Research Activity Report
Supported by "Leading Graduate Program in Primatology and Wildlife Science"
(Please be sure to submit this report after the trip that supported by PWS.)

	2019. 11, 29
Affiliation/Position	Primate Research Institute/D1
Name	Vanessa Nadine Gris

1. Country/location of visit

WRC, Kyoto University, Kyoto

2. Research project

Advanced Laboratory Course

3. Date (departing from/returning to Japan)

2019. 11. 25 – 2019. 11.29

4. Main host researcher and affiliation

KINOSHITA Kodzue (WRC, Kyoto University)

5. Progress and results of your research/activity (You can attach extra pages if needed)

Please insert one or more pictures (to be publicly released). Below each picture, please provide a brief description.

From November 25 to 29 I participated in the Advanced Laboratory Course, held at WRC lab in Kyoto with the supervision of Professor Kodzue Kinoshita. Our main goal was to measure sex steroid hormones and discuss the unique endocrinological features of Yaku-sika deer (*Cervus nippon yakushimae*), a sub-species of deer living in the island of Yakushima, in southern Japan. It is known that Yaku sika deers present a late maturation compared to other Japanese deer. In many species progesterone (P_4) levels increase after ovulation and remain high throughout pregnancy. Previous studies showed that progesterone levels found in male Yaku sika deer are as high as those found in females during pregnancy period. We aimed to measure the concentration of progesterone (P4) and its metabolite pregnandiol-3-glucuronide (PdG) to obtain more information about their unique endocrine profile. We aimed to study three main topics:

1) How levels of progesterone (P_4) and its main metabolite (PdG) are affected by time from defecation to freezing
2) To estimate and compare these hormone level concentrations in each age-sex class
3) To compare concentrations of P_4 and PdG in feces between the estrus season and pregnancy period

On the first day we had a brief explanation of the schedule and work and soon headed to the lab. For the next 4 days we prepared and analyzed the samples. Everything was performed according to written protocols. My group was working with PdG and measurement of P_4 follows almost the same procedure.

1. Extraction of fecal steroid hormones: we used fecal samples that were collected in Yakushima field course (November 2019 – estrus period) and samples from 2017 (January – pregnancy period). We separated a portion of the feces and weighed. Then we started a vacuum freeze dry and left it overnight. Next day, we weighed the dried feces again to allow us to calculate the percentage of water contained in each sample and compare it along the time.

   We transferred crushed feces to the tubes and extract it with 20% methanol. Then vortexed, centrifuged and transferred the important part of the sample (discarding the supernatant) to new tubes.

2. Hormone concentration measurement by double antibody enzyme immunoassay

Each microplate received in duplicate: samples, standards (P4 or PdG), controls, maximum binding wells and blank wells were prepared in duplicate.

2.1 Coating of 2nd antibody - anti-rabbit IgG (H+L): ee used 96-well antibody-coated microplates prepared previously. Plates were sealed and stored in refrigerator until use.

2.2 Sample preparation: Samples prepared on step 1 were vortexed and centrifuged (100 X 100G, 1 min). 5 µl of each sample were transferred to 1.5 ml micro tubes containing 95 µl of EIA buffer.

2.3 Standard curve: a serial dilution was made using standard of P4 or PdG diluted in EIA buffer at the concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195 ng/mL.

2.4 Double antibody enzyme immunoassay

The plate coated with 2nd antibody was washed with wash buffer twice. Then 20 µl of standards (0.195-100 ng/mL), 20 µl of samples and 20 µl of control sample are added to each well in duplicate. Also, 20 µl of EIA buffer is add to maximum binding wells and blank wells in duplicate. After adding 100 µl of 1st antibody (P4 or PdG antiserum) and an equal volume of horseradish peroxidase conjugated (enzyme linked antigen) to the wells (except for the blank wells), the plates were incubated at 4˚C overnight.

Each plate was washed (AMW-8R, BioTec, Tokyo) and after 150 µl substrate buffer was dispensed to each well. Then, plates were incubated (SIC-320LW, AS ONE, Osaka) for 10 minutes at 37˚C to obtain colouring products. Fifty microliters of 6.45N H2SO4 was added to all wells to stop the reaction before reading the plate at a wavelength of 450 nm with a microplate reader (SUNRISE, BIO-RAD Laboratories Inc., Osaka). Hormone concentration was calculated by Microplate Manager Version 6.3 pc (BIO-RAD Laboratories Inc., Osaka).

A total of 58 samples (25 males, 28 females and 5 unknown) were analyzed. Twenty two samples were collected in January of 2017 (pregnancy period) and 36 samples were collected in November of 2019 (estrus period). They included 23 juveniles and 35 adults. One female sample was excluded from the analysis of stability because it rained during the time feces were in the glass plate and they were soaked in water. Water content decreased according to exposing time. Similarly, concentration of P4 and PdG also decreased, reaching stability after approximately 26 hours (Figure 1). Progesterone concentrations were higher than PdG for all age and sex classes. There was no significant variation between age-sex classes for P4 and PdG in either estrus season or pregnancy period (Figure 2). The reason why males have the same progesterone levels as pregnant females is still to be elucidate.

Research Activity Report
Supported by "Leading Graduate Program in Primatology and Wildlife Science"
(Please be sure to submit this report after the trip that supported by PWS.)

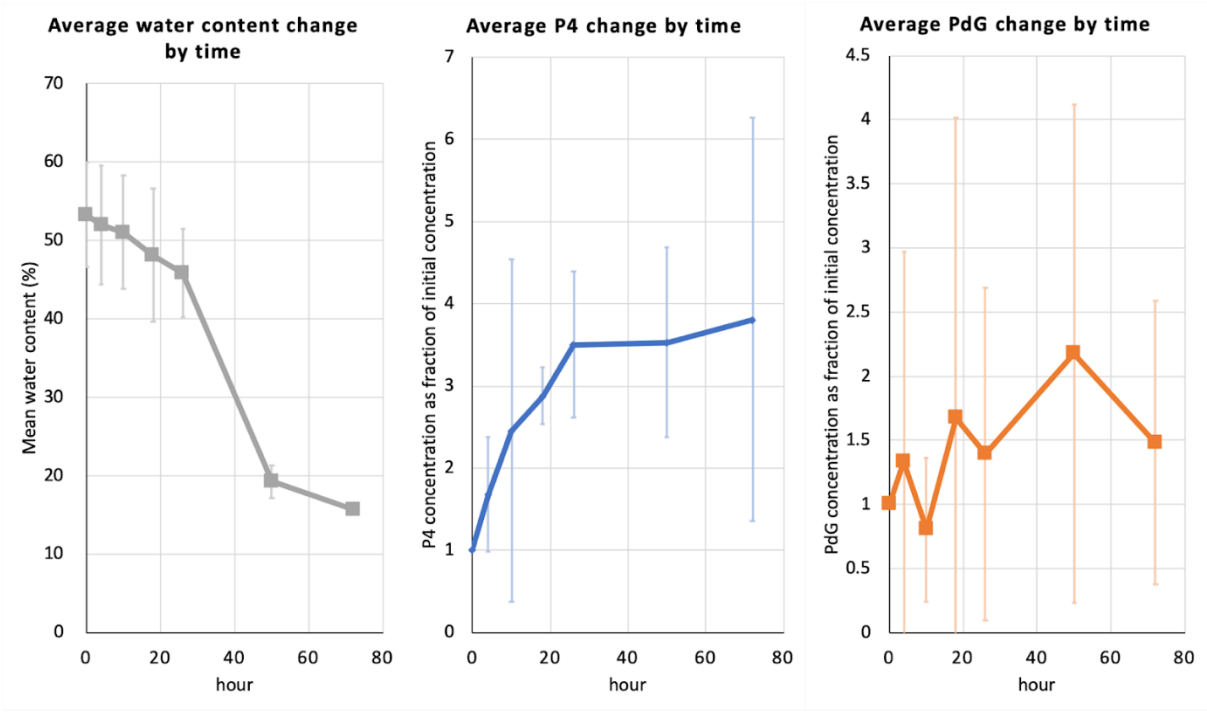
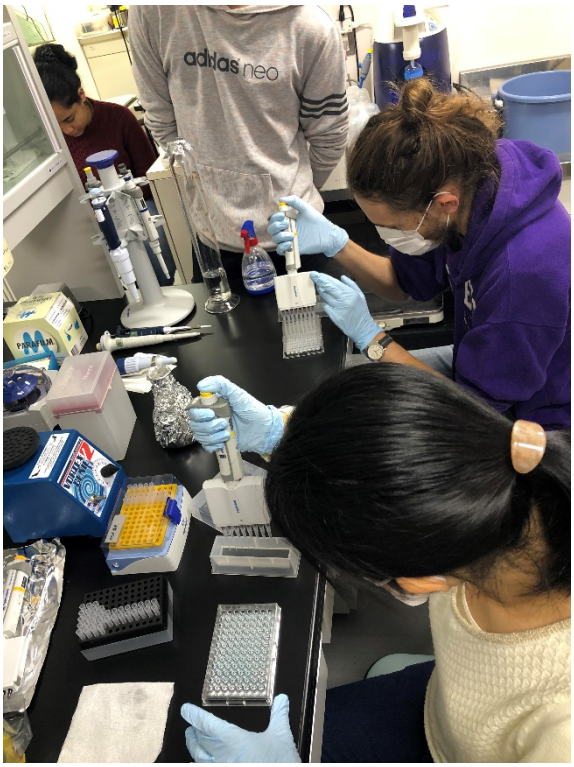


Figure1: Change of mean water content and hormone concentration by time. Error bars represents standard deviation.

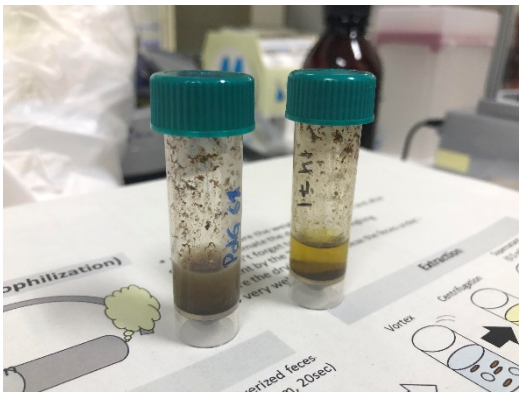
Figure2: Hormone concentration in each age-sex classes. Figures above show the concentration in pregnancy period, and figures below shows concentration in estrus season. Mean values are depicted in box plots.

Submit to : report@wildlife-science.org 2014.05.27 version

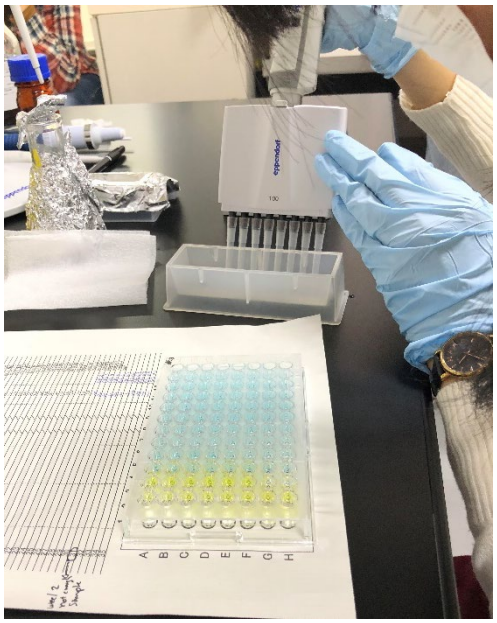
Research Activity Report
Supported by "Leading Graduate Program in Primatology and Wildlife Science"
(Please be sure to submit this report after the trip that supported by PWS.)



Group working on the micro plates to measure Progesterone and its metabolite PdG



Differences in the hormone extraction from fecal samples with methanol 20% for PdG (left) and methanol 80% for P_4 (right)



H_2SO4 is used as a stop solution for enzyme immunoassay changing the color from blue to yellow

6. Others

I would like to thank Prof. Kodzue Kinoshita for conducting the course and my lab colleagues for making this course a very pleasant experience. I am grateful for the financial assistance provided by the Leading Graduate Program in Primatology and Wildlife Science (PWS), Kyoto University. I also thank Professors Hideki Sugiura and Yoshimi Agetsuma-Yanagihara, Takafumi Suzumura and Cathy Lee and all the colleagues of Yakushima Field Science Course for collecting the samples.