Studying stress-associated non-invasive biomarkers in Japanese macaques

（ニホンザルにおけるストレス関連非侵襲的バイオマーカーの研究）

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General Introduction

This thesis explored and developed tools for understanding acute stress in a minimally disruptive way in Japanese macaques (*Macaca fuscata*). A main goal I have been focused on is utilizing saliva as a medium to help increase the temporal resolution of measuring the activity of two primary stress-associated pathways; the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis in this species. The activity of these systems can be measured non-invasively via the salivary analytes cortisol and alpha-amylase, respectively. The steroid cortisol is a well-established marker in assessing activity of the HPA axis in non-human primates. However, the parallel stress-associated system, the SAM axis, is understudied likely due to methodological difficulties. Thus, an added advantage to collecting saliva is the possibility to investigate both systems simultaneously, which was a major goal of this thesis. Stress research is highly applicable to Japanese macaques. Academic institutions, the medical sector, local tourism, and theoretical and applied sciences depend on the health of Japanese macaque populations controlled and studied under human supervision. In this sense, as a wild animal under the supervision of human care, the health of this species on an individual to population level is of concern to human society in general.

Chapter 2 presents captive work to understand the characteristics of saliva collection and the acute responsiveness of salivary alpha-amylase to act as a marker of sympathetic nervous system activity in Japanese macaques. Saliva collection is key to measuring the analytes it contains, though this is difficult to implement in unrestrained and awake monkeys. Training and cooperation were carefully applied and modified to allow saliva collection. Furthermore, the temporal characteristics of salivary alpha-amylase were studied in relation to mild psychological stress.

Chapter 3 is a continuation of the captive work using a more established method to measure stress (i.e., fecal cortisol) to better understand to what extent housing relocation increases the activity of the HPA axis. As a management procedure, Japanese macaques living in captive environments occasionally require the relocation of their housing. Although this species is commonly held in captivity, there is little information available on the effects housing relocation

has on HPA axis activity. Information on monitoring stress throughout such a procedure would be useful in informing management practices. This chapter is an application using concepts of stress to provide a measure of health or well-being in the species.

In **Chapter 4**, I return to saliva as a way for measuring the stress-associate salivary analytes, however, the method has been expanded in two ways; (1) a wild living group of Japanese macaques is studied and (2) two complementary biomarkers are included, i.e., salivary alpha-amylase and salivary cortisol. These analytes correspond to two important physiological stress-associated systems, the SAM and HPA axes, respectively. The endemic monkeys at Koshima were habituated to saliva sampling and short-term changes in both analytes were monitored in relation to behavior. Additionally, I highlight the difficulty of freezer storage in a field environment where electricity may be scarce.

Methods

In the captive saliva study, I utilized principles and techniques reported in the literature on positive reinforcement training to develop a non-invasive method to collect saliva from unrestrained and awake single-housed Japanese macaques. Throughout approximately 600 individualized training sessions, this method allowed me to collect saliva through earned cooperation with the study subjects. Temporal characteristics of alpha-amylase were studied by applying a mild stressor, that is, the introduction of an unknown person in their environment for 20 seconds. Saliva samples were collected minutes before and after this event to better understand the acute responsiveness of alpha-amylase. In the housing relocation study, we monitored HPA axis activity via cortisol metabolites found in fecal samples over a seven-day period. In the field saliva study, I used attractants applied to prepared cotton rope with the aim to habituate these free-ranging monkeys to chew and deposit their saliva into the rope. After the habituation period, individual monkeys were followed and saliva samples were collected while behavioral data was recorded. This was performed with the aim to study the relative activity of each marker in relation to exhibited behaviors. All analytes were analyzed via enzyme immunoassay.

Results

I showed that saliva collection is possible through cooperative training with captive Japanese macaques and that this can be used to study the stress response of an understudied system, the SAM axis. Salivary alpha-amylase concentrations increased minutes after the mild psychological stressor was applied and subsequently returned to baseline levels minutes thereafter. Later I led a study with the same captive group of monkeys to understand to what extent a housing relocation increased fecal cortisol metabolites. This study found no significant differences in fecal cortisol

concentrations throughout the seven-day study period, though on average there was an increase in cortisol on the day of the relocation for the group as a whole. In the field saliva study at Koshima, peanut butter used as an attractant showed to be strongly preferable for most monkeys. Individuals quickly became habituated after their first successful sample or by watching conspecifics chew on the rope. I found that monkeys provided an adequate volume of saliva to later analyze the target study markers. I was able to measure short-term changes in both salivary alpha-amylase and salivary cortisol in relation to behavior. Both analytes showed acute changes, in particular grooming behavior showed relatively decreased concentrations of both analytes.

Discussion

The main findings of this thesis include development of methods to collect saliva in a non-invasive way in both captive and free-ranging Japanese macaques. This allows us to simultaneously measure HPA and SAM axis activity in an acute manner. Notably, the SAM axis can now be investigated in a minimally disruptive way via the correlate marker alpha-amylase to better understand sympathetic activity. Similar to monitoring markers found in blood, the acute nature of these analytes found in saliva allows us to measure short-term changes of both systems. The applicability of monitoring activity of the HPA axis was shown in the housing relocation study. 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. Such information is useful for informing management practices. Saliva can be used in a similar way to assess stress because there is no need for restraint or adding further stress to monkey subjects when sampling. Captivity training can greatly assist in gaining cooperation. However, training is practically impossible in a free-ranging group of Japanese macaques, suggesting the importance of using a reliable attractant to sample saliva. I suggest further testing and validating attractants to reduce uncertainty in measuring salivary analytes of interest. This will not only further facilitate field studies aiming to sample saliva but it is also likely that this would add sampling efficacy in captive groups where training may not be possible. In conclusion, the present thesis advances our knowledge of Japanese macaques by describing and utilizing concepts, methods, and applications found in stress research in an effort to better assess the well-being of this species.