortisol

SD: standard deviation

SEM: standard error of the mean

SNS: sympathetic nervous system

U/mL: enzymatic units per milliliter

y/o: year(s) old

µL: microliter

µg/dL: microgram per deciliter

Chapter 1

General Introduction

This thesis concerns exploring and developing tools for understanding acute stress in a minimally disruptive way in Japanese macaques (*Macaca fuscata*). A main goal I have been focused on is using saliva to help increase the temporal resolution of studying the stress response of two primary stress pathways; the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis in this species. Here in Chapter 1, the concept of stress will be introduced, particularly as I have approached the topic to help answer research questions in the last years. Chapter 2 consists of a published report concerning captive work to understand the characteristics of saliva collection and the acute responsiveness of salivary alpha-amylase to act as a measure of sympathetic nervous system (SNS) activity in Japanese macaques. Chapter 3 is a continuation of the captive work using a more established method to measure stress (i.e. fecal cortisol metabolites) to better understand to what extent housing relocation increases the stress response. This chapter is an application using concepts of stress to provide a measure of health or well-being in the species. By Chapter 4, I return to saliva as a way for measuring the stress response, however, the method has been expanded in two ways; (1) a wild living group of *M. fuscata* is studied and (2) two complementary biomarkers are included (i.e. salivary alpha-amylase and salivary cortisol) that correspond to the two previously mentioned physiological stress-associated systems. In the final chapter, Chapter 5, the main findings and limitations of the previous data chapters are summarized. Some recommendations for further investigation are mentioned. Excluding the general discussion chapter, each chapter has been written as a standalone report;

general concepts are introduced within each chapter and require no particular order of reading.

A brief introduction to stress research

The concept of *stress* can be described in many ways. The root source of the word in English indicates its long history, which originated from the Latin words *strictus*, meaning tight or narrow, and *stringere*, meaning to tighten (Jin 2012). Stress has long been established as a concept to describe a dynamic state of unbalance. Our earliest records show that the ancient Greek physician, Hippocrates (460-375 BCE) established a model of disease based on the balance of, what was thought of at the time, the four basic elements; earth, air, fire, and water (Greenstone 2010). These elements corresponded to blood, phlegm, black bile, and yellow bile, and disease was thought of as the unbalance of these biological material. This model of disease was propagated by Galen of Pergamum (129-200 CE), a prominent Greek physician in ancient Rome, and the theoretical model of maintaining balance to ensure good health was held for many centuries thereafter. Cultural pressures in the ancient world restrained advances of these ideas, particularly for physiological studies (Conner, 2017). Nevertheless, the foundations for understanding how balance or constancy play a large role in the health of organisms were established in our very early written records. By the 17th century, in the English language and notably outside of scientific spheres, stress was used to describe hardship or adversity (Hinkle 1974). The term was adopted by physicists in the 18th century to describe force, pressure,

or strain on physical material objects and it was from here where stress expanded into other scientific fields such as physiology (Brantley & Thomason 1995).

In the 19th century, pioneering work by Claude Bernard brought forth the concept of the *milieu intérieur*, or the internal environment (Gross 1998). Bernard's work focused on the internal physiological systems of organisms and he reasoned that body tissue "intend" to maintain certain conditions such as blood sugar and temperature (Cziko 2000). The ideas of Bernard became central to other scientific fields such as neurophysiology and psychology in large part by the work of Walter B. Cannon, a physiologist at Harvard in the early 20th century (Gross 1998).

In his search to understand the physiological underpinnings of emotion (e.g. Cannon 1915), Cannon was highly influential to advancing concepts of biological stress. His work focused on the multitude of risks that unbalance the physiological functions of organisms, including exposure to cold, hypotensive hemorrhage, severe pain, insulin-induced hypoglycemia, or mental distress, and how these cause the sympathetic nervous system and adrenal medulla to become activated (Goldstein & Kopin 2007). He coined key concepts such as *homeostasis* to describe the natural regulatory ranges of mammalian physiology (Goldstein & Kopin 2007), and the behavioral adaptive response *fight-or-flight*, the instinctive bodily reaction to rapidly defend or flee in a dangerous circumstance (Quick & Spielberger 1994). These terms are commonly used in research today to conceptualize the adaptive physiological and behavioral responses of animals in relation to threats in their environment. Cannon's work is important because it integrated the "internal environment" of Bernard and interconnected their

functions to external phenomena. His work focused on the adrenal medulla and the more direct acute responses to stress (such as fight or flight) to understand the mechanisms of homeostasis. Complementary to Cannon's findings, another researcher improved our comprehension of stress by concentrating on the adrenal cortex and chronic physiological responses to stress in order to make sense of disease.

In a letter to *Nature* in 1936, Hans Selye described 'diverse nocuous agents' that produced a generalized disease syndrome. Experiments on rats showed that a multitude of stressful exposures such as excessive exercise, cold temperature, and pharmacological drug injections resulted in physiological changes including greatly enlarged adrenals, erosions in the stomach and small intestine, and a rapid decrease in size of lymph glands and liver in the hours thereafter (Selye 1936). Selye reported that when relatively lowered proportions of the stressful exposures were applied, the animals built up resistance to the stressor; the *stressor* being that which has caused stress. Conversely at high proportions and sustained for extended periods of time, the animal would succumb to the stress. He termed the process of this phenomenon the general adaptation syndrome (GAS). The syndrome advanced in three stages: the alarm reaction, or the acute initial moments where the body is signaled that the organism has come into contact with a damaging agent; the stage of resistance, or the ability of the organism to adapt and resist the damage; if the organism cannot adapt then the stage of exhaustion will occur, where the organism runs out of its ability to "fight against the damaging agent" and may sustain long-term injury and or expire (Selye 1937). Selye (1950) wrote,

“At first sight it would seem that all these observations have little in common and that there is no reason to attempt their integration into a unified system of physiological and pathological events. Yet most of my research work has been devoted to the construction of bridges between these and many additional facts, since they were thought to be interconnected in nature. Through the comprehension of their unity we hoped to learn how to use them better for the understanding of life and the treatment of disease. The keynote of this unification was the tenet that all living organisms can respond to stress as such, and that in this respect the basic reaction pattern is always the same, irrespective of the agent used to produce stress. We called this response the general adaptation syndrome, and its derailments the diseases of adaptation.**”**

The premise of the GAS model proposed that stress is generalized and leads to the same physiological results regardless of the type of stress. Selye’s theory on the non-specific nature of stress is now largely considered to be false (e.g. McCarty 2016; Nageishi 2015; Mason 1971, 1975). Although stress response profiles may overlap, varying types of stressors (i.e. temperature, psychosocial, pharmacological, etc.) produce non-random physiological responses. Selye mainly studied stress at the glandular level in laboratory animals. Perhaps due to limitations in technological advances such as immunoassay methods, which did not begin to become prevalent until the late 1950s and onward (Tata 2005), much of the work clarifying stress mediators, namely hormones, were not measured. Nevertheless, his ideas stimulated research on the concept of stress, disease, and adaptation. Importantly, the idea that the stress

response protects but also damages an organism, potentially leading to poor health, was established and this concept has permeated the study of biology.

The concept of stress today

I made an effort to briefly highlight the history of stress because the underlying concepts and principles can be more readily understood and appreciated from a stress research perspective. Theoretically, the concept of stress considers the organism in relation to its internal and external pressures. Maintaining a suitable balance through these pressures is a key principle found in homeostasis. To conceptualize stress, we must bear in mind these internal and external factors and whether the organism can meet and overcome these demands in order to maintain an adaptive homeostatic balance. Here I will introduce a contemporary stress model that provides a useful framework when approaching research questions on stress.

Stress is caused by a multitude of factors, on a singular basis or by a combination, occurring simultaneously or over varying periods of time and at differing levels of severity, effecting an organism's ability to adapt to said challenges. The term *allostasis* describes the best use of the organism's resources by enabling a constant reevaluation of demand and a continuous tuning of all parameters toward these changing demands (McEwen & Wingfield 2003). Additionally, anticipation to change was brought forth by the authors of the concept (Sterling & Eyer 1988), for example, if one were to suddenly stand from a sitting position, blood pressure in the head may dramatically fall causing

dizziness. If dizziness were a problem, it would therefore be beneficial to stand slowly when rising from a sitting position. In this way, allostasis considers the experience and predictable challenges for an animal to enhance its adaptiveness. Homeostasis and allostasis are complementary concepts (McEwen & Wingfield 2010). It could be understood that homeostasis is incorporated into the allostasis concept, while allostasis contextualizes stress by considering adaptive responses from widely divergent characteristics of an organism such as social, psychological, and genetic aspects; or in other words, allostasis takes on the complexity that is found in everyday life.

All animals exist in regular rhythms that necessitate, for example, the acquisition of food and water for sustenance, mating for reproductive success, or social behavior for protective qualities related to survival. The lifespan of an animal progresses in developmental stages that are cyclically patterned. Across these factors and at each level, each cycle generates a predictive adaptive response needed for the animal to survive and expand its lifetime reproductive success. The Japanese macaque (*Macaca fuscata*), the focal species of the present thesis, experience cyclical patterns that result in adaptive responses. A thermoregulatory response can be seen in monkeys of juvenile age and older, which experience molting of their fur once a year in the early summer, where relatively shorter hair can be observed in the hotter months and longer hair in the colder months (Hamada & Yamamoto 2010). In mating season, males of each age class tend to show a larger testis size during mating season (Nigi et al 1980) and females exhibit larger teats (Hamada & Yamamoto 2010) than in non-mating seasons, suggesting a shift in physiology in order to prepare for reproduction.

Daily rhythms of cortisol, a steroid hormone largely associated with increased energy, can be found to have the highest levels in the morning and lowest levels in the evening (Suzuki et al 2002). For a diurnal species, increased energy in the morning is an important adaptive function for food foraging and less energy in the evening is adaptive to prepare the body for rest and repair. Allostasis considers that cycles are predictable and the body shifts its needs to what is anticipated for that which is most adaptable for the organism. In everyday life, a complex coordination across many systems, involving a multitude of factors such as molecular messengers, the brain and learning, behavior, and genetics all work together to maintain an organism's adaptive responses, whether to anticipated and or unforeseen circumstances.

The overall burden brought on by aggregated life experiences and long-term stress has been termed *allostatic load* (McEwen & Stellar 1993). This "wear and tear" is the result of the daily and seasonal cycles that organisms use to locate food, acquire mates, and survive, as well as the extra energy needed to migrate, molt, reproduce, along with other adaptive functions, while also coping with unanticipated perturbations (McEwen & Wingfield 2010). In Figure 1, the allostatic load model is presented in the context of humans; however, the principles apply to the non-human primate (NHP). The following are parallel examples from the perspective of a wild living NHP: environmental stressors are extreme temperatures, social conflict, lack of food; major life events consist of developmental stage or age; trauma includes injury from predation or conspecific conflict and so on. Individual differences in genotype, development, and experience effect physiological responses and the cognitive ability of the NHP to

navigate through stress (e.g. Lupien et al 2007, 2009; Marin et al 2011). These factors effect behavioral responses such as fight-or-flight, foraging, grooming which may in turn mediate the physiological responses. Accumulatively, these factors exist simultaneously creating a load that effects whether the NHP can maintain stability through change and successfully adapt to stress. The allostatic load model is a practical conceptual basis for understanding the cost of long-term stress, life events, and the capacity of an organism to cope through them.

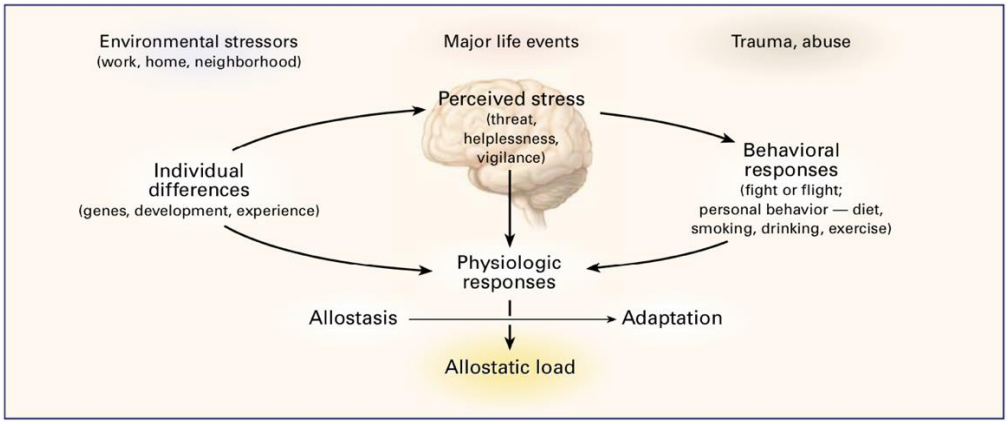

Fig. 1 An illustration of the allostatic load model. Reproduced with permission from (McEwen 1998), Copyright Massachusetts Medical Society.

Stress associated pathways

Stress research is important for determining the health and well-being of NHPs both in captivity and in the wild. For the purpose of this thesis, I refer to stress as any environmental, physiological, and/or psychosocial demand imposed on an organism that unbalances the organism's natural regulatory state (Buijs & Van Eden 2000; Chrousos 2009; Koolhas et al 2011). Organisms produce non-random responses specifically designed to counteract threats to their integrity. These physiological responses can be measured by hormones and proteins which are

needed for quantifying, tracking, and determining the extent of stress in animals. For example, recognizing and avoiding chronic stress is a major concern when considering an animal's overall welfare (Botreau et al 2007; Carenzi & Verga 2009; Fraser 2008). Physiological markers and medical evaluation help assess the degree to which an animal may experience stress. Non-invasive methods are particularly useful because they provide minimal disturbance to the animal and also increase measurement accuracy by avoiding experimentally induced stress (Keay et al 2006; Möstl & Palme 2002; Schwarzenberger 2007). Useful biomarkers can be measured from bodily excreta and fluids that coincide with activity in stress-associated pathways.

The hypothalamic-pituitary-adrenal (HPA) axis is a physiological system that is associated with mediating stress (Kudielka & Kirschbaum 2005; Sapolsky et al 2000; Ulrich-Lai & Herman 2009). Stress activates the HPA axis through a series of hormone release from the brain to the adrenal cortex, energizing the body via glucocorticoids (Herman et al 2005; Lightman & Conway-Campbell 2010; Spinedi & Gaillard, 1998). The HPA axis is often studied by measuring the stress-associated hormone cortisol (Angelier & Wingfield 2013; Cockrem 2013; Romero 2004). The sympathetic-adrenal medulla (SAM) axis has a similar parallel cascade of hormone secretion to the HPA axis but includes electrical signaling from the sympathetic nervous system, leading to the release of the catecholamines epinephrine and norepinephrine in the adrenal medulla (Mason, 1968; Morilak et al 2005; Ulrich-Rei & Hermann 2009). The HPA axis is associated with energizing the body to take on the challenge of more chronic or long-term stress, while the SAM axis is associated with the more immediate

fight-or-flight response. In theory both systems work in tandem, for example, the sympathetic nervous system (SNS) signaling and hormone secretion of the SAM axis acts immediately in a situation where cognitive evaluation and re-evaluation may not be an adaptive response when presented with a sudden predator-prey scenario. The SNS signaling and SAM axis response has evolved in such a way that a coordinated effort to react in an immediate instinctual manner, often involuntarily (i.e. suddenly jumping and running away) is depicted by this physiological system. The HPA axis in this scenario helps generate large amounts of energy in an effort to *sustain* the fight or run away from the predator. These responses come at a cost to the body, particularly if sustained for long periods of time. As Selye's work noted, in one sense the stress response is adaptive but in another it is maladaptive and can potentially lead to disease and failure of the organism as a whole. The predictive and unpredictive fluctuations of these systems are relatively easy to measure in the human subject, but how can they be measured in an undomesticated and essentially wild animal such as the Japanese macaque, particularly in such a way as to cause little to no disturbance?

Hormones, proteins, metabolites and other biomarkers of interest can be measured in the excreta and fluids of all primates. These biomarkers correspond to activity in the HPA and SAM axes. Reviews of methodological approaches have been succinctly reported in recent years (Behringer & Deschner 2017; Novak et al 2013). Blood sampling provides a viable method for measuring activity of glucocorticoids related to the HPA axis and catecholamines of the SAM axis. However, the method is difficult to employ in unrestrained and awake NHPs in both captive and wild conditions. Added stress due to the blood

sampling procedure may further lead to difficulties in interpreting the results. The nature of excreta, urine and feces, accumulates the biomarker of interest until it is expelled by the body. Although this time lag may be highly variable by species, appearance of hormones and respective metabolites may appear on a scale of minutes for blood and saliva, hours for urine, hours to days for feces, and days to weeks for hair (Behringer et al 2017). For example, Takeshita et al. (2018) found a pronounced increase in fecal glucocorticoid metabolite concentration 24 hours after applying an adrenocorticotropic hormone challenge in Japanese macaques. Urine and feces are widely useful, for example, Chapter 3 shows an application of utilizing fecal cortisol metabolites to monitor HPA axis activity; where feces best suited the research question due to the practicality of sample collection and reduced interference in the daily management of the study subjects. For studying the short-term changes of the stress response on a time scale of minutes, saliva sampling provides a viable medium. This is primarily because salivary biomarkers respond acutely to disturbances.

Salivary analytes

Saliva is a medium with a wide application to measure stress and physiological responses (e.g. Steckl & Ray 2018; Urlacher et al 2022). In Chapters 2 and 4, I focused on developing and validating saliva collection in unrestrained and awake Japanese macaques living in captivity or in the wild. The behavioral aspects of Japanese macaques have been studied relatively more in depth than non-invasive saliva collection and salivary stress markers. It was for this reason that I spent

considerable time in this area. My primary aim was to measure activity of the HPA and SAM axes in short successive time periods in relation to behavior to understand some of the proximate mechanisms between behavior and physiology. Two particular salivary biomarkers will help achieve this goal.

Cortisol is a steroid from the glucocorticoid family of hormones found in many mammalian species that regulate functions across physiological systems such as the nervous, cardiovascular, and immune systems (Kadmiel & Cidlowski 2013). A major function of glucocorticoids is to preserve glucose to maximize energy during stress (Kuo et al 2015). Cortisol is measurable from saliva (Hellhammer et al 2009; Kudielka et al 2009; Vining et al 1983). The principal signaling system works via cascading mechanisms of the HPA axis, where cortisol is generated and excreted from the adrenal cortex, permeating out into the body and can be found in fluids and tissues (Chrousos, 2009; Spencer & Deak, 2017). Human studies have shown a consistent strong relationship between salivary cortisol and serum cortisol (e.g. Daniel et al 2006; Dorn et al 2007, 2009; Eatough et al 2009). Cortisol measured from saliva is synchronous with that of cortisol measured from blood.

Salivary alpha-amylase (sAA) enzymes assist in the early stages of digestion by hydrolyzing the 4α-glycosidic bonds of starch molecules (Boehlke et al 2015; Souza & Magalhães 2010). The majority of the proteins found in saliva are created and secreted by acinar cells, a network of cells found in salivary glands (Castle & Castle 1998). These cells appear to be densely innervated by sympathetic and parasympathetic nerves (Garrett & Kidd 1993; Proctor & Carpenter 2007). Although sAA does not play a role in stress regulation itself,

several reports have shown a clear significant correlation with norepinephrine. For example, Petrakova et al (2017) found that human sAA activity significantly correlated with plasma norepinephrine release when pharmacologically stimulated by injection of corticotropin-releasing hormone. In a double-blind study, Warren et al (2017) showed that administration of atomoxetine, a selective norepinephrine transport blocker known to increase central norepinephrine levels, more than doubled sAA from baseline. Kuebler et al (2014) reported that norepinephrine injection stimulated sAA secretion in healthy men. The association of sAA activity with norepinephrine response allows sAA to serve as a biomarker for sympathetic activity (i.e. the SAM axis or SNS).

The Japanese macaque

I began planning the research in this thesis with the primary aim to better understand real-time stress related physiological fluctuations in relation to behavior in Japanese macaques. If non-invasive methods could be developed and at an accessible budgetary cost, then the possibility to answer research questions on a broad spectrum of topics such as health, evolution, and sociality would be enhanced.

Japanese macaques are endemic to the most northern range of NHPs found in their natural habitats. Populations are distributed throughout the three major Japanese islands of Kyushu, Shikoku, and Honshu and some smaller neighboring islands (Fooden & Aimi 2005). The species is highly adaptable living in diverse seasonal habitats such as warm temperate forests to frigid

mountainous areas. Typical group sizes range from 10 to 161 individuals (Izumiyama et al 2003), though provisioned groups can grow as large as 1,255 individuals (Sugiyama & Ohsawa 1988). The society of Japanese macaques is characterized by a strict dominance hierarchy. Females are the philopatric sex and form the center of the group by largely associating with kin-related females, establishing their own linear and stable dominance hierarchies (Yamagiwa & Hill 1998). In this sense, Japanese macaques are often described as nepotistic because matrilineal kin groups form within the greater group and social hierarchical rank is inherited by birth. Male inclusion within a group is often based on their ability to permeate the female-bonded groups while at the same time finding a place within the strict linear male hierarchy. As males become sexually mature, they typically leave their natal groups (Sugiyama 1976). It is common for both sexes to gain or maintain their social rank and status through despotic aggression. Reproduction occurs by promiscuous seasonal breeding. According to the International Union for Conservation of Nature, the species is currently classified as least concern (Watanabe & Tokita 2020).

Stress research is highly applicable to Japanese macaques. In the context of captivity, previous research has shown that Japanese macaques are often reported as a target species of investigation in laboratory experiments (Carlsson et al 2004) and it was expected that the species will become more valuable due to increased need as a bioresource for medical research (Isa et al 2009). In addition to captive research, Japanese macaques are commonly kept at zoos for tourism and public education. Their importance as an economic attraction is evidenced by the provisioning of free-ranging groups at popular monkey parks

spread throughout Japan. In the context of wild populations, the species has been extensively studied for over 70 years from a wide range of scientific perspectives such as sociology, ecology, and reproduction (Yamagiwa 2010). The health of populations managed and investigated under human supervision are thus vital to industries such as academic institutions, the medical industry, local tourism, and applied and theoretical science. In this sense, as a wild animal under the supervision of human care, the health of Japanese macaques on an individual to population level are of concern to human society in general. Methods that can utilize non-invasive approaches to monitor stress in Japanese macaques and assess their well-being as well as act as tools for answering research questions are highly applicable for such reasons.

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Chapter 2

Salivary alpha-amylase enzyme is a non-invasive biomarker of acute stress in Japanese macaques (*Macaca fuscata*)

Broche, N., Takeshita, R. S. C., Mouri, K., Bercovitch, F. B., & Huffman, M. A. (2019). Salivary alpha-amylase enzyme is a non-invasive biomarker of acute stress in Japanese macaques (Macaca fuscata). *Primates*, *60*(6), 547–558. https://doi.org/10.1007/s10329-019-00757-6

Abstract

Salivary alpha-amylase (sAA) enzyme functions as a digestive enzyme in many species, which consume starch in their diet. Human studies have also revealed that sAA enzyme activity levels are positively correlated with the release of the stress hormone norepinephrine, allowing sAA to act as a biomarker for sympathetic nervous system activity. Recent non-human primate studies have incorporated sAA as a physiological stress marker. However, no published reports have investigated the time course of sAA from a stressful event to return to baseline levels in non-human primates. Furthermore, no validation of sAA as a stress biomarker has been reported for Japanese macaques (*Macaca fuscata*). This study had two primary aims: [1] develop a systematic method for non-invasive saliva collection and, [2] investigate sAA as a biomarker of acute stress in *M. fuscata* in order to better understand its acute stress-related characteristics. I developed a non-invasive method for cooperative saliva collection using positive reinforcement training (PRT) and tracked individual progress over 595 trials in 10 individually housed Japanese macaques. I detected sAA enzyme in *M. fuscata* via kinetic reaction assay, then performed 22 acute stress tests. Four tests met conditions for interpreting sAA in response to an acute stressor and these results show that on average sAA activity rapidly increased post-stressor (mean ± SD = 4.2 min ± 0.9) and returned to baseline shortly thereafter (10.4 ± 0.6 min). This report reveals for the first time the temporal dynamics of sAA when applying acute stress to Japanese macaques and could be a useful tool for assessing animal welfare.

Introduction

Stress is unavoidable. The body has evolved responses to stress that are designed to maintain a healthy physiological profile, but frequent stress is detrimental to an animal's well-being – for example, by causing immune dysregulation, impaired cognitive abilities, and self-injurious behavior (Davenport et al. 2008; Glaser & Kiecolt-Glaser 2005; Gunnar & Quevedo 2007; Juster et al. 2010; McEwen & Sapolsky, 1995; Meyer & Hamel 2014). Physiological stress measures such as hormones are vital for quantifying and tracking stress in animal studies, but there is a need to expand on physiological stress markers in order to allow greater understanding of stress response systems in non-human animals. In particular, the development of non-invasive methodologies has proven useful in providing a means to assess naturally-occurring stress among wild animals and those in captivity (Behringer & Deschner 2017; Heistermann 2010; Keay et al. 2006; Möstl & Palme 2002; Schwarzenberger 2007). Furthermore, identifying stress in captive animals is of great interest for researchers aiming to improve the ethical standards in animal welfare (Botreau et al. 2007; Carenzi & Verga, 2009; Fraser 2008).

In the most immediate sense, biological *stress* refers to any environmental, physiological, and or psychosocial demand placed on an organism that compromises the natural regulatory state of that organism (Buijs & Van Eden 2000; Chrousos 2009; Koolhas et al. 2011; McEwen, 1998). Stress is primarily studied by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in the release of glucocorticoid hormones (Capitanio et al. 2005;

Kudielka & Kirschbaum 2005; Sapolsky et al. 2000; Takeshita et al. 2014; Ulrich-Lai & Herman 2009). In the last three decades, human stress research has begun integrating a multi-system approach to studying the system specific stress responses (Bauer et al. 2002; Kyrou & Tsigos 2009; Malarkey et al. 1995). The sympathetic-adrenal-medullary (SAM) axis is one such system that follows a parallel cascade of hormonal releases to the HPA axis via electrical impulses of the sympathetic nervous system and leads to the release of the catecholamines; epinephrine and norepinephrine (Mason 1968; Morilak et al. 2005; Ulrich-Lai & Herman 2009). Compared to the HPA axis, SAM axis activation has been reported to initially respond faster when activated by stress (Engert et al. 2011; Gordis et al. 2006; Nater et al. 2006; Rohleder et al. 2004; Stroud et al. 2009; Takai et al. 2004). Salivary alpha-amylase (sAA) enzyme aids in the initial stages of digestion by hydrolyzing 4a-glycosidic linkages of starch molecules (e.g. Boehlke et al. 2015; Tester et al. 2006; Robyt & French 1967), but human stress studies have also revealed that sAA enzyme activity is positively correlated with plasma norepinephrine release (Kuebler et al. 2017; Petrakova et al. 2017; Warren et al. 2017).

Non-human primate research has begun exploring sAA enzyme as an acute biomarker of sympathetic stress response. Higham et al. (2010) collected saliva non-invasively from semi-free-ranging rhesus macaques (*Macaca mulatta*) and showed that cotton-based collection devices could be used to measure sAA activity in this species. Studies of non-human primates have used sAA activity as an index of sympathetic response by comparing conspecific aggressive encounters versus resting periods (Mandalaywala et al. 2017; Petrullo

et al. 2016). Behringer et al. (2012) demonstrated the presence of sAA in captive bonobos (*Pan paniscus*) and found that sAA levels were significantly higher during stressful conditions, such as birthing, housing transfer, and group integration, than in non-stress conditions.

Japanese macaques (*Macaca fuscata*) have been studied for over half a century in wild populations, provisioned monkey parks, and in captivity across Japan. From our current awareness, there are no studies that have reported the time lag of sAA stress response in a non-human primate, which is needed for accurate sample timing. Understanding the temporal dynamics of sAA as a stress biomarker is necessary if we are to implement this tool for investigating stress in this species in captivity or in its natural free-ranging environment. In the initial stages of the present study I performed quality control experiments using human saliva in order to test differences in potential material absorptivity, water consumption, and starch consumption effects on sAA activity. Next, I verified if the assay kit could detect sAA in Japanese macaque saliva, which was opportunistically collected during a biennial health check. Simultaneously I initiated training with captive Japanese macaques to non-invasively provide saliva in order to study sAA in response to a specific acute stressor. I had two primary aims in this study: 1) to develop a non-invasive methodology for saliva collection with unrestrained and awake Japanese macaques and 2) to investigate the temporal dynamics of sAA in response to acute stress in this species.

Methods

Subjects and Housing

Subjects who underwent positive reinforcement training (PRT) for saliva collection included five adult male and five adult female (age range: 4 – 21 years) Japanese macaques that were individually housed in single cages (850 x 900 x 760 mm) in the same room, at the Kyoto University Primate Research Institute (KUPRI). None of the monkeys were subjected to water or dietary restrictions during training or experiments. Four PRT subjects (two adult males and two adult females; age range: 10 – 19 years) were later chosen from among the most highly cooperative monkeys for acute stress testing. Stress tests were performed in the same housing area where PRT was conducted. During the training period, between 10 to 12 individuals were housed in this room, but the study group was comprised of the 10 individuals who were habituated to each other. Stress testing occurred at least one month after a new individual was introduced to the housing area in order to establish a habituation period for all individuals.

During the initial stages of PRT, I collected Japanese macaque saliva samples from members of one of the open enclosure groups at KUPRI, during a biennial health check conducted in November 2016. The troop, Arashiyama B (PRI), was comprised of 62 individuals (44 females, 18 males; age range = newborn to 23 years) of which I sampled 45. As part of the health check protocol, all individuals were anesthetized with a ketamine (5mg/kg), medetomidine (0.025mg/kg), and midazolam (0.125mg/kg) solution by the veterinary staff.

While the monkeys were under anesthesia, saliva samples were collected using a standardized process, swabbing the outside top and bottom gums then along the inside of the top and bottom gums including the floor of the mouth two times. Medetomidine, which is employed as a safety measure during anesthetic procedures, has been reported to decrease central nervous system activity and inhibit saliva production (Scheinin et al., 1989). Despite the known adverse effects of anesthesia on saliva production, I tried this method first in an attempt to determine whether the sAA assay kit – designed for human use – would detect sAA in Japanese macaques, and also to test the efficacy of collecting saliva during such routine procedures for future studies attempting to measure salivary analytes under anesthetic conditions. My first goal was to ascertain whether I could measure sAA in Japanese macaques prior to performing experimental manipulations, so I collected saliva during routine husbandry procedures.

Saliva collection materials

For our study of captive individuals, saliva was collected using rope swabs for PRT sessions and stress testing. The rope swab was prepared using braided 4 mm diameter 100% cotton rope (Moritoku Co. Ltd.) by cutting the rope into 30 cm long pieces. Both rope ends were tied into overhand knots, one end into two complete overhand knots and the other end into a single overhand knot. After knotting, the ropes became approximately 15 cm

Fig. 1 (a) rope swab, (b) Salimetrics synthetic cotton oral swab, (c) Hakuzo cotton swab

in length. Batches of 60 ropes at a time were then sterilized by placing them in a stainless-steel pot of boiling water for 20 minutes, removing the water and re-boiling again, repeating this process two more times for a total of three 20-minute boils. Boiling three times was done to sterilize the ropes, but in human trials I also found that this method reduced bitterness of the ropes. All water was drained and ropes were placed on covered aerated dish trays for two days. Once dry, the ropes were placed into a water-filled glass jar containing orange flavor (modified from Boyce et al. 1995; Higham et al. 2010; Lutz et al. 2000) sucralose-based drink powder (Fine Japan Co. Ltd.) with a solution concentration of 6 g per 100 mL of water. Ropes were placed in the sucralose solution for 24 hours, dried again in aerated dish trays for two days, at which time they were ready for use. Subsequently I found that a commercially available food drier (Dry Food Maker AFD-550, Apix International Co. Ltd.) could be used to dry a batch of 60 rope swabs within 4 hours, reducing preparation time significantly. This device was used for preparing rope swabs for stress test experiments, initiated after the PRT period. Saliva sampling from anesthetized monkeys was collected with Hakuzo 100% cotton swabs (Hakuzo Medical Corporation, Mfr. #1240156) with extended 15-cm long wooden shafts. The wooden shaft was removed after collection in order to fit the saliva absorbed cotton tip into the double-chamber plastic tube. All saliva samples from monkey subjects were immediately placed in double-chamber polypropylene tubes (Salimetrics #5001.05) upon collection, temporarily stored in an iced cooler box, and then stored at -20° C within an hour of their collection time. Samples were thawed only once on the day of the assay.

Quality control tests

In the initial stages of the present study, I used saliva samples from one author (NBJ) to perform preliminary tests on material absorptivity, starch consumption, and water consumption. I tested three saliva collection material types for potential material absorptivity differences: (a) rope swab (b) Salimetrics synthetic cotton oral swab and (c) Hakuzo 100% cotton swab with an attached wooden stick (Fig. 1). Food and drink consumption were avoided for 2.5 hours prior to saliva collection. Strenuous exercise and major stressors were avoided on the collection day. For each of the three material types, the following method was performed: [1] as a baseline, the "spit" method (Navazesh 1993) was used placing saliva directly into a 1.5 mL polypropylene tube; [2] immediately followed by chewing on the collection material for one minute, or in the case of the Hakuzo cotton swab ("c" in Fig. 1), ran the swab along the top and bottom gums as well as the inside of the gums including along the floor of the mouth two times; [3] immediately followed by providing a second baseline using the spit method in a 1.5 mL polypropylene tube, completing a total of 3 saliva samples per each material type. All samples were collected in sequential order starting with the rope swab, then the Salimetrics synthetic cotton oral swab, and then the Hakuzo 100% cotton swab. Material absorption samples consisted of 9 samples total and were provided within an 8-minute period. All human samples were stored in a -20° C freezer within minutes of their collection and thawed only once on the day of the assay.

Peanuts (*Arachis hypogaea*) are a positive food reward for sustaining motivation for Japanese macaques during PRT, but they also contain starch (e.g. Dieckert et al. 1962; Isleib et al. 2004), which could potentially confound sAA results when interpreting them under stress conditions. Effects of water consumption and starch consumption on sAA activity was tested by using the spit method into 1.5 mL polypropylene tubes. For water consumption, the following method was performed, with time points described relative to the previous time point: [1] a baseline saliva sample was collected and then 5 mL of water was consumed; [2] 30 seconds later a saliva sample was collected and then 50 mL of water was consumed; [3] 30 seconds later a saliva sample was collected and then 100 mL of water was consumed; [4] 1 minute later a saliva sample was collected. All 4 water consumption samples occurred in a 2-minute period. For starch consumption, the following method was performed: [1] a baseline saliva sample was collected 4 minutes after the last water consumption sample; [2] 5 peanuts were consumed within 20 seconds and a saliva sample was collected 1-minute post consumption; [3] the final saliva sample was collected 9 minutes post consumption. Seven saliva samples were obtained for the water and starch consumption tests (Fig. 3b).

Training Methods

Saliva that pools from natural salivation (not stimulated) at the floor of the mouth is often the preferred source for collection in human studies (Bosch 2014; Nagy et al. 2015; Navazesh 1993; Rohleder & Nater 2009). However, this sampling

method is difficult to implement in unrestrained Japanese macaques and therefore gaining their cooperation to chew the rope swab for 30 seconds was the goal of saliva collection training and stress testing. PRT focuses on promoting targeted behaviors by using positive reinforcers, primarily food rewards, and aims to create a cooperative environment for both monkey and researcher (e.g. Coleman et al., 2008; Laule et al., 2003; Reinhardt & Cowley, 1992; Schapiro et al., 2003). Individual preferences for food items were noted such as peanuts, raisins, wheat grains, and other dried fruits. In order to increase initial habituation, juice-soaked rope swabs were used in the first five trials for monkeys who showed no interest in chewing rope swabs.

A 5-step scoring system was implemented in order to track each individual's progress (Table 1). For purposes of analysis, I considered each 'step' as a 'point'. I defined a *trial* as training time for saliva collection spent per individual monkey, usually lasting from 2 to 5 minutes per monkey, which occurred once per training day. One *session* was the accumulative total trials performed per training day, or 10 trials (1 for each monkey) per each training day, which typically lasted 1 hour.

Table 1 PRT progress tracking

Step	Behavior	Description
1	Back housing	Body firmly in back of housing; faces away
2	Front housing	Near researcher; inquisitive; watches training
3	Touch rope	Actively touches the rope, by hand or nose
4	Chew/lick rope	Places rope in mouth, or bites, and/or licks rope
5	Chew rope & return	Chewing performed and allows researcher to retrieve

When the targeted behavior was achieved for at least three consecutive trials, attaining the next step was the goal. For example, if the focal monkey could touch the rope swab (Step 3) on three consecutive trials then on the fourth trial the researcher would wait approximately 2 minutes for the monkey to chew the rope swab (Step 4). Food rewards were given immediately after the target behavior was achieved to promote and sustain the specified behavior. Individual preference in how the saliva collection device was presented to the monkey for chewing was noted. For example, some monkeys preferred that the researcher kneel to eye level with them, while others preferred that the researcher stood and faced away at a 45-degree angle when presenting the rope swab. The researcher also gave verbal cues to the monkey in order to provide a stable predictor of the food reward. Although clicker training has been shown to be an effective training tool in non-human animals (e.g. Chiandetti et al. 2016; Coleman et al. 2005; Fernström et al. 2009; Gillis et al. 2012), I found incorporating clicker training

resulted in a fear response with most monkeys and negatively affected training progress.

Negative stimuli were avoided, including the loud closing of doors, sudden or unusual movements, and direct eye contact. In order to become more easily identifiable for monkey subjects, the researcher wore the same color and style of sterile gown, gloves, and pouch belt each day. Equipment, such as a stopwatch, clipboard, pen, and cooler box were identical during each session. In cases when monkeys responded with aggressive displays toward the researcher, the researcher completely faced away from the monkey for 30 seconds then walked out of the housing room for an additional 30 seconds and moved to the next PRT trial upon returning to the room.

Following Lutz et al. (2000), I used a 60-cm long polymerizing vinyl chloride (PVC) pipe to present the rope swab to the monkey. A nylon string was placed inside the PVC pipe and tied to the rope swab for greater retainability during collection (Fig. 2). The distal end (from the researcher) of the PVC pipe included an elbow-shaped "mouthpiece" which could be removed. For hygienic reasons, the mouthpiece was changed between use for each monkey. After each session, all equipment, including the PVC pipe, mouthpieces, and nylon string were cleaned with warm soapy water. Any training materials in contact with saliva, urine, feces, and or blood were disinfected with Virkon™ and then cleaned with warm soapy water.

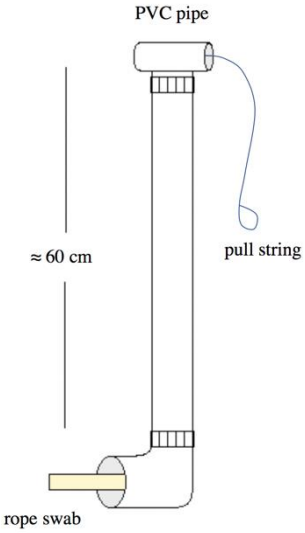

Fig. 2 PVC illustrating removable "mouth piece" (bottom PVC elbow). The rope swab was tied to the pull string within the PVC pipe. The top excess pull string was fastened to the PVC tee for securing the rope swab.

Stress test procedure

The primary purpose of the acute stress test was to perform a clear stressor on each focal monkey in order to study the time lag of sAA in response to acute stress. In humans, the time lag of sAA from the stress event has been reported to peak at around 3 - 10 minutes later and return to baseline levels 10 – 20 minutes post stressor, depending on the type of stressor (Granger et al. 2007; Obayashi 2013; Rohleder et al. 2004). For each test I collected a pre-stress baseline saliva sample and approximately within 5 minutes, applied the 20-second cage restraint stressor, then aimed to collect two saliva samples 3 and again 10 minutes after the stress event. I expected to find a measurable rise of sAA but not necessarily a return to baseline levels. Tests occurred on eight dates between Oct - Sept 2017 and Apr - May 2018 on 2 to 4 target monkeys per experiment day. Sampling occurred prior to their morning feeding in order to avoid potential confounding effects from food consumption. Twenty-two tests (5.5 ± 3.1 tests per individual)

were performed on 4 monkeys, with subjects contributing between 1 and 8 experimental series. Tests were performed individually and to their completion before moving to the next focal monkey. The order of applying the stress test differed for each consecutive series.

Cage restraint is when the researcher or caretaker brings the movable back panel forward to securely restrain the monkey in close proximity to the front panel (Reinhardt et al. 1995). Cage restraining does not physically harm the monkey but rather is a necessary tool used to protect both caretaker and monkey during routine husbandry procedures such as weighing, blood withdrawal, and cage moving. Cage restraint provided the least invasive means of applying an acute stressor and although cage restraint may be familiar to these captive individuals, routine procedures often still elicit a stress response (Balcombe et al. 2004; Buynitsky & Mostofsky 2009; Conrad et al. 1999; Reinhardt et al. 1995). All tests involved a second party who was instructed to enter the room and not to make eye contact with the monkeys. That individual then approached the focal monkey's cage, administered the 20-second cage restraint, released the cage restraint, and then left the room. The same individual participated in all stress testing, so as to maintain continuity throughout.

Enzyme kinetic assay

Saliva samples were tested for sAA enzyme using a commercially available kinetic reaction assay kit (Salimetrics, LLC. Item No. 1-1902) and were analyzed in the Social Systems Evolution Section endocrinology lab at KUPRI.

Salimetrics kinetic reaction assay kits have previously been used for sAA analyzation in a number of other studies (DeCaro 2008; Higham et al. 2010; Mandalaywala et al. 2017; O'Donnell et al. 2009; Petrullo et al. 2016). Alpha-amylase reacts to substrate containing 2-chloro-p-nitrophenol linked with maltotriose yielding 2-chloro-p-nitrophenol, which is then spectrophotometrically measured at 405 nm. Alpha-amylase activity can be measured due to proportionate increased absorption of 2-chloro-p-nitrophenol at 405 nm. Plates were read with a Tecan Sunrise absorbance microplate reader for spectrophotometry and LS-PLATEmanager 2004 software (Wako Junyaku Industries Inc.). The intra- and inter-assay coefficients of variation were 6.12% (n=178) and 11.79% (n=3), respectively.

Statistical analyses

All statistical tests were performed using R statistical software (v. 3.4.3) with the level of statistical significance set at $P < 0.05$. Shapiro-Wilk tests were performed on the data sets to determine normality of their distribution. Kruskal-Wallis test was performed for determining sAA absorptivity among cotton-based saliva collection materials. Spearman's rank correlation coefficient was performed to determine the correlation between age and retrievable saliva volume, chewing time and saliva volume produced, and the estimated flow rate correction of sAA concentration and uncorrected values. Measures of central tendency report the mean ± SD.

Results

Quality control tests

There were no statistically significant differences (Fig. 3a) in sAA absorption among the three cotton-based saliva collection materials (rope swab, Salimetrics synthetic cotton oral swab, Hakuzo cotton swab) using human saliva. Water consumption showed a decrease in sAA activity, suggesting a dilution effect, and the level increased after 4 minutes from the last consumption of water (Fig. 3b). Consumption of five peanuts clearly increased sAA activity, but by the 9-minute post consumption mark sAA activity returned back to the first baseline. These preliminary results showed that food rewards during stress testing should be avoided since they would likely confound sAA results, and further, that water consumption would likely have a dilution effect on sAA activity.

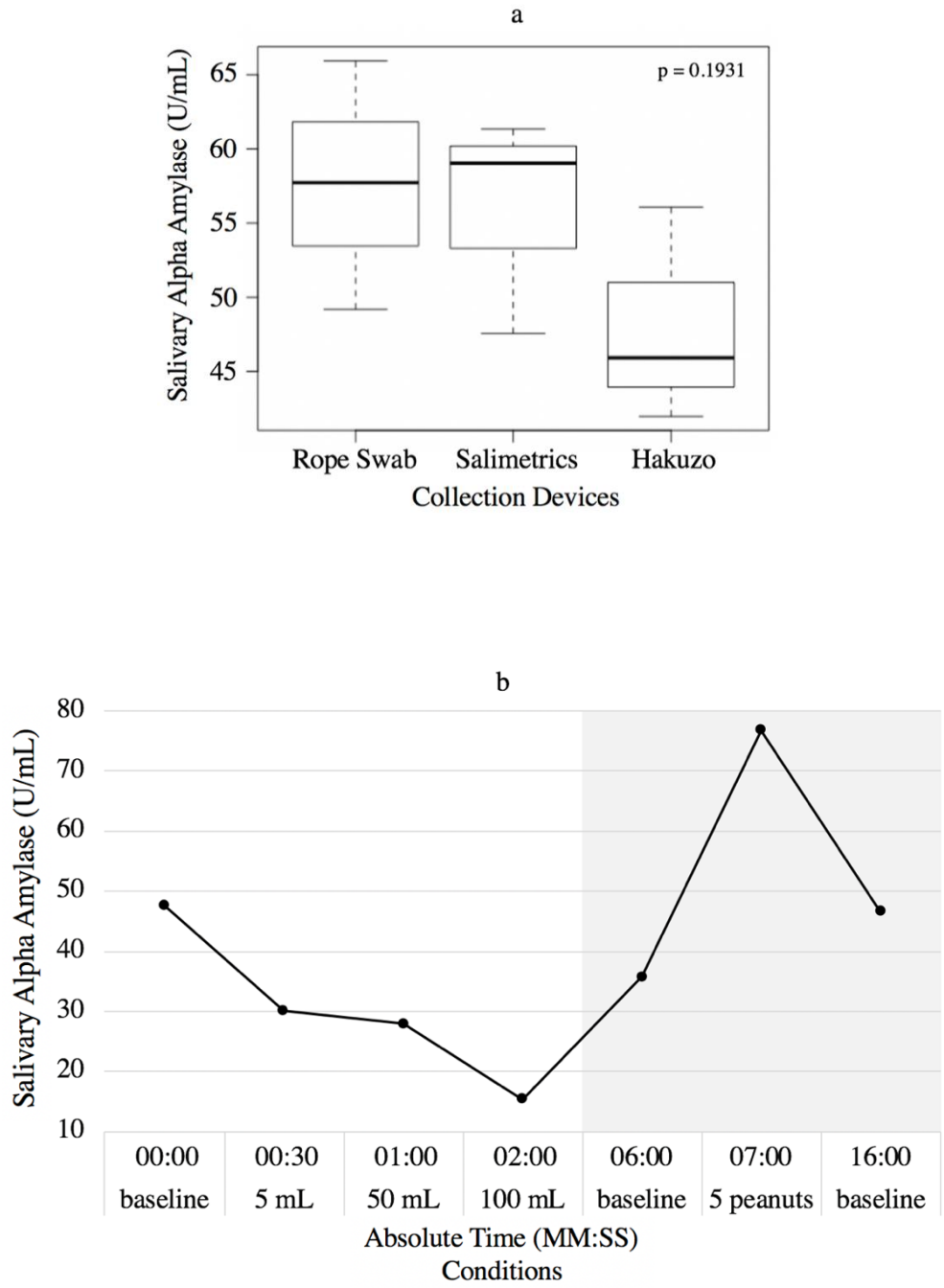

Fig. 3 (a) Comparison of retrievable human sAA per material showed no significant changes (Kruskal-Wallis test, chi-squared = 3.2889, df = 2, p-value = 0.1931). (b) Human results of water consumption (white background) showed a decrease in sAA activity and starch consumption (grayed background) showed an increase in activity illustrating the sensitivity of sAA concentrations to these factors.

sAA activity among group-living monkeys

Out of the 45 group housed monkeys that I saliva sampled during the routine health check, I retrieved at least 10 μL (minimum recommendation from assay kit manufacturers) from only 8 individuals (male = 1, female = 7, age range = 8–19 years). I found a statistically significant correlation between age and saliva volume, which indicates that age is a factor to consider when saliva is retrievable under anesthetic conditions (Fig. 4a). I retrieved 8 μL from 5 individuals (male = 1, female = 4, age range = 6–13 years) and I was able to detect sAA from these samples (range = 374.9 to 753.4 U/mL, median = 492.8). Total sAA readings (n=13) ranged from 94.8 to 1070.6 U/mL with a median of 462.6 U/mL.

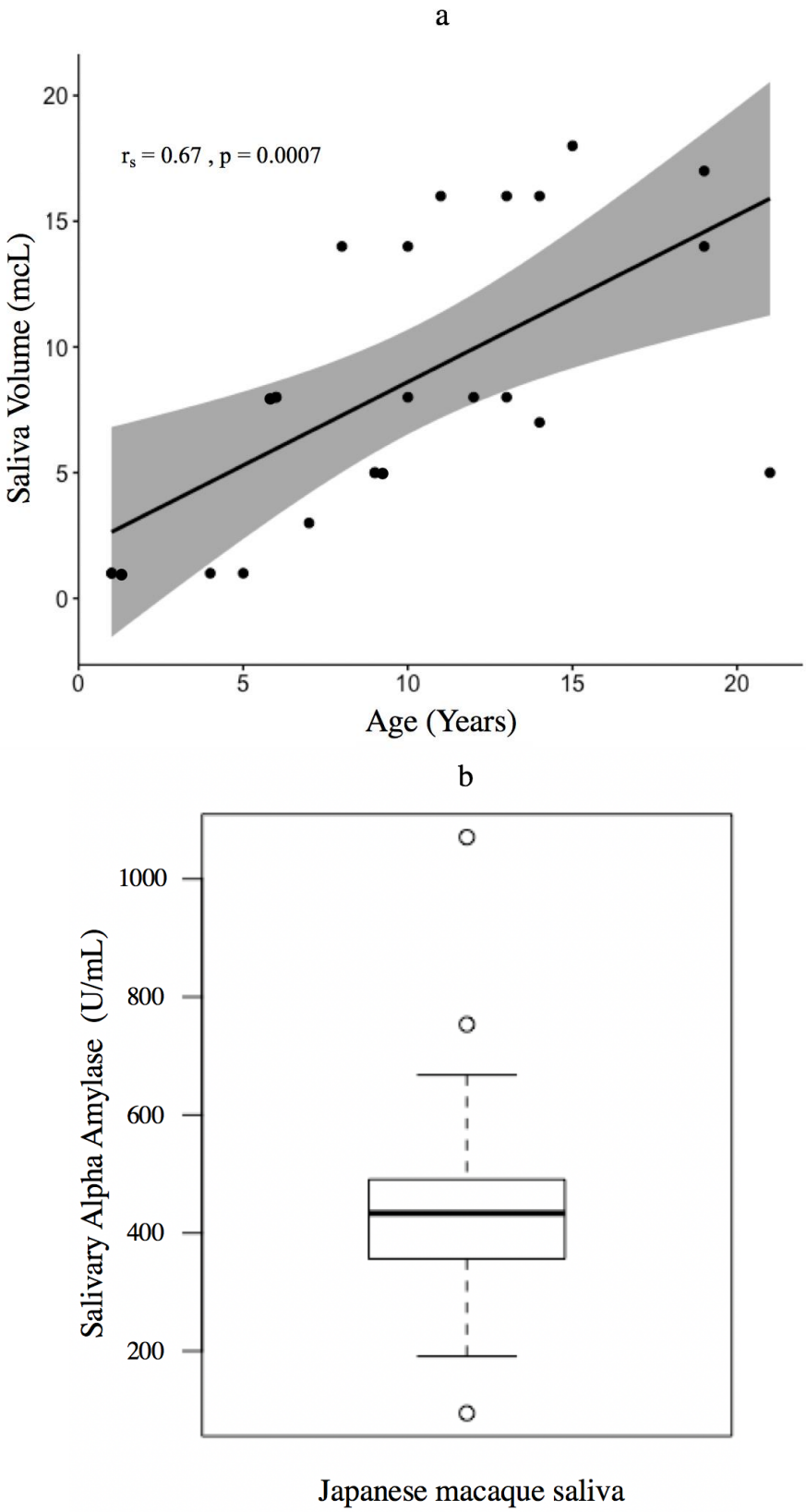

Fig. 4 (a) Retrievable saliva samples (n = 22) collected from anesthetized monkeys showed that age and saliva volume were significantly correlated; (b) Concentration range of sAA in group-housed Japanese macaques (n = 13, sAA range = 94.8 to 1070.6 U/mL)

Positive reinforcement training

Table 2 presents a summary of PRT results and Figure 5 shows sex differences over time. Analysis of the first 15 PRT trials revealed a marked increase toward target behaviors leading to cooperative saliva sampling. Comparing the first and fifteenth trial, using the 5-step progress markers, I found that males (n=5) showed an average 0.4-point increase (8%), and females (n=5) a 1.7-point increase (34%). I found that females exhibited a greater gain in PRT progress in initial trials, but females also tended to produce less saliva over time compared to males (Fig. 5b). In general, for both sexes, PRT scores reached an asymptote at about 15 to 20 trials.

Table 2 PRT Summary

PRT Period	November 2016 – April 2018
Total Training Time	62.5 hours
Average Time Per Session	59 minutes
Total Trials	595
Total Chew Events	276
Total Saliva Samples	177
Room Temperature Range	18.7 – 25.1°C
Average Room Temperature	23.2°C

Note: PRT aimed for one trial per monkey per session day

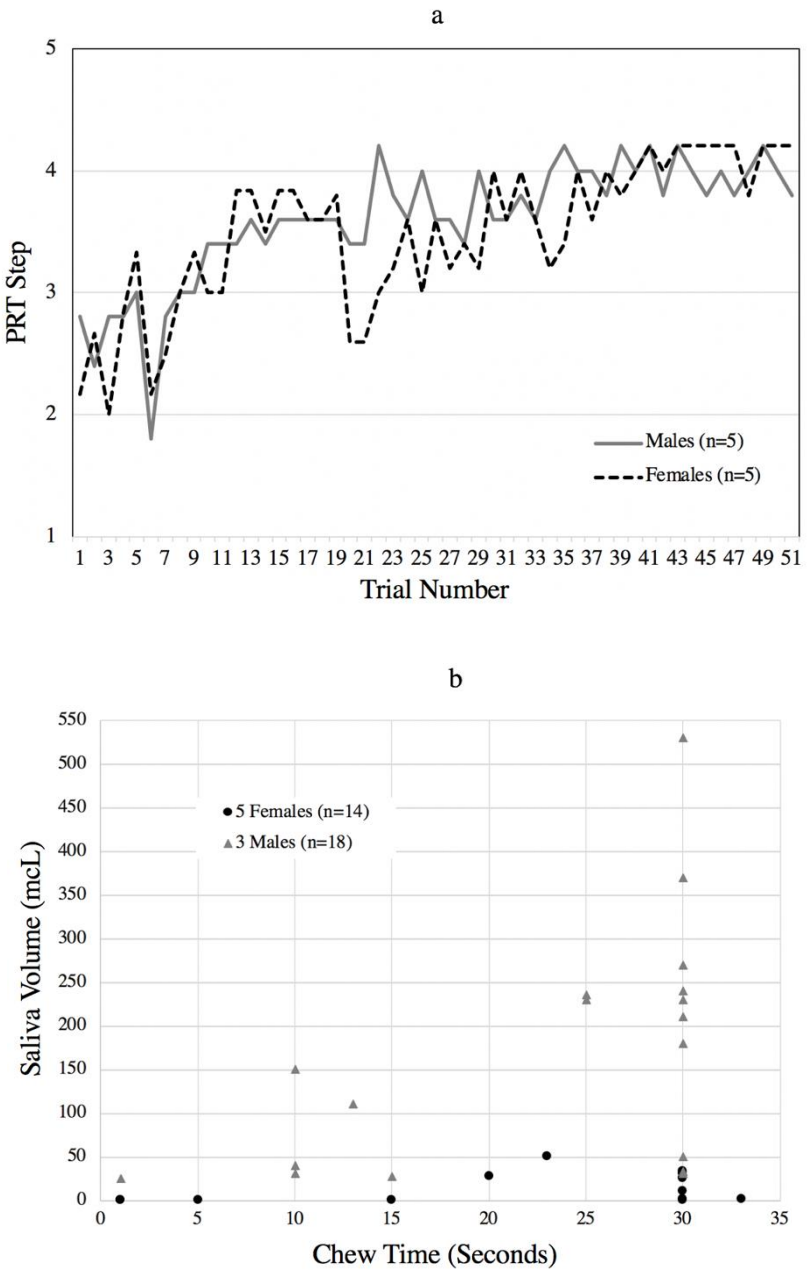

Fig. 5 (a) PRT averages of 51 trials for all trained subjects, by sex and ordered per trial. (b) Chewing seconds and collected saliva volume had a significant positive correlation (r_s = 0.52, p = 0.0022). Subjects were trained to cooperatively chew for 30 seconds.

sAA stress response tests

Testing involved 22 acute stress tests in total, of these, four tests were successful in maintaining suitable conditions for interpreting sAA activity in response to an acute stressor (Fig. 6). On average, sAA returned to pre-stressor levels approximately 10.5 minutes after the stressor was applied. Of 22 tests, 18 tests presented experimental confounds, in some cases overlapping, involving non-cooperation (50%) in collecting three consecutive saliva samples, visible blood contamination (27%) on rope swabs, added conspecific aggression (14%), and one case of water consumption during testing.

One adult male provided saliva during spontaneous episodes of conspecific aggression (Fig. 7a-b). I opportunistically continued the experiments to see if I could compare sAA differences between planned experimental stress and unplanned intense spontaneous social stress. The male began receiving conspecific aggression from a more dominant male at the 8-minute 34-second post stressor mark (Fig. 7a). Post-stressor sAA levels were higher than baseline but the third saliva sample remained relatively high, indicating psychosocial stress was maintained throughout testing. The same male received conspecific aggression on a second occasion, which began moments prior to collecting the pre-stressor baseline and continued throughout the duration of this test (Fig. 7b). The male chewed on the rope swab but later analysis revealed that no saliva could be retrieved from the third saliva collection; in this case the sustained stress might have contributed to a severe loss in saliva production.

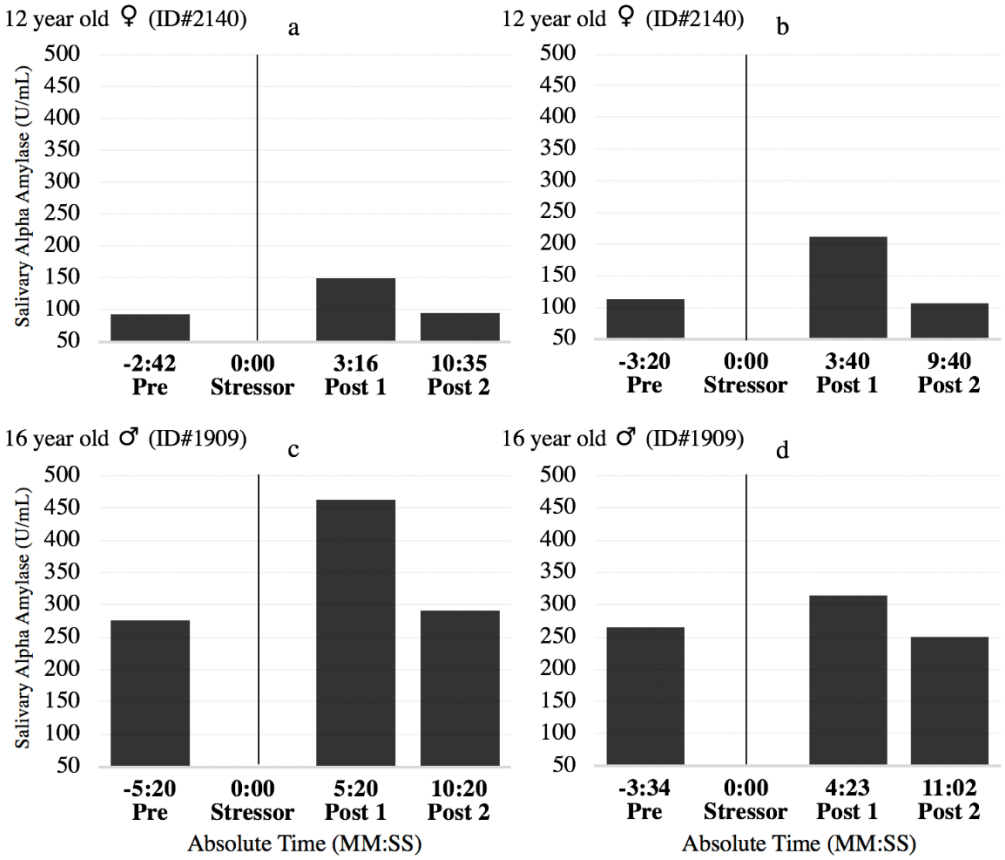

Fig. 6 (a-d) Results of stress tests performed on individually housed Japanese macaques. Absolute time is reported on the x-axis as minutes:seconds (MM:SS), ± 3 second error. Averages for pre-stressor time = (mean ± SD) 3:44 ± 1.12 min, post-stressor 1 = 4:10 ± 0.9 min, post-stressor 2 = 10:24 ± 0.45 min. Average chew time = 26.2 ± 9.6 sec.

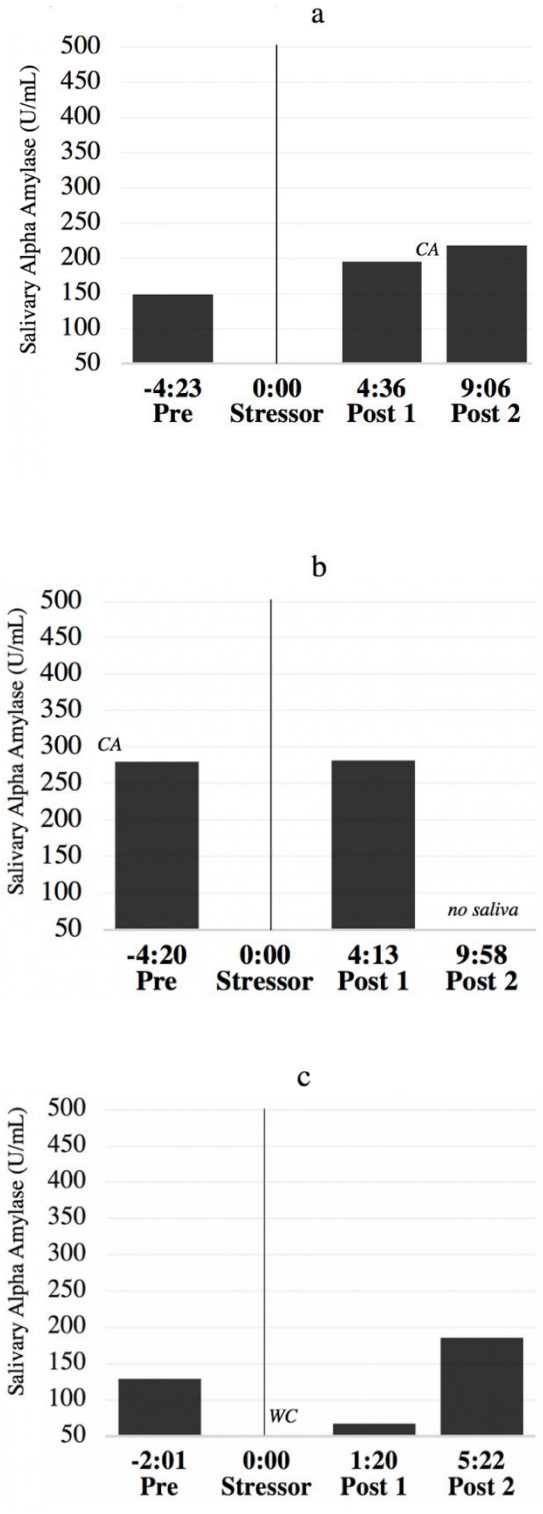

Fig. 7 16 year-old male (ID#1909) (a-b) Results of conspecific aggression (CA). (c) Result of water consumption (WC), which occurred immediately after the stressor. The Post 1 sample showed a clear drop of sAA activity, while the Post 2 sample showed sAA activity rose above the pre-stressor baseline sample indicating that sAA was affected by water consumption but recovered and likely responded to the stressor.

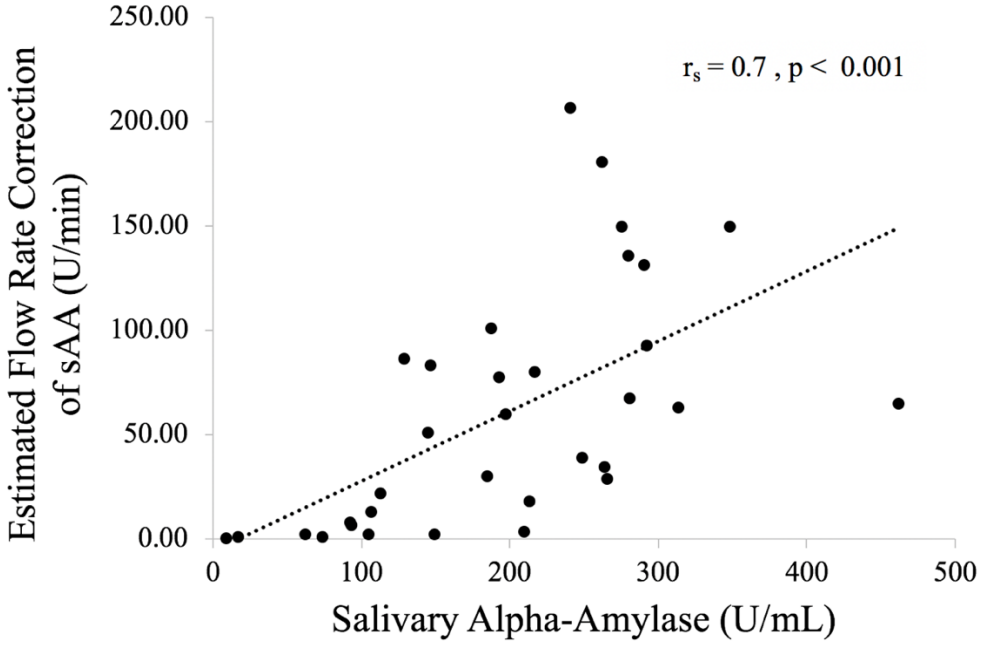

Fig. 8 Correlation between estimated flow rate correction of sAA (U/min) and values uncorrected (U/mL) for flow rate (n=33). Average chewing time = 28.8 ± 13.3 sec.

Results from stress test samples that did not present confounding factors (i.e. blood contamination and water consumption) were analyzed to test if estimated flow rate correction correlated with uncorrected values. Estimated flow rate correction was done by multiplying the estimated flow rate per chewing time (mL/min) with sAA concentrations (U/mL) for each respective sample, resulting in an estimated sAA secretion rate (U/min). The two values were significantly correlated (Fig. 8).

Discussion

I achieved the following aims in this study: 1) develop a non-invasive methodology for saliva collection in unrestrained and awake Japanese macaques and 2) chart the temporal dynamics of sAA in response to acute stress in Japanese macaques. Habituation and minimally disruptive methodologies are two major priorities for studying the stress response (Novak et al. 2013), leading to implementing PRT methodologies that ultimately promoted a cooperative and trust-based relationship between monkeys and researcher. This provided a foundation to investigate sAA in response to an acute stressor, enabling us to examine the time course of sAA levels subsequent to an acute stressor.

This study provided novel information on sAA enzyme in response to an acute stressor in *M. fuscata*, albeit with some limitations. I found pronounced inhibitory effects on saliva production under anesthetic conditions, indicating low efficacy for measuring salivary analytes with this method. During the health check, I sampled 45 individuals but only 8 produced the minimum 10 μL of saliva recommended by the assay kit manufacturer. The detailed effects of anesthesia on sAA activity are unclear, but medetomidine likely explains the low (17.7%) success rate in obtaining sufficient saliva volume. Kimura et al. (2007) reported using reagent strips for measuring sAA, analyzed via a portable calibrated machine in anesthetized Japanese macaques that were sedated using a solution of medetomidine-midazolam. This method may provide a more efficient approach in measuring sAA under anesthetic conditions because strips are placed directly

at a specified place in the mouth for a short period, requiring a small amount of saliva per sample.

I suggest at least four ways that future non-invasive saliva collection from individually housed monkeys could be improved. First, monkeys were sometimes interested in the opening of the mouthpiece and picked at the inside, perhaps looking for food items. Future designs could narrow the opening to where only the rope swab is visible, thereby avoiding an added distraction during saliva collection. Second, *M. fuscata* possesses varying sensitivities to sweetener concentrations (Nishi et al. 2016). In the case of sucralose, low concentrations may not elicit any interest and high concentrations may be aversive (Nishi, pers. comm.). Within the concentration range that does elicit interest, if the sweetened rope swab holds greater motivational value than the food reward, the motivation to continue chewing may be higher than receiving the food reward, mitigating positive reinforcement from food items. I standardized the sucralose concentration added to the rope swab based on initial trials but investigating individual preference of varying sucralose concentrations and their natural loss of interest over time, combined with food reward preference (and their variation) may provide a more efficient training method. Third, although chewing was more prevalent, two male monkeys often preferred to lick the rope swab. Lutz et al. (2000), reported success in a device designed for licking as a means to collect saliva in individually housed rhesus macaques, but also found that licking required greater time for collecting an equal or greater volume of saliva when compared to chewing. In our own trials, I found that monkeys who preferred to lick required considerable effort in *shaping* that behavior to chewing. Fourth,

stress testing resulted in substantial non-cooperation events (50%), most occurring post-stressor. PRT sessions aimed to train subjects for one saliva sample per trial but stress testing included repeated sampling over a longer period of time with an added stressor. Future experimental designs investigating the time course of salivary analytes in response to stress may benefit by expanding training to include repeated sampling that proportionately reflects the time course of the experimental stress test. Furthermore, providing ample PRT in between tests and lessening the frequency of stress testing, such as once per month per individual, may also increase cooperation.

Salivary flow rate has been reported to not confound psychosocial stress induced sAA activation (Rohleder et al. 2006), but the human literature cautions that this potential factor could influence interpretation of sAA results (e.g. Beltzer et al. 2015; Bosch et al. 2011; Nagy et al. 2015). Non-human primate studies have not yet fully investigated this point, so understanding the relative effects of flow rate on sAA concentration should be subject to future detailed research. During our PRT trials and stress tests, I noticed that monkeys often preferred to chew the rope swab in the back of one side of the molar region but intermittently switched chewing to the opposite molar region mid sampling, which might have had an influence on our estimated salivary flow rate. Future non-human primate studies that focus on salivary flow, in addition to annotating time spent chewing, could control for the position of the saliva collection swab in the mouth to more accurately note saliva flow from adjacent salivary glands.

In summary, I applied a 20-second acute psychological stressor in order to better understand the relationship between sAA activity and stress in Japanese

macaques. The stress response of sAA may yield varying patterns due to factors such as the type of stressor (e.g. chronic, thermoregulatory, physical injury), time duration of stress, and the individual's ability to cope within their environment. In addition to already established methods for measuring physiological stress, adding sAA enzyme as a biomarker of acute stress can provide a complimentary non-invasive means for measuring SAM axis activity in *M. fuscata*. I conclude that sAA enzyme can be used to detect acute stress in Japanese macaques.

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Ethical Approval

Experimental designs were approved under permit No. 2016-146, by the Animal Welfare and Animal Care Committee at the Primate Research Institute, Kyoto University and institutional guidelines for the care and use of nonhuman primates were followed.

Conflict of Interest: I have no conflict of interest to declare.

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Chapter 3

Housing relocation does not have to induce a significant stress response in captive Japanese macaques (*Macaca fuscata*)

Abstract

There are few reports that have focused on the stress that housing relocation may place on captive Japanese macaques, which is a species commonly held in laboratory conditions. Consistent high stress can lead to negative health consequences and prevention is key to maintaining the health of captive populations. Non-invasive monitoring of cortisol can help us better understand the degree to which stress is experienced and in turn help inform animal management practices. I investigated the effect a housing relocation procedure had on fecal cortisol metabolites (hereafter 'fecal cortisol') in ten single-housed Japanese macaques. I did not find any significant differences within the study period investigated but on average a small increase was observed on the relocation day. I discuss plausible reasons that likely led to the study outcome, the limitations, and potential future approaches to better understand stress in this context.

Introduction

The management of captive non-human primates (NHP) occasionally necessitates relocating individuals to a new housing facility. This procedure varies in methodology and scope based on the needs and available resources of each institution. Although inducing stress on captive NHPs is usually not the intention of the relocation, studies have reported a clear stress response across widely varying species (i.e. *Otolemur garnetti*: Watson et al 2005; *Pan troglodytes*: Yamanashi et al 2016; *Macaca fascicularis*: Crockett et al 1993). The extent to which housing relocation places stress on the target population is a concern to both researchers and caretakers alike because sustained long-term activation of the hypothalamic-pituitary-adrenal (HPA) axis may have negative health consequences on reproductive, cardiovascular, and immune system responses (McEwen 1998; O'Connor et al 2008). Cortisol has been a useful physiological stress marker for indicating hypothalamic-pituitary-adrenal (HPA) axis activation with the aim of better managing the well-being of captive NHPs (Meyer & Hamel 2014; Novak et al 2013). In the present study, I focus on relative HPA axis activation as an indicator of stress.

The *Macaca* genus represents a large proportion of laboratory NHPs used for medical research, with the *fascicularis* lineage comprising of some of the most commonly reported in the literature (Carlsson et al 2004). For what is essentially a wild animal with complex social needs, living in single-housing conditions may further add a burden to this and related species (Hau & Schapiro, 2007). There is little information available concerning stress induced by housing

relocation in Japanese macaques (*Macaca fuscata*) living in laboratory conditions. Therefore, I sought to monitor changes in cortisol over time to better understand to what extent housing relocation may add stress to the daily life of a Japanese macaque living in captivity.

In the present study, I opportunistically collected fecal samples before and after a relocation from a group of ten single-housed Japanese macaques that were scheduled to be relocated to another building, in order to monitor their cortisol changes over time. My reasoning was that, if housing relocation increased relative cortisol levels, this would indicate HPA axis activation and tell us to what extent these individuals were stressed throughout the days surrounding the procedure. I report my results centered around the relocation day and discuss plausible reasons for the study outcome, as well as point out limitations and share potential approaches for future studies.

Methods

Subjects and housing facility

The study group consisted of ten adult Japanese macaques, five females and five males (age range, 9-23 years) born and housed in captivity for research purposes at the Center for the Evolutionary Origins of Human Behavior, Kyoto University (formerly the Primate Research Institute). Female monkeys (ID#) 2409, 2140, and 2191 have had no pregnancy history and 1644 and 2608 have had four and two normal pregnancies, respectively. Each monkey was single-housed in

stainless-steel cages (850 x 900 x 760 mm) in the same room with an equal number of housing units facing each other. Monkey chow was provided twice daily by caretakers and water was freely available. Their diet was supplemented with weekly sweet potatoes and fruits, and occasional treats such as peanuts. Each individual was provided a variation of daily enrichment devices such as refillable Kong chew balls (The Kong Company, LLC.), hanging wood pieces, or holed plastic pipes for manipulation. Careful efforts were made to individualize the needs of every monkey where applicable, for example, partially opaque curtains for an individual seeking less disturbance from conspecifics. All individuals were in good health.

The housing relocation

At the time of investigation, the monkeys were habituated to each other as well as the researchers, technicians, and caretakers for at least three years. The monkeys were scheduled to be relocated due to construction renovations of their housing room (W 3.5 x D 7.0 m). On the morning of the relocation, the three technicians involved in the relocation each worked with each monkey to move them into their individual carrying cage (W 370 x D 500 x H 460 mm) and a roll cart was used to transport them to a motor vehicle. The monkeys were loaded into the vehicle and driven to a different building where the new housing room (W 3.0 x D 5.0 m) was located. At arrival, the group was unloaded and carted to the new housing room and placed into single-housing units that were the same size as those of the previous housing facility. In total, the entire relocation

procedure occurred in under 30 min. Monkeys were not physically restrained. No anesthesia or medication aimed at sedation was used throughout the entire study period. For several years, this group has been trained through positive reinforcement via food rewards to enter carrying cages for monthly weighing. Typically, the monkeys enter immediately but occasionally the back-housing panel is moved forward halfway to bring the monkey closer to the housing entrance. Management staff made a minor change in the arrangement of the housing units when placing the monkeys at the new facility. Before the relocation, four males were housed in parallel across five females and one male. When the group was introduced to the new facility, five males were housed in parallel across from five females. The environment of the new facility was essentially the same in aesthetics, design, and function. However, a notable difference was that the new housing facility did not have a window. Both housing rooms were temperature regulated, ranging between 25 - 28°C and the outside temperature during the relocation was 29°C. The lighting was regulated to 12h light (07:00-19:00) / 12h dark (19:00-07:00) cycles in both housing rooms. The daily routine of each monkey's care, such as feeding and cleaning, and the time of day they occurred remained the same throughout the study period.

Sample collection

In late August 2019, fecal samples were collected at approximately 09:00 each morning before caretakers performed daily morning health assessments, cleaning, and feeding. I aimed to collect one fecal sample from each individual each

morning. Across the seven days, 66 samples were collected. Four samples were lost due to water or urine contamination, or by lack of sufficient fecal volume during later processing. All fecal samples were stored in a -20°C freezer within an hour of collection.

Hormonal analyses

Cortisol was determined by a previously reported enzyme immunoassay system (Kinoshita et al 2011, Takeshita et al 2018). There was a slight modification in fecal extraction (Takeshita et al 2018) for each sample, that is, after freeze drying, 0.2 g of dried feces was measured and placed into 2 mL of 80% methanol. The samples were then shaken continuously for 30 min at 5,000 rpm, and then centrifuged at 1500 x g for 5 min. The supernatant was stored at -20°C until the day of the assay. All hormonal analyses occurred within 15 months from the sample collection period. Cortisol levels are expressed as per gram of dried feces. The sensitivity of the assay was 0.0133 ng/mL (0.665 pg/well). Recovery of cortisol was 104.8% or as a regression equation $y = 1.048x + 0.1367$, $r^2 = 0.9485$, $n = 4$. Parallelism was assessed by plotting results of three serially diluted samples, which showed to be parallel to the standard curve, and a one-way ANOVA showed no significant mean difference in slopes ($F(3, 28) = 0.087$, $p = 0.966$). Inter and intra assay coefficient of variation percentages were 13.75 and 8.35, respectively.

Statistical analyses

Statistical analyses were performed using R software (v3.6.3; R Core Team 2020). The distribution of the raw cortisol data was visually inspected and normality was formally tested using the Shapiro-Wilk test. The distribution did not meet normality. Log and square root transformations did not adequately normalize the residuals. The Skillings-Mack test (*PMCMRplus* package v1.9.3; Pohlert 2021) was used to detect any significant differences between the repeated measures of cortisol levels across the seven-day study period. The significance level was set to 0.05. For all analyses, cortisol results were time transformed to one day prior, to approximately correct for metabolic time lag effects (Bahr et al 2000; Takeshita et al 2018). All cortisol results are presented with a time shift of minus one day from the day they were collected.

Ethical review

The research described here was approved under permit No. 2019-187, by the Animal Welfare and Animal Care Committee of the Primate Research Institute, Kyoto University and institutional guidelines for the care and use of non-human primates were followed.

Results

No significant differences in fecal cortisol concentrations were found (Skillings-Mack test: $n = 66$, $\chi 2 = 6.95$, df = 6, p = 0.325). The median was highest on the relocation day compared to all other days and the medians of post-relocation days were similar to pre-relocation days (Fig. 1). I more closely examined differences in fecal cortisol concentrations among all individuals ($n = 10$) between the day prior to the relocation (mean ± SEM = 322.23 ± 91.4) and the relocation day (mean ± SEM = 461.77 ± 138.42), which showed that on average cortisol increased on the relocation day relative to the day prior (Fig. 2). Table 1 describes the results per individual including the number of samples collected, the mean and range of concentration of cortisol over the seven-day study period. Additionally, the absolute change of cortisol concentration from the day prior to the relocation day is highlighted in Table 1.

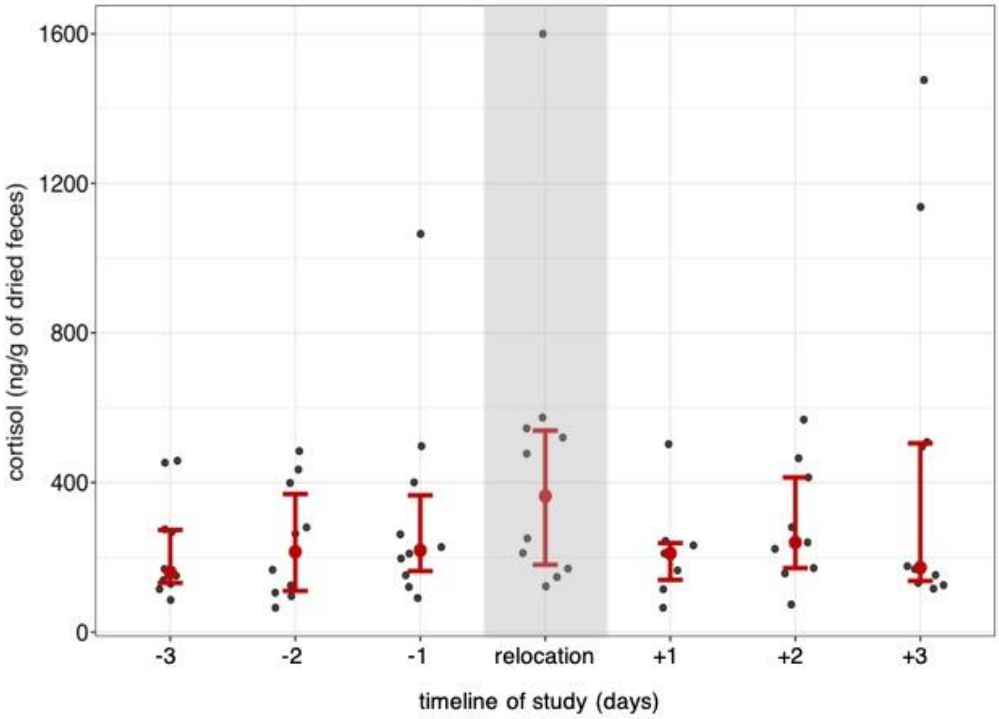

Fig. 1. An overview of all 66 data points plotted per day, one sample per individual. The error bars show the median and inter-quartile range for central tendency changes each day. The relocation day is highlighted in gray.

Table 1. A summary description of all individuals and cortisol results over the seven-day period.

ID	Age	Sex class	Fecal sample *n*	Cortisol (*ng/g* of dried feces) Mean ± SEM	Range *min*	Range *max*	Change *before to relocation*
1909	18	m	6	392.99 ± 64.57	152.8	568.16	119.84
2239	11	m	5	620.19 ± 131.29	434.72	1136.8	76.56
2409	9	f	7	209.85 ± 37.92	96	413.6	14.64
1644	23	f	7	481.56 ± 109.75	138.88	1064.72	-587.6
1791	20	m	7	220.36 ± 22.99	116	280.08	23.12
2140	13	f	6	449.62 ± 237.71	85.76	1599.68	1447.44
2049	15	m	7	463.32 ± 176.38	122.48	1476.48	-87.52
2046	15	m	7	224.28 ± 55.95	129.12	544.96	283.52
2068	14	f	7	100.8 ± 14.58	65.04	169.76	78.56
2191	12	f	7	150.83 ± 15.76	105.44	222.56	26.72

Note: The *Change* column refers to the individual difference in measured concentration from one day before the relocation to the relocation day.

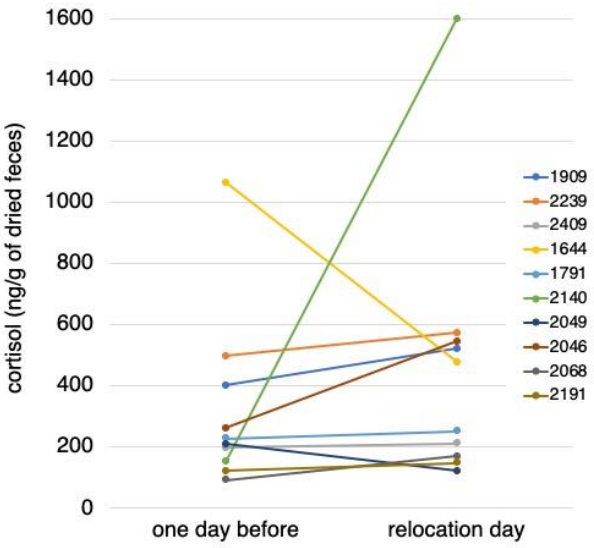

Fig. 2 The raw cortisol values are plotted to visually observe the individual differences between one day prior to the move and the relocation day. The key indicates the ID for each individual. A relative increase in cortisol levels was seen on the relocation day.

Discussion

I measured the fecal cortisol of ten Japanese macaques living in captivity in order to investigate to what extent this group may have experienced stress during a housing relocation. There were no statistically significant differences when comparing the group's cortisol levels over seven days. However, the day prior to the move and the relocation day were more closely compared and an increase of cortisol concentrations in most individuals was observed. These results suggest that the housing relocation procedure caused relatively minimal HPA activation and subsequently was likely minimally stressful for the group as a whole. Although housing relocations may inherently be stressful for captive macaques, discussing the relocation conditions in the present study might be useful for minimizing the stress response of future relocations in Japanese macaques and closely related species managed under captivity.

There was considerable continuity in the monkey's environment before and after the relocation. The group has been housed together for approximately three years and was relatively well adapted to captive living conditions. There were no new individuals that were introduced to the group. Similarly, the group has been habituated to the same caretakers, technicians, and researchers for an equal number of years and the same staff continued to work with the monkeys at the new facility. Minus the new facility lacking a window, the housing conditions were nearly identical in both facilities and there was no change in their daily schedule. Notably, the housing unit of one male monkey (ID 2046) was moved to the opposite side of the new housing room and although I can only speculate

to what extent the relocation or change in housing unit location placed stress on this individual, his cortisol levels on post-relocation days returned to pre-relocation levels beginning on the following day. Two individuals (ID 2140 & 1644), showed a pronounced increase and decrease of cortisol concentrations on the immediate days before and after the relocation. Excluding the relocation procedure itself, there were no particular outlying conditions placed on these individuals that could suggest a clear explanation. It is likely that ID 2140 experienced an increase in HPA activation on the relocation day due to the relocation procedure. Furthermore, it is likely that ID 1644 experienced increased HPA activation the day prior to the relocation due to conditions outside of the present study, such as conspecific conflict. This suggests that monitoring behavior throughout the study could have led to further explaining the individual variation exhibited in the study results. Overall, the social and physical environment remained largely consistent, and this predictability in the environment was likely a contributing factor in the minimal stress response seen in most individuals.

The relocation procedure itself occurred within a short period and monkey-technician cooperative methods were used for placing monkeys in their carrying cages. In total, all ten monkeys were moved to the new facility within 30 min. Longer transportation, for example, 16-18 h to transport *M. tonkeana* and 24 h of simulated transportation in *M. fascicularis* has been reported to be associated with highly elevated levels of fecal and urinary cortisol, respectively (Cinque et al 2016; Fernström et al 2008). Testing differences in relocation time was beyond the scope of the present study, but the short period used to move all

monkeys is consistent with the idea that the amount of time the monkeys remain in carrying cages is an important factor to consider in minimizing stress during relocation. Additionally, technicians used cooperative means to move monkeys into their respective carrying cages for transport and did not physically restrain them. No anesthesia or sedative drug was administered such as ketamine, which has been shown to highly elevate urinary cortisol levels in *M. fascicularis* and *M. Nemestrina* (Crockett et al 1993, 2000) and in plasma in *M. Mulatta* (Elvidge et al 1976). The study results presented in this report add to the notion that sedation may highly increase the cortisol response and continued focus on this factor can help us understand to what extent. Overall, the study outcome demonstrates the benefit of detailed efforts taken by management staff in carefully implementing animal welfare-based practices.

Highlighting the limitations of the present study may help in considering measures of interest for future investigations on stress induced by housing relocation. First is that, due to constraints in time resources and the opportunistic nature of the present study, I could not include a control group, such as comparing a group that did not undergo a relocation procedure. Despite this drawback, my study adds to the existing literature because there is limited information on monitoring stress in *M. fuscata* throughout a housing relocation procedure, though a control group would add validity to future studies. Second, I chose fecal sampling due to its ease of collection and reduced interference in the daily management of the study group. Other biological material such as plasma (Davenport et al 2008), saliva (Lutz et al 2000), or urine (Fernström et al 2008) may provide a higher time resolution of cortisol activity, and hair (Dettmer et al

2012) can determine more long-term stress effects. Fecal sampling took a few minutes each morning and did not require any manipulation to the study group, which illustrates the importance of this medium in monitoring stress-associated analytes. Third, factors more physically apparent such as sleep patterns, behavioral activity, appetite (Crockett et al 1995, 2000), or self-injury (Davenport et al 2008) might be useful indicators to measure when understanding how Japanese macaques cope under stressful periods in their environment.

Lastly, it is important to briefly emphasize the complexity of the stress response found in macaque species. For the purpose of the present study, I defined stress in terms of HPA axis activation, but the sympathetic nervous system is a parallel pathway that responds to stress (Broche et al 2019). Other physiological systems are affected by stress such as the immune system (Nehete et al 2017; Shelton et al 2019), the reproductive system (Bethea et al 2008), and the cardiovascular system (Shively et al 2009). Furthermore, the stress response can be widely modulated, for example, by individual differences in temperament (Linden et al 2018), early rearing environment (Capitanio et al 2005; Sanchez et al 2005), and the predisposition to genetic factors (Pflüger et al 2016). These approaches can also be implemented in helping us understand how captive NHPs adapt through change in their environments.

In conclusion, I found a minimal increase in cortisol levels when a group of captive Japanese macaques underwent a housing relocation. The relocation was short in duration and the physical and social environment remained mostly consistent in both the previous and new environments. Furthermore, cooperative methods were used to move the individuals and no sedative drugs were

administered. These factors may be important when aiming to minimize stress in the relocation of captive Japanese macaques and closely related species. Future studies are needed to look at more long-term relocation scenarios and the benefits of cooperative techniques.

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Chapter 4

Studying stress-associated analytes via saliva in wild living Japanese macaques (*Macaca fuscata*) at Koshima

Abstract

Monitoring short-term changes of endocrine responses in non-human primates living in wild populations is a challenge. Saliva contains enzymes, steroids, and various analytes that can be broadly useful for helping us understand physiological responses to social and environmental sources of stress and other compromises to an individual's health homeostasis. Salivary alpha-amylase and salivary cortisol are known to respond rapidly to stress, which can allow us to use these analytes to monitor stress on a time scale of minutes. I developed a non-invasive methodology for the collection of saliva and verified its applicability by conducting short-term interval sampling of focal individuals under varying social conditions in a group of semi-provisioned free-ranging Japanese macaques (*Macaca fuscata*) living endemically on the island of Koshima, Miyazaki prefecture, Japan. Monkeys were habituated to sampling by using ingestive attractants applied to cotton ropes. Their receptivity and chewing time were recorded during habituation and ad libitum sampling was later performed in relation to social behavior. Salivary analytes associated with stress, alpha-amylase and cortisol, were measured via enzyme immunoassay. Short-term changes in salivary alpha-amylase and salivary cortisol were examined in relation to short-term changes in social behavior. I tested flow rate effects in both analytes and found strong correlations between original sample results and their respective flow rate transformed equivalents. Additionally, temperature effects on samples were tested and both analytes showed nearly the same values when stored at -20, 4, and 30°C conditions for six hours, information expected to facilitate future sampling in field conditions where freezer storage is uncertain. The present study

shows that saliva can be repeatedly sampled in a non-invasive way to investigate short-term changes in stress-associated markers in Japanese macaques living in a free-range environment.

Introduction

Minimally disruptive methods in neuroendocrine sampling are valuable to field studies of non-human primates (NHP). Stress is an important concept for understanding the adaptive mechanisms in how a wild living animal navigates real or perceived threats in their environment. The consequence of physical, physiological, and/or psychosocial demands can elicit adaptive physiological responses mediated by stress-associated pathways to maintain a well-regulated homeostatic balance (Chrousos 2009; Koolhaas et al 2011; McEwen & Akil, 2020).

At present, the most viable material in field research to non-invasively measure acute changes in the neuroendocrine response are urine and feces. Although these excreta are highly useful for field research, within the context of monitoring stress they have two primary limitations; that is, there is little emphasis on stress pathways that are mediated beyond glucocorticoids (Higham 2016), and, markers of interest are accumulations over hours to days (Novak et al 2013) representing a low time resolution from stressful events. Thus, we are limited to investigate one class of hormone and the data we can extract is low in time resolution. However, measuring the stress response via salivary analytes can complement already established methodologies in various ways.

Analytes found in saliva may provide a practical way for us to better understand the physiological stress response in wild living NHPs. Salivary alpha-amylase (sAA) functions as an enzyme that breaks down starch molecules in the initial stages of digestion. The enzyme does not function to regulate stress but by proxy can act as a measure of sympathetic activity (Nater & Rohleder 2009). Human studies have shown strong correlations and significant increased levels of sAA to sympathetic activation, primarily the catecholamine norepinephrine (Kuebler et al 2014; Petrakova et al. 2017; Thoma et al 2012; Warren et al. 2017). Catecholamines respond quickly during the initial stages of stress via the sympathetic nervous system and are released from the adrenal medulla, terming this stress pathway the sympathetic-adrenal-medullary (SAM) axis (Godoy et al 2018). Cortisol is a steroid from the glucocorticoid class of hormones that, in addition to being involved in other bodily functions (Sapolsky et al 2000), assists in regulating the stress response across many mammalian species. The primary signaling pathway occurs from the hypothalamus, to the pituitary gland, and then to the adrenal glands where cortisol is produced and secreted, which is termed the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 2009; Spencer & Deak, 2017). Human studies have reported consistent high correlations between serum cortisol and salivary cortisol (sC) (Daniel et al 2006; Dorn et al 2009; Eatough et al 2009). In these ways, saliva allows us to measure activity in two parallel and integral stress pathways, the SAM and HPA axes.

A key component for saliva collection in NHPs living in a free-ranging context is the application of an attractant to a saliva collection device. Previous studies have reported high efficacy when analyzing genetic material remaining

on chewed or licked items left in the NHP's natural habitat (*Pan troglodytes schweinfurthii*: Inoue et al 2007; *Pan paniscus*: Ishizuka et al 2018; *Gorilla beringei beringei*: Smiley et al 2010), which suggests that an intermediary collection item can be used for saliva collection in NHPs. Typically, hormonal assays require tens of microliters of saliva and thus collection devices capable of high-volume capacity are required. Sample collection devices such as oral swabs and rope prepared for swabbing has been demonstrated in widely varying NHP species studied *in situ* (*Macaca thibetana*: Simons et al 2012; *Cercopithecus ascanius*, *Cercopithecus lhoesti*, *Macaca mulatta*, *Papio anubis*: Smiley et al 2015; *Macaca arctoides*: Toyoda et al 2021). The measurement of salivary analytes sAA and sC via attractants applied to oral swabs has been demonstrated in *Macaca mulatta* (Higham et al, 2010) on Cayo Santiago and the method has provided an approach to investigate dysregulation of the stress response in this population (Mandalaywala et al 2017; Petrullo et al 2016). These studies illustrate how saliva can be used to study the physiological response of NHPs in a dynamic way. Previously, Broche et al (2019) performed validation work with a captive group of Japanese macaques and found that sAA responds acutely to mild psychological stress. I sought to establish a method to study sAA and sC in Japanese macaques living in their natural environment, in order to provide a foundation to investigate multiple stress response systems (i.e. SAM and HPA axes) in relation to social behavior in this species.

In the present study, I performed exploratory research in order to develop a non-invasive saliva collection method in the endemic Japanese macaques of Koshima island, Miyazaki prefecture, Japan. The population on the island has

been studied for approximately 70 years (Watanabe, 2008) and provides an opportunity for researchers to study their behaviors in close physical proximity through provisioning. Monkeys were habituated to saliva sampling via attractants that were applied to prepared rope. Here, I report the success to each attractant, including saliva volume collected and time taken to deposit saliva. Continuous focal sampling was performed to test the viability of monitoring short-term changes in sAA and sC concentrations. Lastly, I evaluated salivary flow rate effects as well as the effects of temperature degradation on the target analytes to help facilitate future studies.

Methods and Materials

Study group

At the time of study, there were approximately one hundred endemic Japanese macaques, forming two groups, on the island of Koshima. The study was conducted on a total of 18 days between mid-December 2019 to late March 2020. Most of the members of the study group were habituated to the presence of researchers and occasional visitors to the island. During this study the group included 46 individuals (32 females, age range: 0 – 21 y/o; 14 males, age range: 0 – 15 y/o). This group was provisioned with wheat grains approximately two to three times per week on the beach, to allow for good visibility while collecting attendance records.

Saliva collection materials

I prepared 100% cotton ropes following the protocol of Broche et al (2019). In brief, a 4 mm diameter braided cotton rope was cut into 30 cm long pieces, tying an overhand knot on both distal ends. Ropes were sanitized by bringing water to a boil in a stainless-steel pot and placing the prepared ropes in the boiling water for 20 min. This was repeated three times, each time removing the previous water and introducing new clean water to the pot. Ropes were then completely dried in a food dryer and then stored in a vacuum sealed plastic bag until later use.

Attractants applied to the ropes (hereafter 'rope swabs') was the primary strategy to entice the monkeys to chew on them, depositing saliva in the process. In the order of attempts, for habituation to the rope swabs, I first used 1g of creamy peanut butter (PB) (Meidi-Ya, Co. Ltd.) lightly applied at one distal end of the rope swab. Next, when most individuals became habituated and were effectively depositing saliva on the rope swabs, I replaced the peanut butter attractant with juice made from a sucralose powder-based drink (Fine Japan Co. Ltd.). The rope swabs were saturated with a sucralose solution concentration prepared by a solution of 1 g per 10 ml of water. After soaking the rope swabs in the solution for 3 min, or enough time to become fully saturated, the rope swabs were completely dried. These rope swabs were prepared one day prior to testing the monkeys' receptivity to this attractant. Additionally, I made attempts to use the sucralose rope swabs dipped into freshly squeezed orange fruit as a comparison of the monkey's receptivity to the original sucralose-based solution. Lastly, 0.5 g of PB was tested as an attractant to compare characteristics such as

the amount of time the monkeys had interest in chewing as well as the volume of saliva obtained.

Sampling habituation, collection and storage procedures

Prior to initiating saliva collection, I spent over 50 observational hours with the study group to habituate them to his presence, identify individuals, and understand their social dynamics. The site manager taught me the monkey IDs. Samples were collected between 09:00 and 14:00; mostly between 10:00 to 12:00. During sample collection, weather conditions, temperature and humidity ranged between 9 – 22°C and 33 – 75%, and the average was 14.19 ± 0.68 (mean ± SEM) with humidity at 55.61% ± 3.33% (mean ± SEM). The weather was mostly cool with partial overcast conditions. Rainy weather was avoided for saliva sampling.

For sampling habituation, my goal was to entice the monkeys to chew on rope swabs containing PB on the distal end. I used 1 g of PB as the initial attractant for sampling habituation. The habituation period was considered complete after five consecutive days of achieving a success rate of 75% or higher using 1 g of PB, which took ten days. I then made attempts using sucralose, orange fruit juice, and 0.5 g of PB attractants, in this order. In total, 31 individuals were approached throughout the habituation period (23 females, age range: 3 – 21 y/o, age mean: 10.7 y/o; 8 males, age range: 4 – 19 y/o, age mean: 9.6 y/o). The habituation period allowed us to understand which monkeys were most receptive to sampling saliva.

Over a period of four days, NB selected such individuals and followed them one at a time for as long as possible, recording all relevant behaviors. In particular, conspecific conflict and grooming behavior was recorded. This was done in an effort to test the feasibility of monitoring short-term changes in sAA and sC and potentially capture acute changes in relation to observed behaviors. During these observations, saliva was sampled at time points where the focal monkey was undisturbed from conspecifics. Only records that could be reasonably linked temporally to recorded events were used in the analyses. Focal sampling ended when the target monkey was no longer visible such as moving into the treetops, or no longer had interest in saliva sampling.

Access to most monkeys of the main group was often possible after provisioning, where monkeys would gather and social interactions could be more readily observed. Monkeys who sat at the periphery of the group were approached for sampling. Monkeys who were high ranking and/or known to be aggressive were not targeted to avoid dominant individuals monopolizing samples. The target monkey was shown the rope swab and the researcher stood nearby presenting it; avoiding direct eye contact with the monkey. The sample was counted as a success if the monkey took the rope swab and began chewing on it within 30 sec. If no interest was shown that attempt was not counted as a success. When the monkey successfully provided saliva, I counted the amount of time the rope swab was placed in the mouth. All sampling occurred on one-individual at a time and rope swabs were retrieved immediately after the target monkey was sampled. No unchewed samples were reused. Typically, a monkey would take the rope swab and chew on it, with saliva being absorbed in the

process. When finished with the rope swab the monkey would drop it to the ground. At this time the rope swab was collected and placed in a double chamber tube (Salimetrics LLC, Item No. 5001.05), labelled, and stored within a double walled vacuum insulated container filled with ice packs.

There is no electricity on Koshima island. To temporarily preserve samples at -20° C, I used a portable commercial freezer (Dometic Group AB publ., model CFX 28) run by a portable 400 Wh battery kept in the sleeping hut. After collection, samples were first stored together with ice packs in a cold storage container, and then shifted to the portable freezer within 30 min after collection; continuing sampling within that time period. When samples could be moved to the Koshima Field Station on the mainland, they were stored there in a -30° C freezer. Finally, samples were transported to the Center for the Evolutionary Origins of Human Behavior on the Inuyama Campus, using dry ice and stored in a -20° C freezer until assays were conducted. All samples were analyzed within 15 months of their collection.

Testing salivary flow rate effects

The human literature on salivary analytes cautions that salivary flow rate may affect the secretion of proteins and steroids (i.e. Bosch 2014), confounding results. Therefore, I sought to investigate flow rate effects on samples by testing the difference between the original untransformed results to their respective secretion rate transformed equivalents. I calculated the secretion rate by multiplying the estimated saliva flow rate (mL/min or dL/min) with original

assay results (U/mL or μg/dL) for each respective sample. This can be expressed as follows:

$$\frac{\text{unit of volume}}{\text{minute}} \times \frac{\text{biomarker concentration}}{\text{unit of volume}} = \frac{\text{biomarker concentration}}{\text{minute}}$$

Temperature tests

Temperature regulated storage was a concern for maintaining the integrity of the salivary analytes contained in the samples, especially during extended stays on the island. On a full charge, the freezer could last approximately 4.5 h. A solar panel was used to extend the life of the battery, however, partly cloudy weather conditions added uncertainty for reliably storing samples during long-term stays on the island. Therefore, testing for temperature related degradation in samples was an aim to help facilitate future sampling on the island. In the lab, I used three saliva samples from the study group to better understand temperature related degradation. These samples were relatively low, medium, and high in values per target analyte. The samples were defrosted and made into three aliquots each. One aliquot from each sample was stored in -20°, 4°, and 30° C for 6 hr away from light. All samples were then stored in a -20° C freezer until assay analysis.

Salivary analyte analyses

Saliva samples were tested for sAA and sC by commercial assay kits (Salimetrics, LLC. Item Nos. 1-1902, 1-3002, respectively). Samples were determined by using a 1:200 dilution rate for sAA, which I found performs well from previous lab work using *M. fuscata* saliva (Broche et al 2019). I ran samples for cortisol at a 1:2 dilution rate, which was preliminarily tested and a 1:2 dilution rate was found to perform nearly the same to their respective neats. I performed dilution recovery tests with three samples that had relatively low, medium, and high values per each respective analyte. Serial dilutions were assessed for sAA at 1:50 to 1:400 and for sC from the neat to 1:8. Total average recovery for sAA was 94% ± 7% (mean ± SD) and sC was 97% ± 7% (mean ± SD). Cortisol dilution results were parallel to the standard curve when plotted. A one-way ANOVA was performed and there were no significant mean differences for sAA ($F(3, 8) = 0.163$, $p = 0.918$) and sC ($F(3, 8) = 0.021$, $p = 0.995$). I performed a spiking test with three saliva pools that were made from relatively low, medium, and high value samples. The sAA samples were spiked with controls and the sC samples were spiked with three separate standards. Recovery of sAA was 107% ± 6.1% (mean ± SD) and sC was 116% ± 5.4% (mean ± SD). The inter coefficient of variation (CV) was calculated by averaging the high and low controls ($n = 3$ plates per analyte), which was 5.63% for sAA and 10.04% for sC. The intra CV was 2.19% for sAA and 5.02% for sC, which was calculated by averaging the CV of all samples per respective analyte. All samples were run in duplicate.

Statistical analyses

All statistical analyses were performed using the base functions of R statistical software (v3.6.3; R Core Team 2020). Spiking test results were mean compared by a one-way ANOVA. To test salivary flow rate effects, I applied the Pearson correlation test.

Results

Testing receptivity to attractants

Peanut butter showed to be a strong attractant with most monkeys immediately providing samples when approached (Figure 1). Monkeys who were initially hesitant to sample, quickly became habituated after their first successful sample or by watching conspecifics chew rope swabs. Typically monkeys took the rope swab and first placed it in their mouth, while holding the opposite end of the rope swab, pulled outward using their front teeth to scrape off PB from the surface of the rope swab several times, then chewed the rope swab in the molar region of the mouth. When the monkey lost interest in further chewing, the sample was dropped to the ground. I found that sampling at the periphery of the group provided low disturbance from conspecifics, though there were a few cases where the target monkey was physically displaced by a more dominant individual. I had zero success when implementing the sucralose attractant on rope swabs. On the following sample day, I used the sucralose rope swabs and lightly saturated them with juice from a freshly cut orange fruit, which resulted in 54.5% success in saliva collection attempts. The monkeys were more receptive when orange juice

was added, suggesting that olfactory stimulation likely plays an important role for initial motivation. I ended saliva collection by using a 0.5 g of PB attractant to compare characteristics such as saliva volume and the amount of time spent chewing to the 1 g PB condition. Saliva was saturated into the rope swab at the halved amount of PB, but this also resulted in less time depositing saliva and less saliva volume collected (Table 1).

Table 1. Summary description of each attractant used for saliva collection

	attractant type			
	PB [1g]	PB [0.5g]	sucralose	orange fruit
number of attempts	120	36	12	11
number of individuals	31	9	10	7
success rate (%)	83.3	97.2	0	54.5
saliva volume (μL) mean ± SEM	86.7 ± 10.7	54.1 ± 8.6	na	na
saliva deposit time (min) mean ± SEM	2.10 ± 0.13	1.62 ± 0.22	na	na

Note: The 'number of individuals' were unique individuals approached for saliva sampling.

Fig. 1 An adult female Japanese macaque deposits saliva on a prepared rope swab

Short-term changes of sAA and sC by focal sampling

During the sampling habituation period, I found that individuals began to provide repeated saliva samples by the thirty-seventh overall attempt among all individuals approached (Fig 2a). In the final days of the study, I was able to follow focal individuals and record their behavior while periodically saliva sampling. This allowed us to measure short-term changes in both sAA and sC in relation to their social environment. Focal individuals showed both sAA and sC fluctuated rapidly on a scale of minutes. Concentrations of sAA decreased following grooming behaviors (Fig. 2b, Fig. 3, Fig. 4). Social conflict showed increased sAA concentrations in Figure 2a and Figure 4 but social conflict did not increase sAA concentrations in Figure 3a. Salivary cortisol concentrations

had fluctuations after conspecific agitations in Figure 2a, Figure 3a, and Figure 4. However, in the case of Figure 3b, although this male did not experience social conflict during or near the time of sampling, sC concentrations fluctuated and increased in the last sample. There was a decrease in sC concentration after reciprocal grooming in Figure 2b, though there was no clear decrease in Figure 3.

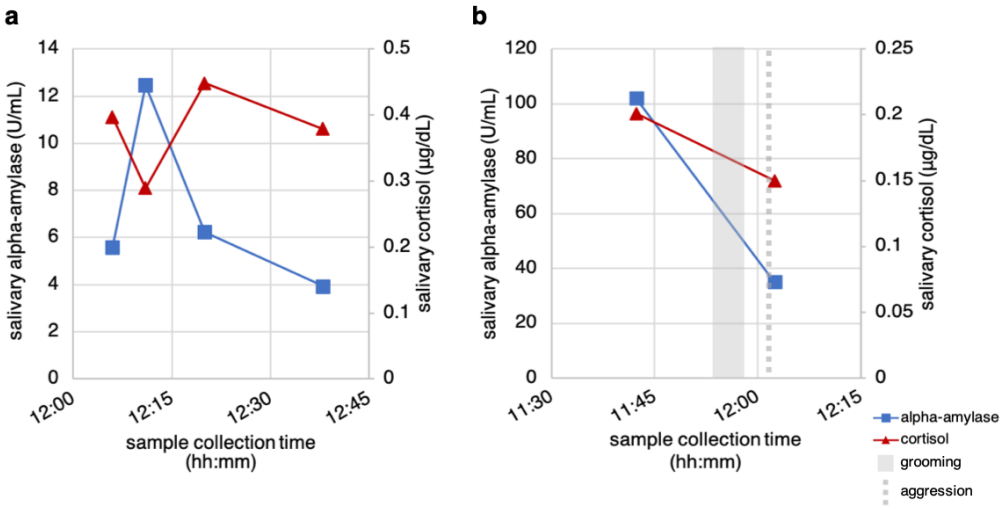

Fig. 2 Line plots of two individuals during the habituation period, demonstrating short-term changes in alpha-amylase and cortisol. (a) By the thirty-seventh overall attempt among all monkeys approached, a 6-year-old female was the first to provide repeated samples within minutes. Although no behavioral data were concretely recorded, there were several conspecifics nearby she aggressively displayed toward periodically, which may account for the concentration fluctuations seen here.; (b) During the same period opportunistic behavioral data began to be recorded. Here a 4-year-old male provided a baseline sample while sitting after provisioning, then engaged in a reciprocal grooming session depicted in gray. Several seconds before sampling he made a brief aggression call toward an approaching conspecific.

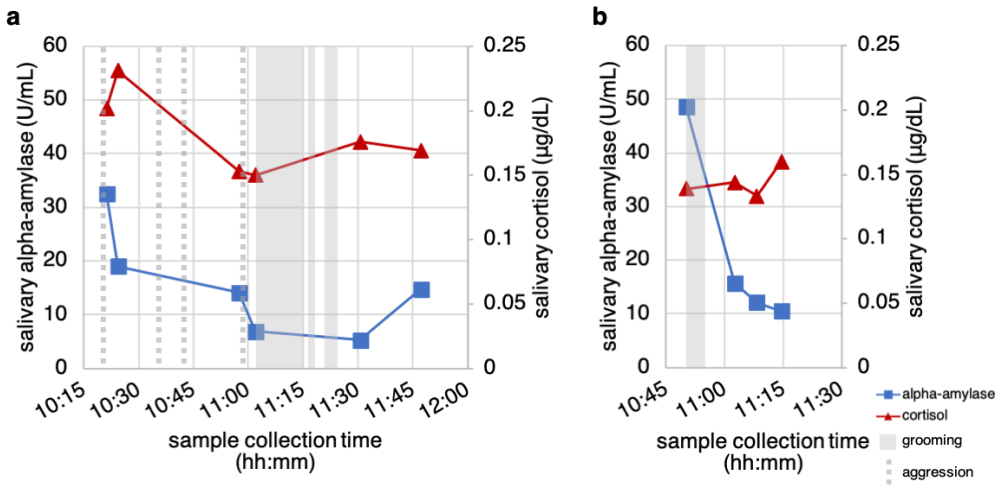

Fig. 3 Line plots of a 6-year-old male. (a) He joined provisioning from approximately 10:25 to 10:49. He received aggression displays from higher ranking females during the first three events. At 10:49, he moved to the periphery of the group and sat alone. He made a brief display to a lower ranking female, then minutes later he engaged in reciprocal grooming bouts with two conspecifics. (b) On this day, he became a focal individual after provisioning. The first sample was taken as he was sitting at the periphery of the group, then his mother approached and groomed him for approximately five minutes. They both sat nearby each other for the remaining samples.

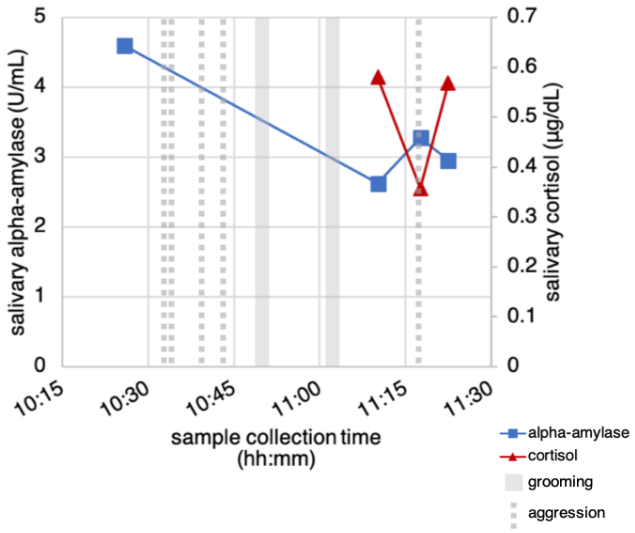

Fig. 4 A line plot of a 12-year-old female with an infant. The baseline sample was taken seconds before provisioning began. The first two aggression encounters began with her receiving a brief aggression display, she then returned a brief aggression display (second dotted vertical line). This pattern occurred again, where she received a brief display and then returned aggression minutes later (third and fourth dotted vertical lines). She stopped feeding at 10:54, moved to the periphery of the group and self-groomed then moved to a more secluded rocky area and self-groomed again. The fifth aggression event was the most pronounced that was recorded. She made intense aggressive calls and displays to an adult male that lasted 30 s. Notably, the first sample only contained 12 µL, which was enough only to assay sAA. Relatively low saliva volume was a common occurrence in her samples.

Testing of salivary flow rate effects

I found sAA as a unit of time (U/min) and their original values (U/mL) were strongly correlated ($r(32) = .85$, $p = < 0.001$, $n = 34$). In addition, sC as a unit of time (µg/min) and their original values (µg/dL) were strongly correlated ($r(26) = .81$, $p = < 0.001$, $n = 28$). The scatterplots of these correlations are illustrated in Figure 3. These results suggest that salivary flow rate effects likely do not confound the results.

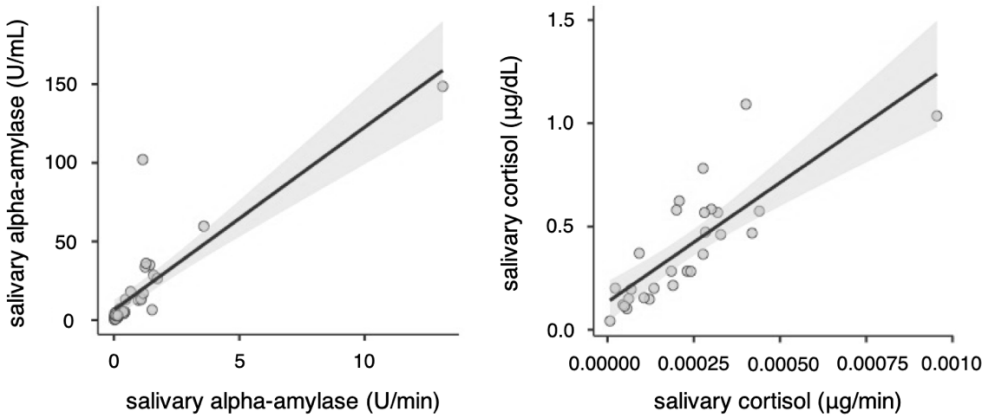

Fig. 3 Scatterplots of (left) alpha-amylase and (right) cortisol comparing their respective time transformed values to their original values. The x-axes represent salivary results as a unit of time to approximately account for salivary flow rate effects. The y-axes represent the original untransformed values. Each plot contains a linear regression line with standard error depicted as grayed areas

Temperature degradation tests

The temperature degradation test resulted in both sAA and sC remaining stable for six hours at -20°, 4°, and 30°C temperature conditions (Figure 4).

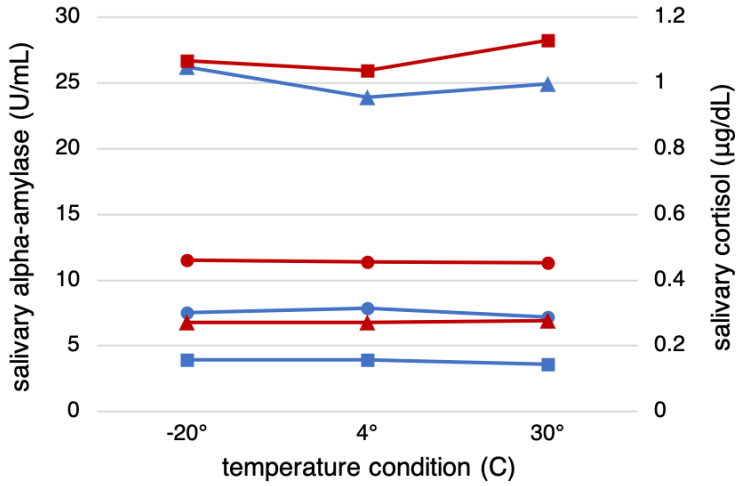

Fig. 4 A line plot of temperature test results from three individual samples stored for 6 h out of direct light. Blue lines correspond to alpha-amylase and red lines to cortisol. Point shapes correspond to the individuals of the same sample (square: 19 y/o female; circle: 15 y/o male; triangle: 10 y/o female)

Discussion

Saliva collection

I developed and validated a non-invasive saliva collection method, testing its accuracy on a group of Japanese macaques endemic to the island of Koshima and analyzed the short-term changes of sAA and sC in relation to behavior.

The attractants applied to rope swabs were suitable to extract adequate saliva from monkeys and this allowed us to examine short-term changes of sAA and sC on a timescale of minutes. Typically, saliva collection occurred after provisioning on monkeys found around the periphery of the beach and rocks on the forest edge. During initial habituation, some monkeys were hesitant to take the rope swab, though in subsequent sampling days the monkeys gradually habituated to the rope swab. In most cases, target monkeys immediately took the rope swab and began chewing when the rope swab was presented to them by the researcher. Collection attempts were made in the surrounding forest but in almost all cases the monkeys showed no interest in sampling and continued foraging on the natural vegetation. There were a few cases where the target monkey ran off with the sample into tree tops and high rock crevices. Most of these samples were retrieved. Securing the rope swab by a string held by the researcher may be a more effective approach for retrieving a chewed rope swab. From the sampling habituation period, I found that provisioning brought the monkeys together, which provided the most efficient period to observe social behavior and attempt saliva collection.

The receptivity of attractants

On the first day of sampling habituation, there was over a 75% success rate in saliva collection attempts using PB and high success continued on most days thereafter using this attractant. I tested the receptivity of the sucralose-based attractant; however, no saliva was obtained. Essentially there was no interest in sucralose. In a few cases, the monkeys smelled the sucralose rope swab but thereafter dropped it on the ground. On the following sampling day, I cut a ripe orange fruit and lightly dipped unused sucralose ropes in the juice in order to add its noticeable scent. Visibly the ropes appeared the same as the previous sampling day, though contained the scent of orange fruit. Briefly saturating the rope swabs in orange juice resulted in 54.5% success in the monkeys chewing the rope swabs, which suggests that olfactory cues play an important role for enticing monkeys to chew on rope swabs. Although orange fruit juice was likely a viable attractant, particularly if dried to avoid dilution effects in the samples, I did not investigate this attractant further because it was unclear how the acidity would affect the cortisol assay, which recommends a pH range of 3.5 to 9.0 to maintain accuracy. However, future studies may benefit from testing these effects.

Throughout the study, I did not interfere when the target monkey began chewing, instead, I stood nearby until the monkey's interest in chewing naturally extinguished and the sample was dropped to the ground. My reasoning was that the amount of attractant could act as the signal to end sampling without interference from the researcher. The amount of attractant determined chewing

time and consequently the volume of saliva. I compared these characteristics in the PB attractant. Using 1 g of PB attractant, I found that monkeys chewed on rope swabs on average for 2.10 min yielding an average of 86.7 μL of saliva. By comparison, using a half gram of PB, monkeys chewed on average for 1.62 min and deposited 54.1 μL of saliva. The volume of saliva decreased by 37.6% on average when the PB attractant was halved. If the chewing period is too short in duration, this may in turn yield too little saliva volume for assaying multiple analytes simultaneously. For example, the assay kit recommended 10 μL and 25 μL of sample neat run in duplicate for alpha-amylase and cortisol, respectively. Likewise, if the period of depositing saliva is too long, this may confound results by measuring overlapping short-term changes because the study analytes respond rapidly. Additionally, long sample collection times increase the chance of disturbance from dominant conspecifics. In these ways, maximizing saliva volume while minimizing the collection time needed may be important in facilitating saliva collection in a free ranging context.

Salivary flow rate effects

I found a fairly strong positive correlation in both analytes when comparing their original values to their respective transformed equivalents as an output unit of time, suggesting that salivary flow rate likely did not confound the results. Previous studies in the human oral biology literature have emphasized the potential confounding effects of salivary flow rate on the target analytes (e.g. Beltzer et al 2010; Bosch et al 2011, 2014). Although this literature largely

involves humans as the study subjects, it is important to note that macaque salivary glands are similar in anatomical structure to that of humans (e.g. Jacobsen & Hensten-Pettersen 1976; Nagato & Tandler 1986; Paul & David 1958) and thus general principles may apply in the study population. The primary concern here is that salivary flow rate might fluctuate due to the stimulatory effects of sampling itself. Concentrations of salivary analytes could show marked increases if saliva output were to decrease and likewise decreased concentrations if saliva output were to increase. There has been particular interest concerning sAA due to its localized production in the salivary glands. Acinar cells, a network of cells present in salivary glands, produce and secrete the majority of proteins contained in saliva, which includes alpha-amylase (Castle & Castle 1998). These cells appear to have extensive sympathetic and parasympathetic nerve innervation (Garrett & Kidd 1993; Proctor & Carpenter 2007). Proteins along with sAA are stored in granules within the salivary glands until neuronally activated to secrete into saliva (Bosch et al 2011). In theory, sC is relatively more independent from salivary flow rate effects than sAA. This is due to the physiological mechanisms of sC that distinctly differ from sAA; in that cortisol is produced and secreted by the adrenal cortex and transported via blood to the capillaries surrounding the salivary glands (Kaufman & Lamster 2002; Quissell 1993). Cortisol is then released into saliva through the secretory cells of the salivary glands. These factors should be considered when measuring salivary analytes.

Short-term changes in sAA and sC

We performed focal sampling to test the feasibility of monitoring short-term changes of sAA and sC. Behavior was recorded ad libitum to help validate whether each analyte responded to external stimuli, particularly to aggression and grooming. My assumption was that aggression is inherently stressful and grooming is inherently calming for the monkeys. Thus, my reasoning was that concentrations of the target salivary analytes would likely result in patterned associations to these events. Grooming was associated with lowered sAA concentrations but aggression did not show an increase of sAA concentrations in every instance. Exhibited aggression consisted of brief bouts of vocalizations and posturing, which typically lasted less than 5 s. However, in one case the aggression event lasted for approximately 30 s (Fig. 4, fifth aggression event). In all events, no physical altercations were observed such as grabbing, biting, or slapping. The intensity of most aggression displayed was mild. In a previous captive study of Japanese macaques, sAA concentrations increased on average 4.2 min after exposure to an unpredictable 20 s psychological stressor (Broche et al 2019). In the case of the 6-year-old male (Fig. 3), the exhibited social stress was acute (lasting between 2 – 4 s) and likely was not enough to elicit a measurable sympathetic response. Acute social stress may need to exceed a sufficient threshold of intensity in order to have a measurable sympathetic response such as the case of the 30 s aggression event in Figure 4. Interestingly, the 6-year-old male showed increased and asymptotic sC responses, which suggests there was ongoing HPA activation during these sample periods. Having

reached sexual maturity, this male is at a critical developmental stage where males typically must leave their natal groups (Sugiyama 1976). This ongoing HPA activation might reflect the energetic demands needed to navigate his compromised social position. In order to further validate sAA and sC as reliable markers of stress, future studies will need to carefully discern the type of stress (e.g. physical, psychosocial, injury) along with duration, frequency, and intensity while considering the effects of outlying pressures (e.g. developmental stage, social status, season) on the individual.

Daily fluctuations of sAA and sC

The analytes presented in this report are known to follow a distinct natural daily rhythm and this factor should be carefully considered when interpreting results. At present and as far as we are aware, there are no extensive studies on the diurnal fluctuations of sAA in NHPs. In healthy adult humans, salivary alpha-amylase has been shown to have the lowest mean concentration at 09:00 in the morning with a gradual increase peaking at 18:00 (Nater et al 2007). I can only speculate the natural diurnal variation in the study subjects, however, if Japanese macaques as a species follow a similar pattern to that of humans then this would add validity to the findings because sAA was associated with a decrease in the minutes after grooming behavior was observed. Although the daily rhythms of sAA are currently unspecified, it is likely that grooming has a relaxing effect on monkeys and the subsequent lowered concentrations seen in focal monitoring likewise reflected lowered sympathetic activity. The daily fluctuations of cortisol in

Japanese macaques is more understood. Suzuki et al (2002) measured the daily fluctuations of plasma cortisol in 4-hour cycles throughout a 24 h period in captive Japanese macaques. Their study found that cortisol concentrations were highest at 08:00 in the morning, then decreased by 12:00 and again decreased by 16:00. Thus, it is likely that the sC results were affected by the natural daily rhythmic decline of cortisol since the collection periods were within this decline period. Clearly separating the daily rhythm of cortisol apart from external influences is difficult at present. However, it is reasonable to speculate that asymptotic or increased responses of sC during the sample collection time indicated ongoing activation of the HPA axis.

Temperature degradation

Field conditions present a unique challenge for preserving the integrity of samples. I was able to use a portable freezer to store samples at -20°C while on the island but the main drawback was the inconsistent availability of electricity. In cases where a portable freezer, battery, and solar panel set up is possible, the weather conditions may be too variable and the lack of freezer storage may lead to bacterial degradation in collected samples. A viable alternative is to test the samples in relation to temperature conditions, which can be used as an indicator of the hardiness of the analytes to withstand degradation in the field. Both sAA and sC performed well at a typical refrigeration temperature (4°C) and a relatively high temperature (30°C) when compared to the freezer temperature (-20°). Such testing provides an approximation for understanding if ice pack

containers (*i.e.* 4°C) help maintain the integrity of samples or, for example, to what extent samples degrade when electricity is not available (*i.e.* 30°C) for several hours. Future efforts in this area will assist sample collection in field studies where consistent freezer storage remains a difficult challenge.

Limitations

I recommend three ways salivary analyte research can be expanded upon to facilitate NHP field studies. First, it was clear that PB was a powerful motivator for monkeys to deposit saliva, but residue oil was sometimes difficult to avoid when processing samples. I noticed samples that required re-running in the assay system also contained specks of oil. To avoid this issue, a powdered or dried form of PB as an attractant will likely allow for more efficient assay analysis. Second, biochemical work is needed to understand the effects of attractants, if any, on target analytes. In theory, salivary alpha-amylase hydrolyzes α-1,4-glycosidic linkages (van der Maarel et al, 2002) and the extent these glycosidic bonds are present in the attractants used, primarily PB, is unclear. Such studies would optimize saliva sampling by reducing uncertainty in target analytes. Notably, it was the attractants themselves that allowed saliva collection in these free-ranging monkeys. Studies aiming to compare differences between plain swabs and those containing an attractant might best be suited with captive groups where training techniques can be employed (e.g. Broche et al 2019; Lutz et al 2000). Third, diurnal variations are present in salivary analytes (Heintz et al 2011; Nater et al 2007). Understanding the diurnal rhythms of sAA and sC in the study population

was outside the scope of the present study, however, investigating the circadian characteristics of target analytes will benefit the interpretation of results.

In summary, I described a method for the non-invasive collection of saliva in the monkeys endemic to Koshima island. Short-term changes in sAA and sC that corresponded to the monkeys' social behavior were found. These salivary analytes will be important to future research aiming to measure activity of the stress pathways, the SAM and HPA axes, respectively. Both analytes showed a strong correlation between their estimated flow rate and their respective untransformed equivalents, suggesting that salivary flow rate likely did not confound sAA and sC in the present study. The target analytes remained stable when samples were stored at a relatively high temperature for six hours, and though preliminary, these results indicate a viable method for further testing to facilitate studies of NHPs in their natural habitat. I conclude that saliva collection can be applied as a tool to better understand the physiological stress response at a high time resolution in Japanese macaques living in a free-ranging context.

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Conflict of interest

I have no conflicts of interest to report.

Ethical approval

The research described here was approved (permit no. 2019-001) by the Field Research Committee of the Center for the Evolutionary Origins of Human Behavior (formerly the Primate Research Institute), Kyoto University

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Chapter 5

General Discussion

Introduction

To briefly summarize, Chapter 1 introduced the concept of stress and a modern theoretical framework to investigate this phenomenon in Japanese macaques (*Macaca fuscata*). In Chapter 2, I began the development and validation of saliva collection to understand its applicability in the species. Chapter 3 involves a direct application of using a common and useful biomarker of stress, fecal cortisol metabolites, to help assess the extent that stress may have been experienced in this group. In Chapter 4, the monkeys of Koshima were studied in an effort to establish saliva collection in a population living in the wild with the aim to measure the two primary stress axes. In the present chapter, I will discuss the main findings and limitations of each study and attempt to bring them together to conclude this thesis on studying the physiological stress response of Japanese macaques using non-invasive biomarkers.

Saliva collection in a captive, highly managed population

The development and validation of saliva collection in the Chapter 2 captive study was important to understanding its applicability. With careful judgement in applying a positive reinforcement training regime, I was able to utilize a non-invasive approach for the collection of saliva from awake and unrestrained Japanese macaques, and that allowed me to track the temporal dynamics of salivary alpha-amylase in response to acute stress. Positive reinforcement training (PRT) techniques encourage a cooperative and trust-based interaction

between the monkeys and the researcher. Habituation to sampling in a minimally disruptive way allowed the investigation of the stress response. I found that salivary alpha-amylase responded acutely when minor stress was applied. The study was practical in providing a foundation in gaining insight on salivary alpha-amylase enzyme in response to short-term mild psychological stress in this species.

During the health check of the open enclosure monkeys, there were likely some effects due to anesthesia. In Chapter 2, pronounced inhibitory effects on saliva production under anesthetic conditions were found, indicating that this factor may add difficulty in collecting adequate saliva. Out of the 45 individuals sampled, only eight produced the minimum 10 μL of saliva, recommended by the assay kit manufacturer, to determine the concentration of one sample. Under ideal conditions 10 μL of saliva is typically used for the assay, however, several times more saliva volume would be useful for re-assaying samples for additional testing. For example, it is common to run samples in duplicate or triplicate to add validity in determining analyte concentrations, and for that reason double or triple the amount of saliva is required. Additional assay testing includes dilution and spiking tests, which help validate the performance of the assay system. Several other tests could be performed such as testing temperature effects on collected samples (as in Chapter 4). Thus, health checks could provide an opportunity to collect saliva to study the foundational aspects of salivary alpha-amylase or other analytes of interest. It is therefore important to understand why saliva was difficult to collect in a health check context because unlike awake and unrestrained Japanese macaques, monkeys placed under anesthesia remove the

potential difficulty of training and the great effort involved therein. The salivary inhibitory effects of the drug medetomidine might explain the low (17.7%) success rate in obtaining sufficient saliva volume. In cases where anesthesia is required, perhaps reagent test strips, which require minimal saliva volume, could be used for an estimation of salivary analytes of interest.

The systemic effects of anesthetic drugs should be considered in study designs. Ketamine is known to cause an increase in saliva secretion (Pawson & Forsyth 2008), while medetomidine reduces saliva secretion (Scheinin et al 1987). Furthermore, ketamine is a drug that acts as a N-methyl-D-aspartate receptor antagonist, which reduces responsiveness in the nervous system (Anis et al 1983), and medetomidine is an alpha$_2$-adrenoreceptor agonist that inhibits the sympathetic nervous system (Sinclair 2003). In these ways, each anesthetic drug has varying systemic effects on the body and require careful consideration for measuring and interpreting stress-associated salivary analytes. Procedures that require the NHP to be physically restrained and undergo needle injections likely adds further stress on the NHP. If cooperative methods can be safely utilized and soundly applied when working with captive Japanese macaques (e.g. Chapter 2), saliva may provide a viable alternative for interpreting and quantifying stress markers on an acute basis.

Improving method to test acute temporal characteristics of salivary markers

Throughout the PRT sessions, I noted ways to help improve this method of investigation for future studies. I would like to share the main points that may

apply to other macaque species and NHPs here. During training, occasionally some monkeys would pick at the interior of the mouthpiece's aperture, perhaps in search of food or as a form of exploratory behavior. This may reduce the efficiency of testing, for example, in Chapter 2 understanding temporal characteristics of salivary alpha-amylase was a main aim. Testing timed responses may improve if future designs could remove the open part of the mouthpiece so that only the rope swab is visible, which will eliminate a further source of distraction when collecting saliva. Secondly, the initial trials led me to standardize the sucralose concentration added to the rope swab, however, the sensitivity of *M. fuscata* to sugar concentrations varies (Nishi et al. 2016). Sucralose may not pique interest at low concentrations and may be repulsive at high concentrations (Nishi, pers. comm.). A key factor during PRT training is to sustain motivation via food reward. There are two important motivational factors to note here: motivation from the rope swab attractant and motivation from the food reward. On the one hand, the rope swab contained flavoring that likely sustains the chewing behavior. On the other hand, a food reward was given immediately following the behavior to promote and sustain cooperation with the monkey. The motivation to keep chewing on the rope swab may be larger than obtaining the food reward, dampening the positive reinforcement effects from the food items in the process. The key here is that it would be best if the monkey discontinued chewing the rope swab on verbal cue. In the Chapter 2 study, there was an attempt to standardize the chewing time to 30 seconds, which was primarily aimed at facilitating the later timed stress response testing. A detailed investigation into the tradeoff between the monkey's interest to chew on the rope

swab in relation to the motivational value that food reward items offer may be useful. Third, some monkeys preferred to lick the rope swab and gradually that behavior was shaped into chewing through careful PRT, though this required much effort. A device designed for licking has been previously reported (Lutz et al 2000) in captive rhesus macaques (*Macaca mulatta*) but importantly licking required more time than chewing to obtain an equivalent or greater volume of saliva. I did not systematically study chewing versus licking, however, I can confirm that licking the rope swab usually consisted of sampling that took several minutes longer than chewing. Stress related salivary biomarkers respond quickly and thus it may be important to efficiently sample within a time frame that can accurately capture the intended measurement. Related to this point, the collection device should not become fully saturated to maximum capacity because sampling beyond this point is likely not meaningful (i.e. the ceiling effect). Anticipating that some monkeys will require careful attention to shaping their behavior early on in training may assist future study designs. Lastly, PRT sessions were designed to prepare subjects for one saliva sample, however, the actual stress testing itself included sampling three times in a short successive period with a minor stressor added. Additionally, during the time the Chapter 2 study progressed to this stage, the frequency of performing PRT with the study group largely decreased in order to focus on stress testing. I believe these factors likely led to the substantial 50% non-cooperation events during the stress tests. It may be best to provide several PRT sessions in between each stress test day and space out the frequency of stress testing, for example, to once every two weeks or a month per monkey. Furthermore, if the stress test requires three samples, the PRT

sessions should also perform training in the same manner, that is, three samples in approximately the same time frame as aimed for in stress testing. I predict that these improvements in study design will likely result in greater cooperation and thus yield better data to study temporal characteristics of alpha-amylase and cortisol.

Salivary flow rate

The human literature on salivary stress biomarkers cautions that salivary flow rate, that is the volume rate of saliva produced from the salivary glands, may affect proteins and steroids found in saliva (e.g. Beltzer et al. 2015; Bosch et al. 2011; Nagy et al. 2015). Flow rate is calculated by noting the time taken in seconds to deposit the saliva. The seconds recorded can be later transformed into their equivalents in minutes as a decimal form. When processing the sample, the saliva volume is measured. The saliva volume is divided by the time in minutes to calculate the flow rate, expressed as: the unit of volume / minute. The biomarker result can then be expressed as a function of time by multiplying the concentration of the biomarker result with the flow rate (unit of volume / minute). The formula is as follows here:

$$\frac{\text{unit of volume}}{\text{minute}} \times \frac{\text{biomarker concentration}}{\text{unit of volume}} = \frac{\text{biomarker concentration}}{\text{minute}}$$

To my knowledge, reported studies measuring salivary stress biomarkers in NHPs have not investigated this factor in great detail. In the human literature, the

continuous variables of flow rate transformed results are typically plotted against the original results using a scatterplot to visualize the relationship and a correlation test is performed to determine the strength of the relationship. When comparing the original values of both analytes to their corresponding transformed equivalents as an output unit of time, a fairly strong positive correlation was found in the Chapter 2 and 4 studies, indicating that salivary flow rate probably did not affect our results. The potential confounding effects of salivary flow rate on our target analytes have been highlighted in prior investigations in the human oral biology literature (e.g. Beltzer et al 2010; Bosch et al 2011, 2014). General principles may apply to our study population because macaque salivary glands have a similar anatomical structure to that of humans (e.g., Jacobsen & Hensten-Pettersen 1976; Nagato & Tandler 1986; Paul & David 1958). The uncertainty here is that salivary flow rate might change as a result of the stimulatory effects in sampling. The reason this is important is because if saliva output were to decrease, the concentrations of salivary analytes may rise. Inversely, if saliva output were to increase, the concentrations could fall. It is this interaction that testing flow rate helps to rule out.

The physiological mechanisms of each analyte and how they are secreted into saliva should be considered for testing potential salivary flow rate confounding effects. The majority of the proteins found in saliva, including alpha-amylase, are produced and secreted by acinar cells, a network of cells found in salivary glands (Castle & Castle 1998). These cells have been reported to be heavily innervated by sympathetic and parasympathetic nerves (Garrett & Kidd 1993; Proctor & Carpenter 2007). Prior to being neuronally activated to be

secreted into saliva, proteins including alpha-amylase are stored in granules within the salivary glands (Bosch et al 2011). Theoretically, salivary cortisol is more resistant to the effects of salivary flow rate than salivary alpha-amylase. This is because cortisol is synthesized and secreted by the adrenal cortex, which is located away from the salivary glands and above the kidneys. This steroid is then delivered by blood to the capillaries surrounding the salivary glands (Kaufman & Lamster 2002; Quissell 1993). The secretory cells of the salivary glands then allow cortisol to be released into the saliva through passive diffusion. These are important factors to consider when ruling out confounding effects. It may be possible that in the Chapter 2 and 4 studies the saliva collection period was not long enough to have greatly altered flow rate, though a sampling collection time of 5 minutes or longer perhaps could confound these analytes. Continued efforts in this area will help us determine the role salivary flow rate plays in accurate measurement.

The receptivity of attractants

In human studies, the cooperation of subjects is relatively easy to gain in comparison to non-human animals where some form of enticement is necessary. At present, the most suitable saliva collection method in an unrestrained, awake, free-ranging NHP is to present a swab with an attractant applied to gain the subject's cooperation. In the captive study of Chapter 2, a variety of attractants were initially used. In the first training sessions, juice, such as commercially sold grape or apple juice made for human consumption, was saturated into the rope

swabs to entice chewing and gain habituation. After initial training sessions, rope swabs were prepared with a commercial sucralose-based attractant made for human consumption. The reasoning being that alpha-amylase interacts with glycosidic bonds found in starches such as sugars, cleaving them. In theory, fruit and sugar itself contain these bonds and it was, and still is, unclear exactly how this could affect salivary alpha-amylase levels when sampling. Biochemical analysis in this area would help clear this potential confounding factor because in the field study at Koshima, monkeys were not enticed by the sucralose-based attractant. In the Chapter 4 study, the monkeys were highly motivated by peanut butter and to some extent freshly squeezed orange juice. Each attractant type requires more quality control testing to ensure accurate measurement. For example, orange juice may be a viable attractant but the assay kit manufacturer recommended avoiding low pH samples, which may confound results. In this case, the pH of the attractant should be tested and preliminary samples ran through the assay for quality control. Problematic results here would best be worked out in the lab, for example, increasing pH to a suitable level if too low.

Peanut butter is a strong motivating attractant for monkeys. The field study at Koshima showed consistent reliability to gain the cooperation of these free-ranging monkeys. Similarly, peanuts as a food reward item quickly gained the attention of the monkeys in the Chapter 2 captive study. Used as a saliva collection attractant, there are several problems that must be resolved. First, the oil found in peanut butter was difficult to avoid when pipetting the sample after centrifuge. This caused re-running the samples in the assay. Plate wells containing tiny specks of oil were problematic. The spectrophotometer utilizes

light at the specified wavelength to measure the concentration. Oil that is caught in the suspended sample may interfere with assay measurement here. A powdered type of peanut butter used as an attractant for saliva sampling likely is best suited to avoid this. Furthermore, the effects of starch found in peanut butter are unknown and more testing is needed in this area to reduce uncertainty in interpreting salivary alpha-amylase.

Attention to several key factors during saliva collection may provide efficient sampling. There is a positive correlation between the amount of time taken to saliva sample and the saliva volume collected in the process. The saliva collection device has a maximum capacity of volume saturation. Too little time taken for saliva sampling may result in a sample that does not meet the minimum volume required for assaying. Likewise, excess time taken to saliva sample may not be meaningful should the volume of saliva exceed the maximum capacity of the collection device. Furthermore, the amount of attractant can act as the signal to end sampling by naturally extinguishing the monkey's interest to chew. Therefore, key factors such as the amount and kind of attractant used with the time needed to extinguish motivation, the type of swabbing device used, and the resulting average volume collected are important for collecting an adequate volume of saliva that measures a meaningful level of the salivary analyte. Additional factors to avoid for sampling over long periods of time include reducing the possibility of overlapping measurement of the acute responsiveness of the salivary analytes and reducing interference from conspecifics that can lead to a missed opportunity to sample.

The application of monitoring stress for well-being

This thesis primarily concerns advancing methods in salivary stress markers in Japanese macaques. However, this does not exclude the usefulness of more established methods such as stress fecal markers. In Chapter 3, I was able to investigate the extent that a housing relocation procedure may have placed stress on this group. The study was opportunistic in nature, occurring around a planned management movement of the group to a new location. There were no statistically significant differences found when comparing the group's cortisol levels over seven days. There was an increase of cortisol levels found the following day of the move when more closely examined. The results suggested that the housing relocation in this study caused relatively minimal HPA activation and subsequently was likely minimally stressful on the whole. Captive housing is inherently stressful, however, monitoring stress can help assess and ultimately reduce this burden.

Consistency and the predictability in the environment were likely key factors that influenced the study outcome. From the perspective of the monkeys, the social and physical environment remained largely the same at both locations. Additionally, this group has been managed by the same caretakers, technicians, and researchers for several years prior. The disruption in predictability in the environment due to the housing relocation was short in time duration. The procedure occurred within a 30-minute period. Highly elevated levels of cortisol have been reported in related macaque species between 16 to 24 hours in transport (Cinque et al 2016; Fernström et al 2008). The short time needed to

relocate the monkeys is consistent with the idea that the amount of time in carrying cages is an important factor to minimize stress during relocation. Additionally, technicians used cooperative means to move monkeys, that is, the monkeys were trained via positive reinforcement techniques to enter carrying cages. The study group was not physically restrained nor placed under anesthesia. Ketamine is known to highly elevate cortisol (Crockett et al 2000) and thus from a research design perspective, anesthesia makes interpreting stress results difficult. However, it is important to note that this study group had been trained for several years by using positive reinforcement techniques to enter carrying cages for monthly weighing and health assessment procedures. The management staff's efforts to work cooperatively with the study group was a positive contributing factor in reducing the stress burden throughout the relocation procedure.

There were several key takeaways. Fecal sampling was a useful method to efficiently study the changes in cortisol because of its ease of collection while avoiding interference with the daily management and procedures throughout the study period. Predictability in the social and physical environment, the short period effectively used to reduce the unpredictable nature of the relocation, cooperative methods used by the technicians to move the monkeys, and the lack of pharmacologically induced unconsciousness (i.e. anesthesia) all likely played a large role in reducing stress from the housing location procedure. This study illustrated how non-invasive biomarkers can be applied in monitoring the animal's well-being during potentially stressful events.

A wild living population of Japanese macaques native to the island of Koshima were studied in order to develop non-invasive saliva collection techniques to study short-term changes in stress markers. I next examined the short-term variations in salivary alpha-amylase and salivary cortisol in relation to behavior. Attractants applied to rope swabs allowed the investigation of minute-to-minute fluctuations in salivary alpha-amylase and salivary cortisol, which correspond to SAM and HPA axes respectively.

Saliva was collected after provisioning on monkeys found near the forest edge. Provisioning attracted the monkeys together and provided the most effective time to monitor social behavior and make saliva collection attempts. However, in the forest, monkeys were more interested in foraging and/or remaining in the treetops. In a few instances, the target monkey fled with the sample into steep rock crevasses and treetops. String fastened to the rope swab and secured to the researcher may provide a practical way to retrieve rope swabs, though lost samples were not a common occurrence. Typically, the monkeys took the rope swab and chewed on the distal end containing the attractant until interest was lost. The sample was dropped and collected.

Monkeys were receptive to peanut butter and orange juice attractants but not the sucralose-based attractant used in the Chapter 2 captive study. On appearance the rope swab was identical in both the captive and field saliva studies reported in this thesis. Why did the sucralose-based rope swab work successfully in the captive group but not in the free-ranging group? In the captive study,

training was performed through positive reinforcement of food reward items. This encouraged the sampling behavior and allowed for cooperative saliva collection. The sucralose attractant was originally implemented to help sustain interest for extended periods in early trials of saliva sampling in captivity. Anecdotally, the study group showed preference to this attractant. When I tested the receptivity of rope swabs without any attractant, most monkeys chewed the swabs but only for a very brief period and yielding almost no saliva, suggesting a much lower motivation to provide saliva. In other words, in a captive setting, perhaps some kind of attractant sustains interest in the rope swab and PRT using food reward reinforces the behavior. In a wild setting, saliva collection largely relies on the motivation aroused from the attractant alone. The sucralose-based rope swab contained no smell and unlike peanut butter it was perhaps visually plain in appearance. The orange juice attractant applied to the same rope swabs had a rich citrus aroma and led to interest in chewing. In the studies presented in this thesis, the key to saliva collection was the attractant itself in the free-ranging population studied. However, in the captive group, training could be implemented and therefore the attractant was not as important in gaining interest to chew.

The need for field-adapted sample storage

Maintaining sample integrity in a field setting is difficult. While on the island, I was able to utilize a small freezer to keep samples at -20°C, but the main problem was inconsistent available electricity using a portable freezer with a solar panel

and battery. In theory, lack of freezer storage may cause bacterial deterioration in the samples that have been collected. Testing the resistance of samples to deterioration at vary temperatures might assist in facilitating field storage. A preliminary test was devised from the samples I collected with the population on Koshima island. The samples were subjected to temperatures simulating temperature scenarios the samples would undergo. The baseline comparison temperature was -20°, a commonly recommended long-term storage temperature. Additionally, 4°C was tested to simulate a refrigeration or sample container temperature. The high temperature of 30°C was included to simulate a hot day. The samples were equally aliquoted and left in these temperatures out of the sun for 6 hours. The measurement of alpha-amylase and cortisol remained relatively stable at all temperatures throughout this period. The sample size is small but further study might reveal the hardiness of the analytes to remain intact when proper temperature regulated storage is not consistently available.

General limitations

It is important to not overly emphasize the HPA and SAM axes as the sole physiological systems responsible for mediating stress. The allostatic load model in Chapter 1 as a whole may provide insight into the general complexity of stress. In this thesis, I have looked at stress from the perspective of HPA and SAM axis activation. In the *Macaca* genus, other physiological systems have been shown to be affected by stress as well. For example, the immune system (Nehete et al 2017; Shelton et al 2019), reproductive system (Bethea et al 2008), and

cardiovascular system (Shively et al 2009) are reported to be affected by stress. There is also wide modulation of stress, for example, individual differences in temperament (Linden et al 2018), early rearing environment (Capitanio et al 2005; Sanchez et al 2005), and genetic factors (Pflüger et al 2016) all play a role in the ability of the macaque to overcome stress. These and other factors should be considered to understand the allostatic load placed on NHPs maintaining adaptable stability through change in their environments.

Chapters 2 and 4 were exploratory and Chapter 3 was opportunistic. I regret that control groups were not adequately realized in these studies to add validity to the results. In Chapter 2, testing the acute time lag was performed with three samples and a minor stressor applied. The study could benefit from a comparative control group where no stressor is applied while three samples are collected. In Chapter 3, validity can be added by measuring a separate group that does not undergo housing relocation. In the Chapter 4 field study, the study design was aimed at monitoring short-term changes of salivary analytes and the nature of studying a wild group does not easily allow a control group without serious manipulation. However, the level of the salivary markers in relation to behavioral comparisons would benefit by distinguishing clear definitions of what constitutes high or low stress, for example, severe physical injury versus resting, respectively.

More emphasis on behavioral comparison is needed to interpret physiological stress activity, so that one could potentially predict and inform the other. Physical indications of stress have been reported in macaques such as altered sleep patterns, behavior, and appetite (Crockett et al 1995, 2000).

Importantly, for captive Japanese macaques, linking physiological measures to maladaptive behaviors such as self-injury (Davenport et al 2008) would be a useful indicator of health, particularly if this could be predicted. The application of stress research in NHPs has great potential in this area.

Future studies on stress related salivary analytes

In humans, diurnal variations are present in salivary analytes (Heintz et al 2011; Nater et al 2007). It is common for neuroendocrine research to account for the natural diurnal variation of biomarkers. Diurnal rhythms are understood to exist in the Japanese macaque. At present, the diurnal rhythms of salivary alpha-amylase and salivary cortisol are not well understood in the Japanese macaque, though detailed study of this topic will benefit the interpretation of results.

Future studies focused on the Japanese macaque may find that activity of stress-associated salivary markers will yield predictable response patterns based on the type of stressor. For example, in humans, a physical stressor such as exposure to extreme cold has shown a significant salivary cortisol response but not salivary alpha-amylase (O'Donnell et al. 2009). Psychosocial stress such as public speaking has shown acute sensitivity in the salivary alpha-amylase response but was less prominent in relation to cortisol activity (Rohleder et al 2004; van Stegeren et al 2006).

The timing found in the response of each axis is an important consideration. The time lag, or different time dynamics, of salivary cortisol and salivary alpha-amylase likely respond to stress on varying time lengths. Although

salivary cortisol has been shown to measurably increase following an acute stressor in humans, reaching peak levels approximately 20 minutes after the stressor and remaining elevated for 50–90 minutes thereafter, salivary alpha-amylase has been shown to be particularly psychogenic. It exhibits a more acute response during acute stress, peaking approximately 10 minutes after exercise and returning to baseline approximately 20 minutes after exercise (Engert et al 2011; Gordis et al 2006; Nater et al 2005; Stroud et al 2009; Takai et al 2004). These time dynamics will need to be studied in greater detail for the Japanese macaque and related species to better apply this method and interpret results.

The use of video technology as a tool to assess physiological changes

In the last years, my research interest has been aimed to advance our ability to measure "real-time" physiological changes in Japanese macaques but under the condition that it is done so in a minimally disruptive way. Although this thesis has focused on biological material as a source to measure physiological correlates, we need not only look here. The application of non-invasive stress monitoring can be extended to other areas. The development of a technology utilizing Eulerian mathematical algorithms to magnify changes in recorded digital video has promise. This technique, Eulerian Video Magnification (EVM), magnifies video content in relation to color and movement to reveal subtle changes that are normally not visible to the naked eye (Wu et al 2012). The color magnification corresponds to heart rate fluctuations in the skin, while movement magnification can allow for measuring respiration. Heart rate and respiration rate are known to

increase with stress (e.g. Atkins et al 2018; De Vente et al 2003; Gross 1983; Hopster & Blokhuis 1994; Jouven et al 2009). The possibility to measure these physiological phenomena at a distance would allow us to gain useful data without disturbance to monkey subjects.

To test the feasibility of EVM, I performed a pilot study from the platform of an open enclosure at Inuyama Campus, Kyoto University. The video camera used was a Nikon B500 secured to a tripod to stabilize the video and recording was performed between 10 – 15 meters away from subjects. Here I will share some observations from the small sample size of videos I collected and their results when EVM was applied. It was possible to apply respective EVM algorithms for color and movement magnification. In Figure 1, color magnification corresponds to heart rate and movement magnification corresponds to detecting chest movement. In the case of color magnification, a two second sample shows a frequency of two heartbeats over a one second sample. In the case of movement magnification, one full respiration cycle is shown in a two second sample; the movement can be seen in the chest area. Although this method has great potential in detecting heart and respiration rate, there was one main drawback. Since EVM magnifies almost undetectable changes, movement from the subject or camera itself created large noise in the videos. During such cases, it was difficult to gain any meaningful data. This will require further work in order to successfully apply the technology with awake and unrestrained monkeys. We must also validate the method to accurately detect heart and respiration rate. Due to research budget and time constraints I could not perform extensive developmental testing of EVM in Japanese macaques, though

there is great potential in this tool because it is a completely non-invasive, inexpensive, and it is a practical way for acquiring heart rate and respiration rate in the species.

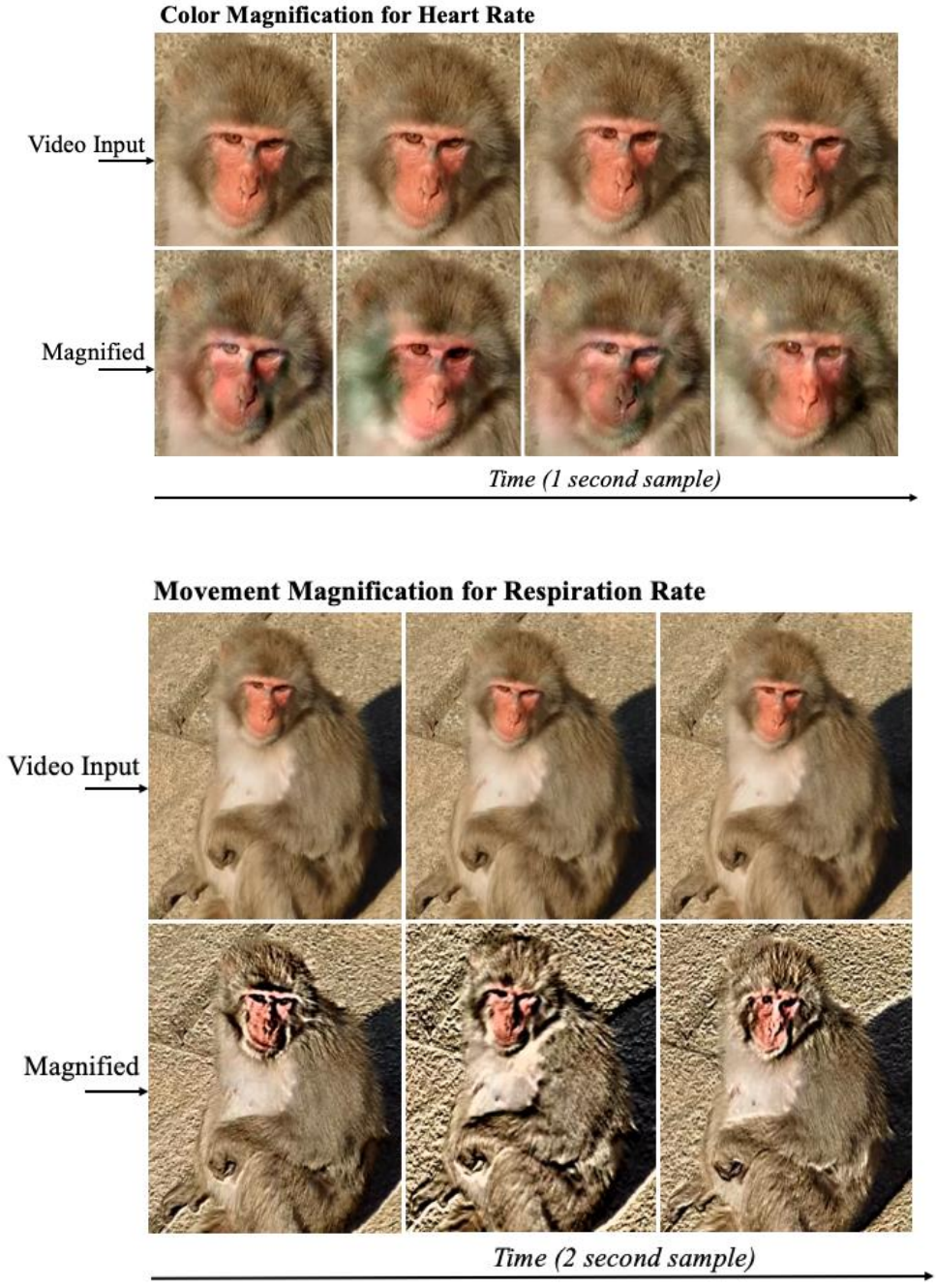

Figure 1. An example of EVM applied to a Japanese macaque living in an open enclosure; (top) an example of two heart beats as seen via color variation on the nose area; (bottom) video magnification example of one full respiration cycle.

In summary

Here I would like to provide a brief summary of the data chapters and conclude this thesis on studying non-invasive stress-associated markers in Japanese macaques. In Chapter 2, I applied a 20-second acute psychological stressor in order to better understand the relationship between salivary alpha-amylase activity and stress in Japanese macaques. There is not much data about alpha-amylase in the context of investigating stress in this species, however, it is likely that future studies will find varying response profiles as a consequence of the type of stressor (e.g. chronic, thermoregulatory, physical injury), time duration of stress, and the individual's ability to cope within their environment. In addition to already established methods for measuring physiological stress, adding salivary alpha-amylase enzyme as a biomarker of acute stress can provide a complimentary non-invasive means for measuring SAM axis activity in *M. fuscata*. In Chapter 3, a minimal increase in fecal cortisol metabolites was measured when a group of captive Japanese macaques underwent a housing relocation. The relocation was short in duration and the physical and social environment remained mostly consistent in both the previous and new environments. Furthermore, cooperative methods were used to move the individuals and no sedative drugs were administered. These factors may be important when aiming to minimize stress in the relocation of captive Japanese macaques and closely related species. Future studies are needed to look at more long-term relocation scenarios and the benefits of cooperative techniques. This chapter shows how stress research can be applied to help us understand the extent

to which environmental manipulations may or may not place stress on captive individuals. In Chapter 4, a method for the non-invasive collection of saliva in the Japanese macaques endemic to Koshima island was described. Short-term changes in salivary alpha-amylase and salivary cortisol were found that corresponded to the monkeys' social behavior. These salivary analytes will be important to future research aiming to measure activity of the stress pathways, the SAM and HPA axes, respectively. Both analytes showed a strong correlation between their estimated flow rate and their respective untransformed equivalents, suggesting that salivary flow rate likely did not confound salivary alpha-amylase and salivary cortisol in the study population. Target analytes remained stable when samples were stored at a relatively high temperature for six hours, and though preliminary, these results indicate a viable method for further testing to facilitate studies of NHPs in their natural habitat. Saliva collection can be applied as a tool to better understand the physiological stress response at a high time resolution in Japanese macaques living in a free-ranging context.

The present thesis broadly advances several notions that should be taken as key points for future studies. The first is that saliva is a viable medium to study stress-associated analytes in awake and unrestrained Japanese macaques. For populations in captivity, a careful training regime will allow for saliva sampling and subsequently this will enable the study of analyte characteristics such as diurnal variation, responsiveness to internal/external stimuli, and time course. In wild populations, saliva collection will heavily rely on the attractant itself and thus extensive testing should be performed to understand these effects on target analytes. Additionally, free-ranging populations present many uncontrollable

variables, for example group dynamics, and therefore researchers should spend considerable time to understand the group as a whole in order to maximize sampling. The second key point of this thesis is the advancement of utilizing not only one stress-associated pathway but both of the main stress pathways simultaneously. Although the HPA axis is associated with stress due to its adaptive response to provide and sustain energy, we must also consider the role of the SAM axis. In this context, this is perhaps where saliva sampling could be most useful because research using non-invasive sampling methods currently lacks a way to measure hormones related to the adrenal medulla or SAM axis. The third and final key point is related to the application of stress research in Japanese macaques. If stress research is to be utilized in a practical way in the future, it will be critical for theoretical work on stress in this species to work toward its application to help assess the well-being or health in populations. At present, there is not a large literature on stress in Japanese macaques, which makes it difficult to determine normal ranges of stress-related physiological markers despite the need for a general consensus that could be implemented in assessing health. I conclude that the tools developed and presented in this thesis will be useful for future work on stress in Japanese macaques and closely related species. This will ultimately enhance our ability to quantify stress broadly on topics such as health, evolution, and sociality.

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