Innovation of Non-Invasive Endocrine Assays for Primates in the Wild

野生霊長類の非侵襲的内分泌測定法の開発

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Abstract

The present thesis aimed to develop non-invasive methods to monitor the reproductive status and endocrine changes of female primates in the wild. Since steroid hormones are excreted in both urine and feces, measuring these in excreta is an effective way to monitor endocrine status. However, the metabolic mechanisms can vary among primate species, steroid hormones, and excreta types. Hence, it is necessary to validate whether the profiles measured in feces and urine accurately reflect the reproductive status and endocrine changes in each species. Moreover, the challenge of stably storing samples arises in remote sites where the utilization of electrical equipment for sample storage can be impractical. Thus, I investigated practical methods for monitoring the reproductive status of wild female primates using samples from captive Japanese macaques and chimpanzees, to validate protocols.

First, I developed enzyme immunoassays (EIAs) for estrogen and progesterone metabolites (E_1C, E_3G, and PdG), and adrenal androgen (DHEAS) in Japanese macaques. For fecal sample extraction, aqueous reagents that reduce complex extraction steps were used as extraction reagents instead of volatile organic solvents. The results revealed that fluctuations in E_1C and PdG in extracts using aqueous reagents were consistent with those using volatile organic solvents, indicating that the profiles showed typical ovulatory cycles and pregnancies. Moreover, I found that E_3G levels in excreta just before parturition increased significantly, suggesting the increase of E_3G serves as an indicator of parturition in Japanese macaques. The changes in DHEAS were measured in blood, urine, and feces after administering DHEAS to examine the relationship between blood and excreted DHEAS. The results demonstrated a significant correlation between DHEAS levels in blood and excreta, affirming that measuring DHEAS in excreta is a

valid alternative for blood samples. In addition, the method for the long-term storage of chimpanzee urine samples was developed by drying them on filter papers without freezing. The comparison of E_1C, PdG, and E_3G concentrations in frozen urine and dried urine on filter paper showed a significant correlation between both storage methods, resulting in accurate estimation of the day of ovulation and detection of pregnancy. Chorionic gonadotropin was detected in dried urine, indicating protein hormones can also be preserved on filter papers over extended periods of time.

Finally, I used these methods to examine reproductive status such as puberty, pregnancy, and resumption of the ovulatory cycle post-parturition in long-term study sites of wild primates. I revealed the relationship between face redness and reproductive status in wild Japanese macaques, and long-term fluctuations in E_1C levels before and after the timing of female dispersal and during maximal sexual skin swelling towards the next pregnancy from parturition in wild bonobos. Thus, these methods were key for revealing the relationship between hormones and behavior in wild primates. The developed DHEAS EIA and filter paper preservation methods may contribute to the elucidation of the endocrine status of wild primates during adolescence and post-menopause in the future.

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Acknowledgments

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Chapter 1

General Introduction

1.1 Female Reproductive Hormones and Behavior in Primates

Reproductive hormone secretion in female primates changes throughout life with development and aging. In particular, their secretion fluctuates dramatically over relatively short periods, such as during the ovulatory cycle (Shimizu, 2008; Buse et al. 2008), pregnancy (Baird et al. 1991; Rigaill et al. 2015; Hashimoto et al. 2022), parturition (Shimizu et al. 2003), and perimenopause (Gould et al. 1981; Shideler et al. 2001; Bellino and Wise 2003). Behaviors and physical characteristics also change significantly in response to these fluctuations. Motta-Mena and Puts (2017) reported that hormonal changes directly or indirectly influence reproductive behaviors such as sexual orientation, gender identity, mate preference, mate competition, sex drive, and maternal behavior in humans; a species living in a variety of complex social systems. On the other hand, in non-human primates, the effects of hormonal changes on female reproductive behavior and phenotype can vary across species and individuals, and they are not always constant and direct (Anestis, 2010). Additionally, Stephen and Wallen (2013) revealed that social and environmental factors significantly influence the timing of puberty onset, whereas hormone levels possibly modulate the frequencies of sexual behaviors, but not the timing of puberty onset and pubertal events. Female social rank was related to age at puberty onset in wild savanna baboons (Bercovitch and Strum, 1993), and the season was related to the timing of first ovulation in outdoor-housed female rhesus macaques (Wilson and Gordon 1989). These factors should be elucidated by monitoring hormonal and

behavioral changes adding social and environmental factors in a natural setting. However, such comprehensive studies are still limited because of several challenges in accurately monitoring endocrine changes over time in wild primates.

1.2 Field Endocrinology of Female Primate Reproduction

Generally, assessing physiological status involves measuring blood components. However, obtaining blood samples from wild primates is highly invasive, thus the use of non-invasive sample material is better for monitoring physiological status. Practical techniques for pregnancy detection from human urine and feces were introduced by Adlercreutz and others (Adlercreutz and Martin 1976; Adlercreutz et al. 1977). Subsequently, these endocrinological analyses from excreta have been applied to wild primates by Risler et al. (1987) and Wasser et al. (1991). Excretory analysis as a non-invasive method has recently been utilized not only for assessing reproductive status but also for exploring its connection with behavior in wild primates (Deschner et al. 2003; Rigaill et al. 2015, 2017; Douglas et al. 2016; Corley et al. 2017; Hashimoto et al. 2022; Toda et al. 2022). However, measurements from excreta involve complex methodological issues distinct from those encountered in blood and saliva, which contain the native hormones. Thus, numerous challenges, including the stability of sample materials, time lag for the excretion process, biological validation, and assay validation adapted to each primate species, require resolution in the laboratory (Touma and Palme 2005, Behringer and Deschner 2017).

1.3 Reproductive Hormones in Excreta

Most steroid hormones in the blood are not excreted in their original form. After steroid hormones are biosynthesized from cholesterol in various organs, they circulate and act on target cells, after which they are inactivated in the liver (Schiffer et al. 2019). For example, physiologically active progesterone in blood undergoes irreversible reduction, converting the double bond in A ring to 5-β and the 3-keto group to a 3-α hydroxyl group. Subsequently, it is further reduced to 20-hydroxylated pregnanediol by 20-reductase. Particularly, the 20-α reduction of progesterone terminates its progestogenic activity (Penning et al. 2000). Therefore, many of the steroid hormones found in excreta exist in inactive forms different from their blood. Moreover, inactivated steroid hormones are primarily metabolized into water-soluble metabolites via glucuronidation in the liver (Schiffer et al. 2019). They are then excreted via two routes: with bile in the feces, and in the urine via the kidneys. Conjugates excreted from the kidneys are directly eliminated in urine. However, those throughout the intestine are deconjugated due to the action of intestinal bacteria in the large intestine to free-form steroids again. Some of those are reabsorbed into the portal system, undergoing further metabolism in the liver, and the rest are excreted in the feces in their free form. Shideler et al. (1993a) report that most fecal steroids in macaques are un-conjugate forms and urinary steroids are conjugate forms.

Considerable inter-species and inter-hormonal differences were measured in both quantities of steroid metabolites excreted in feces and urine (Palme et al. 1996). In rhesus macaques, approximately 55% and 42% of exogenously administered carbon 14 labeled estradiol (E_2) and progesterone, respectively, were excreted in the urine (Shideler et al. 1993b). In contrast, in humans, 80% of administrated E_2 is excreted in the urine (van Kammen et al. 1975).

Another important question is the origin of excretory sex steroid metabolites. In humans,

more than 70% of estrogen in premenopausal women and 100% after menopause is synthesized in peripheral tissues that express aromatase, such as adipose tissue, using DHEA/S secreted from the adrenal glands (Labrie et al. 1998; Labrie, 2015; Schiffer et al. 2019). Although excreta is a simple, non-invasive collectable material, reproductive hormone metabolites in the excreta are not a direct reflection of only ovarian function. In bonobos, changes in estrogen levels in feces do not correspond to changes in blood or urine (Heistermann et al. 1996; Jurke et al. 2000). Additionally, the urinary profile of slow loris does not yield useful data for monitoring the ovulatory cycle (Perez et al. 1988). Thus, the process of hormone excretion varies depending on the species, excreta, and hormones. However, what is important is to assess whether those changes adequately represent changes in the reproductive status of a given species.

1.4 Technical Issues of Enzyme Immunoassays for Metabolites

When utilizing excreta, researchers must determine the analytical methods for measuring steroid hormones and establish protocols for sample processing. Emery Thompson et al. (2012) have shown the relationship between sample size and individual variation, indicating that cross-sectional studies require sufficient sample size to obtain accurate data. On the other hand, longitudinal studies also need to collect a sufficient number of samples per individual throughout the study. This issue of analyzing large numbers of samples at one time has been resolved using enzyme immunoassays (EIAs). Furthermore, EIAs are particularly suitable for assaying excreta including conjugated or unconjugated variable forms, because they provide immunoreactive group-specific assays with antibodies. However, while the use of commercial EIA kits is easy to handle and saves time, it is generally difficult to obtain information on the composition of the

reagents or modify experimental conditions to suit the unique requirement of each animal or sample material under investigation. The epitope of the antibodies and the form of immunized and enzyme-linked antigens in kits are largely unknown. Moreover, commercial kits are expensive compared to in-house assay systems. Consequently, in-house assay systems offer both economic and scientific advantages, as they can be custom-made and adapted to specific research needs. For developing assay systems, I tried to adapt to the various changes in primate excreta analysis and to use less harmful materials, such as non-toxic reagents or non-mercury-containing preservatives, in order to protect the environment and the health of the researcher.

1.5 The Collection, Storage, and Transportation of Excreta in Remote Field Sites

For endocrine analysis using excreta, it is necessary to determine and collect the appropriate sample material and process it according to the research purpose, the research target species, the environment, and research site conditions. For example, the collection of feces is more reliable and continuous compared to collecting urine excreted on sandy soil or from animals situated high up in the tree canopy (Ziegler and Crockford, 2017). However, the selection of the appropriate sample material is not solely based on the ease of collection; it should align with the research purpose. For example, as discussed in the previous section, urinary estrogen changes are more distinct than feces for monitoring the ovulatory cycle of bonobos (Heistermann et al. 1996), whereas fecal samples are more informative for monitoring the reproductive cycles of slow lorises, given that they excrete estrogens primarily through feces rather than urine (Perez et al. 1988).

In addition, some research sites have no electricity to freeze samples, calling for simple and reliable methods aimed at long-term storage. Monitoring endocrine changes in wild primates at remote field sites necessitates the development of suitable storage methods, adapted to the specific sample type.

1.6 Purpose of these Studies

In this thesis, my primary objectives were to develop an accurate non-invasive monitoring method for assessing ovarian and adrenal status through endocrine analysis of excreta, and to demonstrate the effectiveness of practical sample processing methods; a fecal extraction method that avoids the use of toxic reagents and a stable urine storage method suitable for remote field sites where frozen storage is difficult or impossible. This was done with the intent to link behavior with physiological events of animals living in the wild.

1.7 Outline

The following is a brief description of each chapter of this thesis.

In Chapter 2, EIAs for estrogen and progesterone metabolites using feces extracted without volatile organic reagents such as ether and alcohol were developed to monitor ovarian function and pregnancy, noninvasively and accurately in Japanese macaques. Further, the developed EIAs were adapted to measure urinary metabolites accurately.

Chapter 3 describes an EIA for the measurement of dehydroepiandrosterone and its sulfate (DHEA/S). I developed a sensitive DHEA/S assay system using a synthesized heterological enzyme conjugate. This study revealed how the circulating DHEAS is excreted in feces and urine of Japanese macaques. Furthermore, I revealed the

administered DHEAS effects on estrogen and progesterone synthesis in Japanese macaques.

In Chapter 4, I validated a long-term storage system using filter papers adsorbed with the urine of chimpanzees that does not require freezing. This chapter also discusses the measurement of steroid hormones using the in-house developed EIA.

Finally, Chapter 5 offers a summary of Chapters 2 through 4. In addition, it presents studies that used these developed methods to monitor the reproductive status of primates in remote field sites and revealed the relationship between endocrine dynamics and behavioral and physical changes.

Chapter 2

Enzyme immunoassays for water-soluble steroid metabolites in the urine and feces of Japanese macaques (*Macaca fuscata*) using a simple elution method

2.1 Abstract

A simple, non-alcoholic extraction method for measuring estrogen and progesterone metabolites in excreta using enzyme immunoassays (EIAs) was developed in Japanese macaques. The obtained detection limits of EIAs using estrone conjugates (E_1C), pregnanediol glucuronide (PdG), and estriol glucuronide (E_3G) polyclonal antibodies with cross-reactivity to urinary and fecal steroid metabolites were 6.6 pg/ml, 2.1 ng/ml, and 0.35 ng/ml, respectively. These assays allowed the determination of E_1C, PdG, and E_3G from the excreta with good reproducibility and accuracy. Thereafter, urine and fecal samples of two menstrual cycles and six pregnancies from eight female Japanese macaques were assayed. A typical increase in urinary and fecal E_1C in follicular phase and PdG in luteal phase were shown during non-conceptive menstrual cycles. Urinary E_3G levels also showed a preovulatory increase; however, fecal E_3G levels were very low throughout the non-conceptive menstrual cycles. Levels of E_1C and PdG in the urine and feces of pregnant females were gradually increased until parturition, while fecal E_3G levels were low and reached detectable levels after the mid-pregnancy period. Although the extraction rate of estrogen and progestogen metabolites by developed method was lower compared to those of the previous extraction method using an alcohol-containing buffer, the method was simple, and the correlation coefficients for the relationship

between two methods were found to be statistically significant. The results presented here are of great practical value for a non-invasive method of monitoring ovarian function and pregnancy in Japanese macaques.

2.2 Introduction

The non-invasive measurement of sex steroids in captive and free-ranging animals provides valuable information for understanding their current reproductive status and is widely applied to zoological research (Lasley and Kirkpatrick 1991; Shimizu, 2005; Hodges and Heistermann 2011; Behringer and Deschner 2017). In the beginning, the method was directed at measuring sex steroids in urine samples. However, in free-ranging species, collecting urine samples is difficult, and fecal samples are easily collected from the ground (Shideler et al. 1993a). Consequently, the measurement of steroid hormones in fecal samples has been developed (Behringer and Deschner 2017).

The measurements of urinary and fecal estrogen and progesterone metabolites as an alternative to blood have been developed to monitor concentrations of estrone conjugates and progesterone metabolites, mainly in the form of pregnanediol-3-glucuronide (PdG), in free-ranging animals (Lasley, 1985). In most mammals, steroids are metabolized by the liver, and afterward excreted via the kidneys into the urine, or via the bile ducts into the gut (Behringer and Deschner 2017). Additionally, metabolic products can be conjugated to various degrees to glucuronides and/or sulfates (Bahr et al. 2000). A previous study reported that in general, circulating bioactive estradiol (E_2) and progesterone (P_4) were metabolized to their conjugated forms in urine, and unconjugated forms in feces (Lasley and Kirkpatrick 1991). Furthermore, Shideler et al. (1993a) administered ^{14}C-steroids to macaques, and clarified that E_2 was metabolized to estrone

conjugates (E_1C) in urine but existed as free E_1 form in feces. Additionally, they clarified that P_4 did not metabolize to authentic pregnanediol conjugates, but to complex conjugated pregnane-like forms in urine and free pregnanediol in feces of macaques (Shideler et al. 1989; 1993a; 1993b). In the case of Japanese macaques, O'Neill et al. (2004) also reported that free E_1 and pregnanediol forms were found in their feces. Thus, because the primary metabolite form is different between urine and feces, using appropriate antibodies is important for the effectiveness of the immunoassay.

Though fecal samples are easy to collect, the extraction process of feces is indispensable for the measurement of steroids by immunoassay. The fecal steroid metabolites of macaques have been measured by immunoassays with various extraction methods, commonly with highly concentrated alcohol or volatile solvents (Risler et al. 1987; O'Neill et al. 2004; Ziegler and Wittwer 2005; Palme et al. 2013; Wallner et al. 2011). Though these solvents allow for a high extraction rate, highly concentrated alcohol or volatile solvents can interfere with the enzymatic and/or immunometric reaction. Therefore, it is necessary to remove the blockage from the extraction. This complicated extraction process in fecal samples requires special equipment and can lead to some errors, which will then bring incorrect results. Consequently, a simpler method of obtaining accurate results from fecal extraction is desirable.

The present study described the establishment of 1) a simple and rapid method for fecal extraction without a large quantity of alcohol or volatile solvent, and 2) EIAs using appropriate polyclonal E_1C, PdG, and E_3G antibodies with cross-reactivity to urinary and fecal conjugated and unconjugated steroid metabolites in Japanese macaques. 3) Then, I measured these steroid metabolites using this method in both the urine and fecal samples of female Japanese macaques.

2.3 Materials and Methods

Experimental subjects

Eight sexually mature female Japanese macaques (*Macaca fuscata*); with body weights ranging from 7.2–11.5 kg (mean = 9.2 kg, SD ± 1.4) were used in this study. All females were housed individually at the Primate Research Institute (PRI), Kyoto University, Inuyama, Japan. They were provisioned commercial monkey chow daily and supplemented with sweet potatoes. Water was available *ad libitum.* They were maintained in a natural lighting-conditioned room at a temperature of approximately 20℃. All females were multiparous, and daily vaginal bleeding was monitored. After confirmation of the onset of menstruation, all females were mated with males between approximately 10–15 days from the beginning of menstruation. The use of laboratory subjects adhered to the Guide for Care and Use of Laboratory Primates (1986) of the Primate Research Institute, Kyoto University.

Sample collection

Urine and fecal samples were collected two times per week during the entire breeding season. Large collection trays were placed beneath the individual cages, and a stainless-steel mesh was used to separate the feces from urine to minimize contamination. Morning urine samples were aspirated from the tray using a disposable syringe. Feces were collected and placed into plastic collection bags. All samples were immediately frozen at -30℃ and stored until the assay.

At the end of the breeding season, six of eight females conceived, and five pregnancies resulted in the birth of healthy offspring. However, one offspring died five days after birth

by negligence. One other pregnant female had a premature stillbirth. The urine and fecal samples of pregnant females were collected over the entire gestation period. The menstrual cycles of the two remaining females who had not conceived were monitored until the end of July.

Sample preparation

Urinary samples. Urinary samples were unprocessed, and diluted with deionized water between 100–5,000, 1–50, 1–20, 50–100, and 1–20 times for the E_1C, PdG, and E_3G assays of pregnant females: and the E_1C, and PdG assays of non-pregnant females, respectively. For the E_3G assay, urinary samples were used without dilution.

Fecal samples. Fecal samples were thawed and dried using a vacuum drier at 50℃ for approximately 12 hr. Dried samples were pulverized, and foreign substances such as rough fiber and seeds from the dried fecal powder were removed. A fecal sample of the powder representing 0.25 g was placed into a 14 ml polypropylene test tube (Stockwell Scientific Inc., Scottsdale, AZ, USA), and 2.5 ml of extraction buffer which contained 0.1 M phosphate buffer (pH 7.0) 0.1% BSA, with 0.05% Tween 20 and no alcohol were added, and then rotated on a test-tube rotator (Labinco, B.V., Breda, Netherlands) for 24 hr at room temperature. Following centrifugation at 4℃, 1,500 × *g* for 10 min, the supernatant was decanted into microtubes and stored at -30℃ until the assays.

Enzyme Immunoassays

Antibodies. Previous studies have described that major estradiol (E_2) metabolites were two types of estrone-monoconjugates (E_1C) in urine and unconjugated estrone (E_1) in feces. Progesterone (P_4) metabolites were various 20α-hydroxy C21 compound-

monoconjugates in urine and unconjugated pregnanediol in feces (Braasch et al. 1988; Shideler et al. 1993b; O'Neill et al. 2004). Moreover, during pregnancy, it has been reported that large quantities of estriol (E_3) are produced from the placenta of humans and great apes (Reyes et al. 1975; Czekala et al. 1983). Therefore, I employed group-specific polyclonal antibodies against estrone-3-glucuronide-BSA, pregnanediol-3-glucuronide-BSA, and estriol-6-carboxymethyloxime-BSA raised in rabbits by Dr. A. Kambegawa.

Plate preparation. Anti-rabbit IgG (H+L) goat serum (Rockland Immunochemicals Inc., Limerick, PA, U.S.A.) was diluted to 15 µg/ml in pH 9.6, 0.05 M carbonate buffer, and 50 µl was added to microtiter plates (Maxisorp flat-bottom, Thermo Fisher Scientific, Waltham, MA, U.S.A) for the E_1C, PdG, and E_3G assays. Then plates were left for one or two nights at room temperature (<24℃). The plates were washed off the unbound anti-rabbit IgG with wash buffer (0.15 M NaCl, 0.05% Tween20), and blocked with blocking buffer (0.05 M borate buffer, pH 7.8, 0.1% BSA, 3% sucrose) overnight. The following day, the blocking buffer was discarded, and the plates were dried, sealed, and stored at 4℃ until the assays.

E_1C EIA. The E_1C polyclonal antibody was diluted with assay buffer (0.05 M borate buffer, pH 7.8, 0.2% BSA) at an appropriate dilution rate (1:8,000,000), and 50 µl of the antibody was added to IgG-coated plates and left overnight at room temperature. The following day, the plate was washed to remove the unbound first antibody, and all emptied wells were filled with 50 µl of assay buffer immediately. Afterward, serially diluted 50 µl of standards (range 0.001–10 ng/ml), internal controls, and unknown samples were added to each well. Correspondingly, 50 µl of estrone 3-carboxymethyl ether (CME)-HRP diluted with assay buffer (1:300,000–700,000) was added to each well.

The plate was sealed and incubated overnight at room temperature. The following day, the plate was washed, and 100 µl of substrate solution (0.2 M citrate buffer, pH 4.5, 0.05% *o*-phenylenediamine, 0.025% hydrogen peroxide solution) was added to each well. The plate was incubated on the reciprocating shaker for approximately 30 min at room temperature. After the plate color was developed, 50 µl of acid was added to each well to stop the enzyme reaction, and absorbance was measured at 492 nm on an automatic plate reader (Sunrise Rainbow; TECAN, Zurich, Switzerland). The concentrations of each sample were calculated by fitting the absorbance for a standard curve using a four-parameter logistic model (LS-PLATE Manager 2004; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

PdG and E_3G EIAs. The PdG and E_3G antibodies were diluted at 1:200,000 and 1:55,000 with assay buffer, respectively. Other processes were the same as those used for E_1C EIA. Pregnanediol-3-glucuronide-HRP for PdG EIA (1:300,000–500,000), and estriol-3-carboxymethyl ether (CME)-HRP for E_3G EIA (1:50,000) were used. Serially diluted standard ranges were 1–1,000 ng/ml for PdG and 0.03–300 ng/ml for E_3G EIA.

Urinary steroid metabolites were compensated creatinine concentrations measured using the Jaffe reaction (Taussky, 1954).

Assay validation

The cross-reactivity of steroids and steroid metabolites structurally related to estrone, pregnanediol, and estriol were assessed with antibodies against estrone-3-glucuronide-BSA, pregnanediol-3-glucuronide-BSA, and estriol-6-carboxymethyloxime-BSA by EIA (Abraham, 1956). The sensitivity of the assays was estimated as the minimum detectable dose by mean - 2 SD of the optical densities from the zero concentration of the standard.

Linearity was assessed in the recovery rates by serially diluted urinary and fecal extractions from three randomly selected female Japanese macaques. Accuracy was examined by determining the recovery of a known amount of steroid added to three randomly selected urinary and fecal samples before their extraction. The precision of these EIAs was assessed in high and low controls of each hormone. The intra- and inter-assay coefficients of variation (CV) were calculated by urinary and fecal sample assays from eight females, and the high and low control values.

Comparison of two extraction methods

To evaluate the extraction method, hormonal profiles were compared between my extraction and the previous extraction method using 50% ethanol and diethyl ether (Strier and Ziegler 1997; O'Neill et al. 2004) with some modifications. In brief, the dried fecal sample was divided into two portions, and the first 0.25 g of dried feces was extracted by my simple method (previously described). The remaining portion was extracted with 5 ml of 50% ethanol and the aliquot of 500 µl of supernatant was extracted with 5 ml of diethyl ether. Thereafter, the ether layer was decanted and reconstituted in the assay buffer.

2.4 Results

Assay validation

The specificity of the polyclonal antibodies against estrone-3-glucuronide-BSA and pregnanediol-3-glucuronide-BSA were tested. Cross-reactions of various steroids and their related metabolites with the E_1C, PdG, and E_3G antibodies are summarized in Table 2.1. Validations of the three EIAs were summarized in Table 2.2. The sensitivities of the

EIAs for three steroids were 6.6 pg/ml, 2.1 ng/ml, and 0.35 ng/ml for E_1C, PdG, E_3G, respectively. Serial dilutions of urinary and fecal extractions from three randomly selected individuals produced displacement curves parallel to those of the standard curves in the EIAs (Fig. 2.1). The endogenous E_3 levels of fecal extractions in female macaques were very low. Therefore, the displacement curves from fecal E_3 extractions were parallel to those of the low dose-response standard curves (Fig. 2.1). The recoveries for the accuracy of each steroid from the urine or feces were assessed by determining the recovery of known amounts of the added hormone. Average recoveries are listed in Table 2.2. Mean ± SD recovery values ranged from 94.1 ± 12.6% for urinary E_1C and 108.0 ± 7.5% for fecal E_1, 113.4 ± 23.8% for urinary PdG and 119.1 ± 25.4% for fecal pregnanediol, and 90.7 ± 11.7% for urinary E_3G and 103.5 ± 18.0% for fecal E_3. The intra-assay coefficients of variation for E_1C, PdG, and E_3G were 5.0%, 5.4%, and 6.2%, respectively. The inter-assay coefficients of variation for E_1C, PdG, and E_3G, with internal high and low controls, were 14.0% and 9.0% for E_1C, 18.7% and 13.5% for PdG, and 18.2% and 13.6% for E_3G, respectively.

Evaluation of the simple extraction method

My method was compared with the previous extraction method using 50% ethanol and diethyl ether, and the results are depicted in Fig. 2.2. Fecal E_1C and PdG levels could be measured using my simple extraction method within the detectable range. On the other hand, E_3G levels in the fecal extract of the non-conceptive cycle and early pregnancy period were very low and almost undetectable in both extraction methods. Although the extraction rate of my method was 10–12% of the previous extraction method, the results of the assays showed a similar pattern in the E_1C, PdG, and E_3G profiles. The Spearman's

rank correlation coefficients ρ (r) for comparison of the two methods were 0.412 (df = 43, $p < 0.01$) for E_1C, 0.632 (df = 43, $p < 0.001$) for PdG and 0.538 (df = 16, $p < 0.05$) for E_3G from the fecal extraction of female Japanese macaques. The correlation coefficients for the relationship between the two methods were found to be statistically significant.

Hormonal profiles in urine and fecal samples

The representative profiles of urinary and fecal E_1C, PdG, and E_3G during a non-conceptive menstrual cycle of two females are illustrated in Fig. 2.3. Urinary E_1C levels transitorily increased during the follicular phase, from baseline levels of 11.16–30.15 ng/mg creatinine (Cr) to preovulatory peak values of 75.93–184.87 ng/mg Cr. Then, urinary E_1C levels decreased to the nadir. Urinary PdG levels were low during the follicular phase (0.10–0.25 μg/mg Cr) and increased after the E_1C peak to reach their maximum levels of 0.71–1.40 μg/mg Cr during the mid-luteal phase. Thereafter, PdG levels decreased to their baseline levels. Urinary E_3G levels gradually increased during the follicular phase from their baseline levels of 2.46–11.01 ng/mg Cr to their preovulatory peak values of 13.33–31.38 ng/mg Cr. Thereafter, urinary E_3G levels decreased to their baseline levels.

Fecal E_1C levels also increased 1.3 to 3-fold from their follicular phase levels of 1.92–3.60 ng/g to a preovulatory peak of 4.82–9.36 μg/g on approximately day 10, and thereafter gradually declined. Fecal PdG profiles showed a well-defined pattern throughout the menstrual cycle, with low levels of 1.02–3.02 μg/g during the follicular phase and increased with a maximum of 5.43–12.69 μg/g in the luteal phase. Thus, the profiles of E_1C and PdG showed similar fluctuating patterns between the urinary and fecal

samples. On the other hand, fecal E_3G levels were observed at low and barely detectable levels during the menstrual cycle.

Profiles of E_1C, PdG, and E_3G levels in the urine and feces of one pregnant female during the entire gestation period are shown in Fig. 2.4 (left). Urinary and fecal E_1C levels during the conceptive cycle gradually increased as gestation progressed until parturition. Urinary and fecal E_3G levels were low during the early pregnancy period, particularly fecal E_3G levels, which were barely detectable. Afterward, both urinary and fecal E_3G levels were increased during the late pregnancy until parturition. In comparison to E_1C and E_3G, urinary and fecal PdG showed a peak at early pregnancy, and then maintained a modest increase until parturition. Levels of all hormones decreased abruptly to the nadir within a few days of parturition. Fig. 2.4 (right) illustrates the average urinary and fecal E_1C, PdG, and E_3G levels during the early, mid, and late pregnancy in the six females. While urinary and fecal E_1C and E_3G increased as gestation progressed, PdG levels in urine and feces were maintained at nearly constant levels during gestation. The mean ± SD levels of urinary E_1C during the early (E), mid (M), and late (L) pregnancy were 172.00 ± 72.36 (E), 326.34 ± 175.67 (M), and 611.28 ± 276.11 ng/mg Cr (L), and fecal E_1C levels were 257.09 ± 112.05 (E), 359.33 ± 180.99 (M), and 518.85 ± 184.83 ng/g (L), (n = 6), respectively. The mean urinary E_3G levels were 15.27 ± 4.73 (E), 29.54 ± 17.82 (M), and 49.44 ± 33.85 ng/mg Cr (L), and fecal E_3G levels were 7.72 ± 3.01 (E), 19.82 ± 16.23 (M), and 38.48 ± 30.53 ng/g (L), (n = 6). The mean urinary PdG levels were 1.68 ± 0.68 (E), 1.32 ± 0.31 (M), and 1.73 ± 0.35μg/mg Cr (L), and fecal PdG levels were 3.08 ± 1.45 (E), 2.11 ± 0.51 (M), and 2.78 ± 1.04 ng/g (L), (n = 6), respectively.

2.5 Discussion

The present study described a simple and practical extraction method for measuring fecal steroid metabolites by EIAs in female Japanese macaques. As mentioned above (see Introduction), the concentrations of fecal steroid metabolites in non-human primates have been measured by immunoassays following their extraction, commonly with highly concentrated alcohol and volatile solvents (Risler et al. 1987; Heistermann et al. 1993; O'Neill et al. 2004; Ziegler and Wittwer 2005; Wallner et al. 2011; Palme et al. 2013). However, there is a risk that highly concentrated alcohol, detergent, or volatile solvents could interfere with the enzymatic and/or immunometric reaction. Therefore, Heistermann et al. diluted the methanol extractant to avoid the risk before RIA (Heistermann et al. 1993). However, the complicated extraction process of fecal samples takes labor and sometimes can lead to errors. To shorten the amount of labor involved by providing an easy procedure would decrease artificial error and improve the working efficacy. My extraction method, using an aqueous buffer to measure fecal hormone metabolite, reduced the number of steps for extraction and gave us accurate results.

Studies on Old World monkeys have focused exclusively on measuring steroids in the unconjugated form in excreta (Bamberg et al.1991; Wasser et al. 1991). Profiles of fecal extractions in the previous HPLC study suggest that there are at least 8–10 fecal P_4 metabolites, and many of them contribute to the immunoreactive profile (Shideler et al. 1993a). The previous study also suggested that assessing the C21 PdG-like metabolites provides useful information when applied to solubilized macaque feces (Shideler et al. 1993a). Because the steroid metabolite form in excreta is not only single and different between urine and feces, using the appropriate antibody is important for the efficiency of the immunoassay. It is known that major E_2 metabolites are two types of estrone monoconjugates (E_1C) in urine and unconjugated E_1 in feces. Progesterone (P_4)

metabolites were various 20α-hydroxy C21 compound-monoconjugates in urine and unconjugated pregnanediol in feces (Braasch et al. 1988; O'Neill et al. 2004; Shideler et al. 1993b). Therefore, I employed group-specific polyclonal antibodies against estrone-3-glucuronide-BSA, pregnanediol-3-glucuronide-BSA, and estriol-6-carboxymethyloxime-BSA for the EIAs raised by Dr. A. Kambegawa.

In the present study, the results of sensitivity, linearity, accuracy, and precision were quite satisfactory in the E_1C, PdG, and E_3G EIAs. The profiles of E_1C and PdG levels in the urine and feces showed a similar trend. The important difference between the urinary and fecal profiles was the large amount of P_4 metabolites in the feces. The previous study described that the fecal profiles were superior in their correspondence to serum P_4 when compared to that in urinary PdG, presumably because the fecal compartment contained the majority of hormone metabolites, and consequently, the fecal measurements were more accurate (Shideler et al. 1993b).

Because of my low sampling frequency, the lag times between hormone levels in urine and feces could not be detected; however, fecal peaks appeared slightly later than urinary peaks. Shideler et al. (1993a) performed an experiment to characterize the time course and metabolic rate of circulating E_2 and P_4 in their *in vivo* study of ^{14}C labeled steroids. The observed lag times between hormone levels in feces and blood of macaques were 1.0–2.3 days behind in E_2 and 1.3–2.0 days behind in P_4 (Shideler et al. 1993b). Likewise, in their results, lag times between testosterone levels in feces and blood of Japanese macaques showed, approximately, a 48 hr delay (Barrett et al. 2002). Wasser et al. also reported similar results in baboons (Wasser et al. 1993). Similar to macaques, urinary PdG and E_1C levels lagged behind that of serum by an average of one day in women (O'Connor et al. 2003).

Pregnanediol-3-glucuronide is the primary progesterone metabolite excreted by humans and accounts for approximately 30% of P_4 in blood (Czekala et al.1988). In comparison to humans, macaques excrete very low levels of pregnanediol. The major P_4 metabolite in macaques is a compound immunoreactivity similar to 20α-OH-progesterone, which is believed to reflect the same ovarian activity as that of pregnanediol in other anthropoids (Czekala et al.1988).

Furthermore, the urinary and fecal E_3G levels were low and barely detectable in the non-conceived cycle and early pregnancy period of Japanese macaques. It is not elucidated in the present study, but this may be because these hormones are released from the fetal zone and are of adrenal origin. Unlike most mammals, primates possess a fetal adrenal composed of a definitive zone and a fetal zone. A previous study reported that the fetal zone produces the precursor of E_3, and the placenta then converts this precursor to E_3. Estriol was the major estrogen during the last month of gestation because the formation of E_3 in anthropoid primates is largely dependent upon 16α-hydroxylase activities on fetal dehydroepiandrosterone before aromatization (Faiman et al.1981). In this study, E_3 levels increased during the late pregnancy period until parturition. Although further study is needed to assess this in more detail, the measurement of E_3 levels in urine or feces can be considered as a reliable pregnancy diagnosis method in Japanese macaques.

In conclusion, the present study demonstrates a new extraction method using an aqueous buffer for measuring fecal E_1C, PdG, and E_3G in female Japanese macaques. The method presented here is simple and practical and has reliable applications to detect the reproductive status of Japanese macaques, and probably that of other non-human primates.

Table 2.1 Cross reactivity (%) of relevant steroids in the three EIAs

Steroid	%	Steroid	%	Steroid	%
Estrone-3-glucuronide	100.0	Pregnanediol-3-glucuronide	100.0	Estriol-3-glucuronide	100.0
Estrone	32.4	Pregnanediol	100.0	Estriol[a)]	100.0
Estrone-3-sulfate[a)]	15.0	20α-OH-Progesterone	74.7	Estrone[a)]	5.5
Estradiol[a)]	0.7	Progesterone[a)]	2.3	Pregnenolone[a)]	2.5
Estradiol-3-glucuronide[a)]	0.6	5β-Pregnane-3-α,20β-diol[a)]	0.8	Testosterone[a)]	1.2
Cortisol[a)]	0.1	5β-Pregnane-3-β,20α-diol[a)]	0.2	Estrone-3-sulfate[a)]	0.7
		5β-Pregnane-3-β-ol-20-one[a)]	0.1		
		Pregnenolone[a)]	0.05		

a) Data were provided by Dr. A. Kambegawa.

Table 2.2 Validation of three steroid EIAs

			E_1C	PdG	E_3G
Sensitivity[a)]			6.6 pg/ml	2.1 ng/ml	0.35 ng/ml
Linearity Mean ± SD (%) (Dilution factor)		Urine	89.0 ± 7.4 (1:20–320)	112.8 ± 15.5 (1:2–32)	86.6 ± 6.1 (1:6–50)
		Feces	107.9 ± 14.5 (1:1–32)	95.5 ± 11.3 (1:1–8)	94.4 ± 10.0 (1:1–60)
Accuracy Mean ± SD (%) y = ax + b (r^2)[b)]		Urine	94.1 ± 12.6 y = 0.8276x + 0.0166 (r^2 = 0.97)	113.4 ± 23.8 y = 0.9537x + 6.6217 (r^2 = 0.93)	90.7 ± 11.7 y = 0.9564x - 0.2656 (r^2 = 0.98)
		Feces	108.0 ± 7.5 y = 0.9739x + 0.0084 (r^2 = 1.00)	119.1 ± 25.4 y = 1.1837x + 0.7159 (r^2 = 0.90)	103.5 ± 18.0 y = 1.187x - 0.4577 (r^2 = 0.98)
Precision	Intra-assay CV %		5.0% (n = 712)	5.4% (n = 723)	6.2% (n = 452)
	Inter-assay CV %	Low control	14.0%	18.7%	18.2%
		High control	9.0%	13.5%	13.6%

a) determined from the mean-2SD of the absorbance (0 concentration).

b) observed amount = slope × added amount + intercept (r^2)

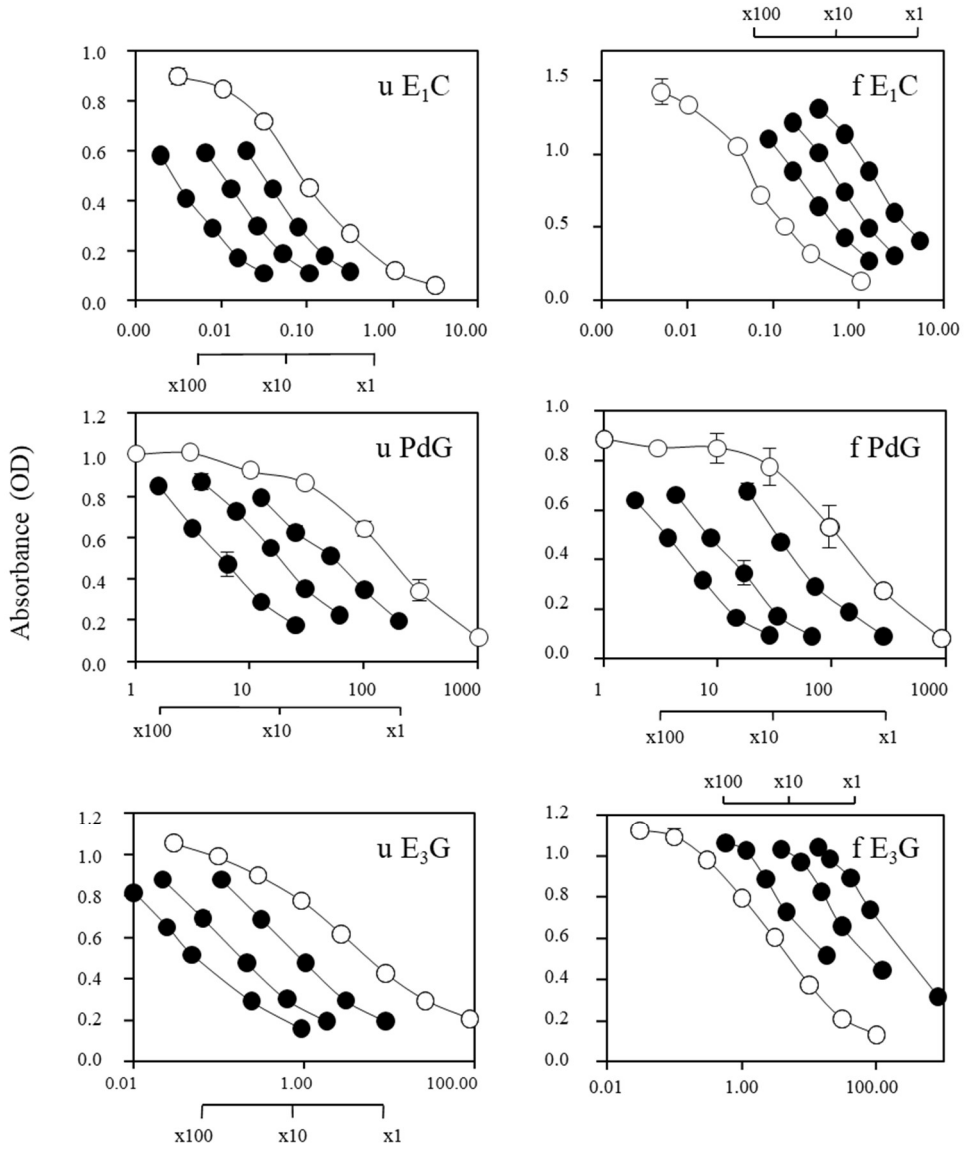


Fig. 2.1 Linearity of the three steroid metabolites

Standard curves and dose-response curves of serially diluted urine and feces. Each figure shows urinary E_1C, fecal E_1C, urinary PdG, fecal PdG, urinary E_3G, and fecal E_3G. Closed circles (●) showed the displacement curves of serially diluted urine or fecal extracts from three randomly selected female Japanese macaques. They were generated parallel to the dose-response standard curves, which depicted open circles (○). Absorbance is depicted as means ± SD.

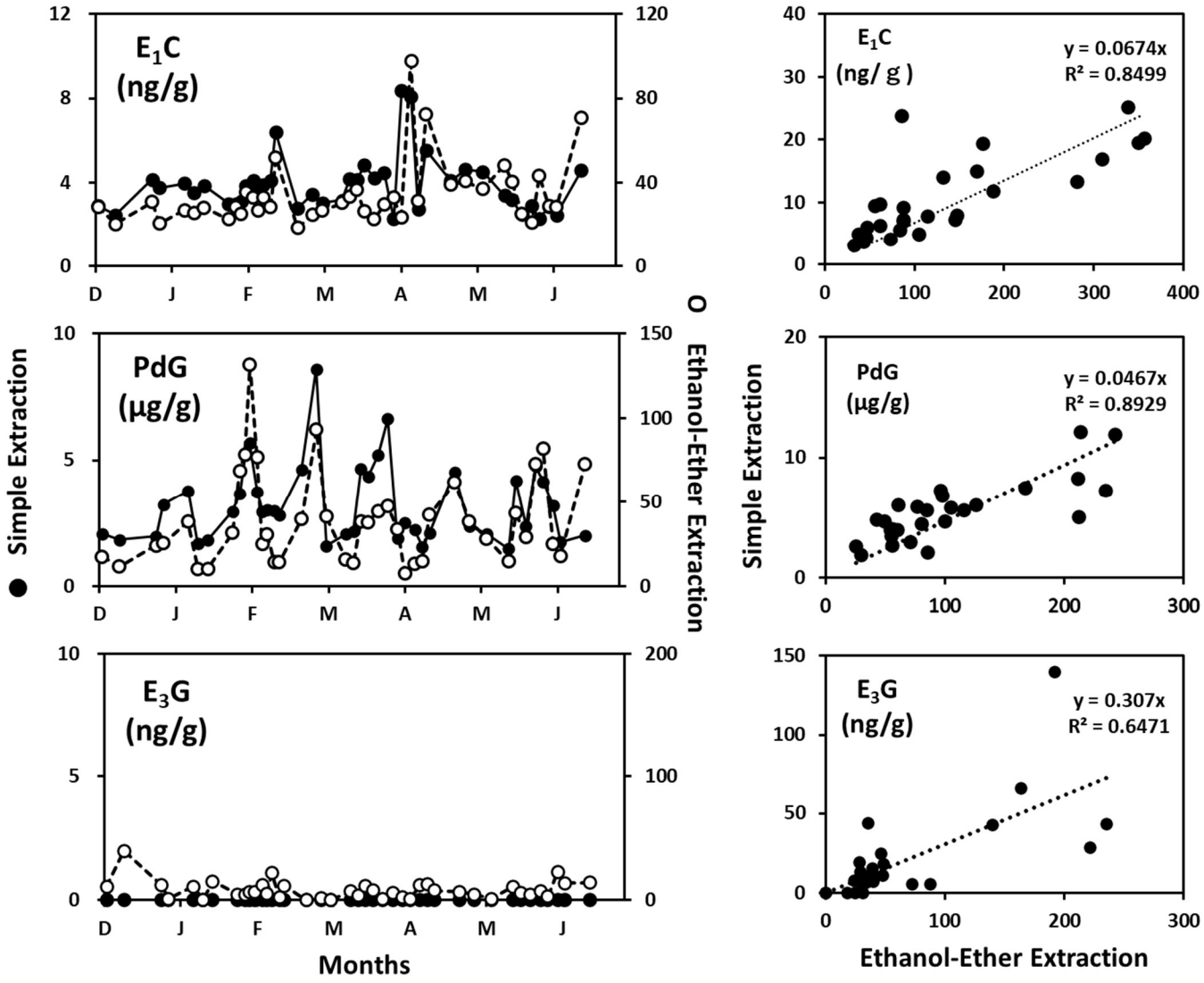


Fig. 2.2

Hormonal profiles of fecal E_1C (top), PdG (middle), and E_3G (bottom) during the non-conceptive menstrual cycle using the simple extraction method (●) and ethanol-ether extraction method (○) in a non-pregnant female Japanese macaque (left). The relationship between fecal E_1C (top), PdG (middle), and E_3G (bottom) levels using the simple extraction method and ethanol-ether extraction method in the samples of 6 pregnant Japanese macaques (right).

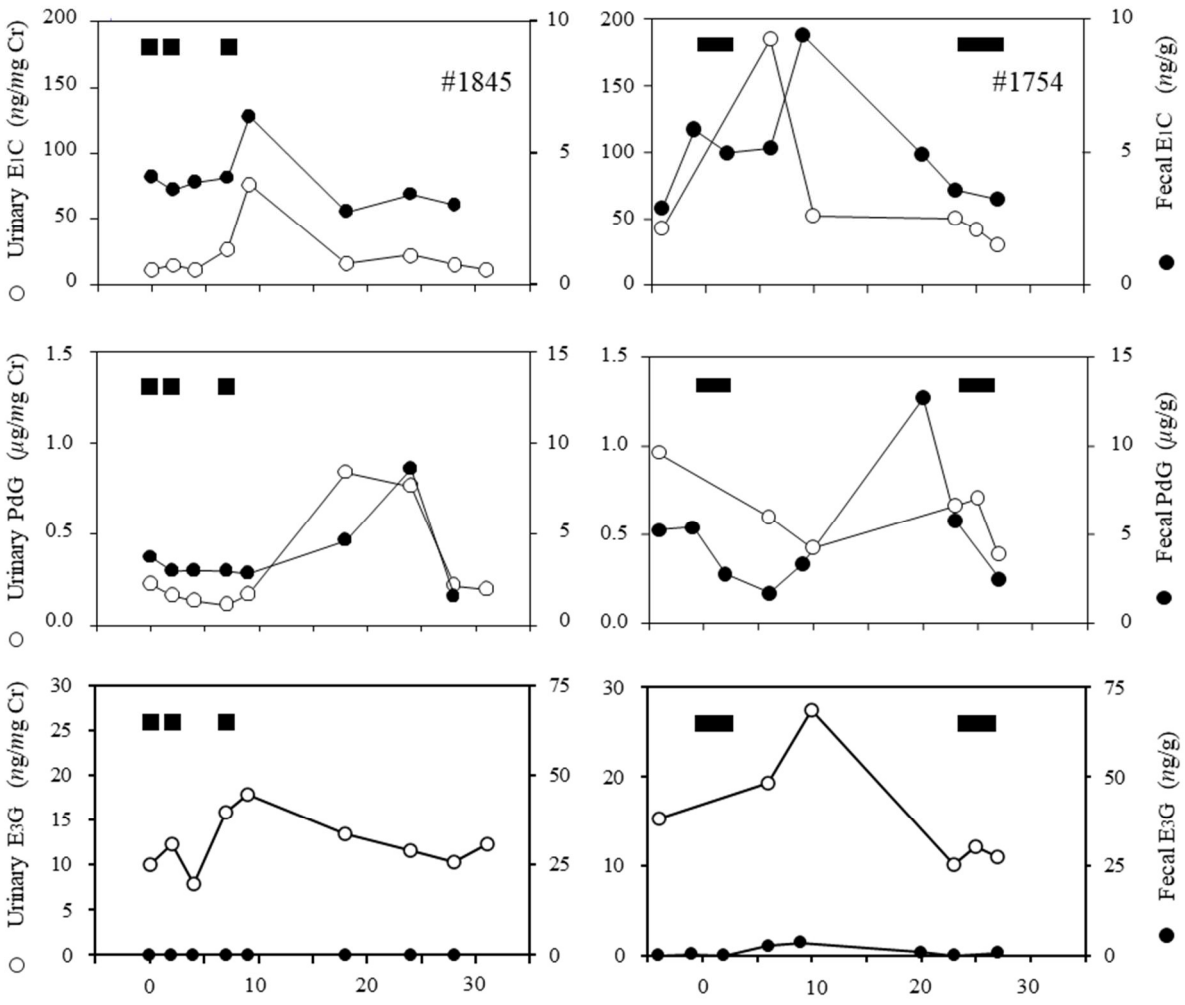


Fig. 2.3

Hormonal profiles of urinary (○) and fecal (●) E_1C (top), PdG (middle), and E_3G (bottom) during the non-conceptive menstrual cycles of two female Japanese macaques. The X-axis shows days from the first day of menstrual bleeding. Horizontal black bars at the upper part of each figure indicate menstrual bleeding.

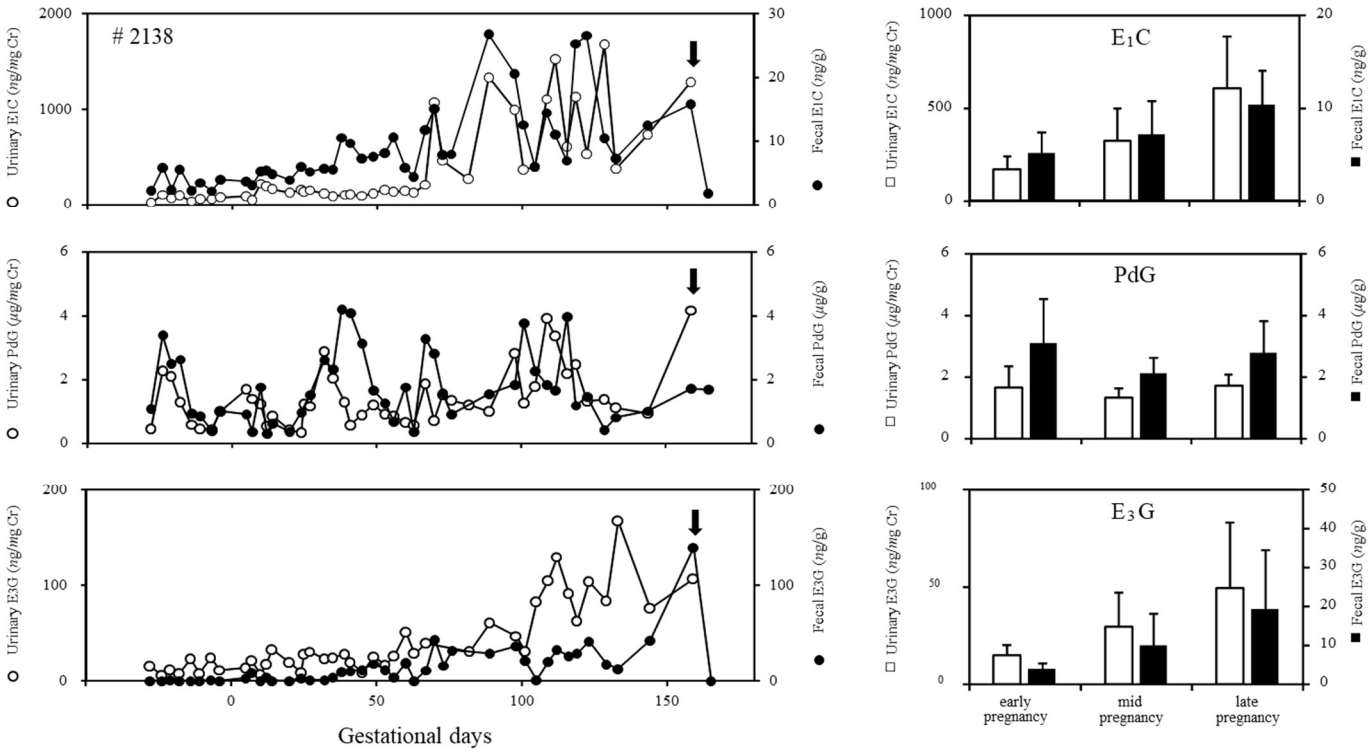


Fig. 2.4

Hormonal profiles of urinary (○) and fecal (●) E_1C (top), PdG (middle), and E_3G (bottom) during the full-term pregnancy of an individual female Japanese macaque. Arrows indicate the day of parturition. Day 0 indicates the estimated conception day (left). Mean (± SD) levels of urinary (□) and fecal (■) E_1C (top), PdG (middle), and E_3G (bottom) during the early, mid, and late pregnancy periods of six female Japanese macaques (right).

Chapter 3

Dehydroepiandrosterone sulfate (DHEAS) in excreta is a good indicator of serum DHEAS in Japanese macaques (*Macaca fuscata*)

3.1 Abstract

A microplate enzyme immunoassay (EIA) to measure dehydroepiandrosterone sulfate (DHEAS) in the blood, urine, and feces of Japanese macaques was developed and the relationship between serum DHEAS and excreta DHEAS was evaluated. My DHEAS EIA using heterological DHEA derivatives conjugated with enzyme was highly sensitive, and linearities and recoveries for all matrices of Japanese macaques were reliable. For the biological evaluation of the metabolism of DHEAS in Japanese macaques, dissolved DHEAS was injected into the subjects, and consecutively collected serum, urine, and fecal samples were analyzed. The peaks of serum DHEAS were observed 6 hours after the administration, while those of urine and feces were observed after 24 hours. The fluctuation of those in urine and feces was significantly correlated with serum DHEAS levels. In addition, I measured pregnanediol-glucuronide (PdG), and estrone-conjugate (E_1C) in urine and fecal samples to investigate the effects of administrated DHEAS on these progesterone and estrogen metabolites. The peak of PdG was observed 24 hours after administration, then declined sharply. The concentration of E1C increased 1 week after administration in two out of three subjects. My results suggest that measuring urinary and fecal DHEAS with my EIA provides a non-invasive alternative to assessing DHEAS levels in the serum of Japanese macaques.

3.2 Introduction

Dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) in primates are steroid precursors secreted mainly from the adrenal glands and converted to sex steroids in other organs and peripheral tissues. Most of their circulating DHEA/S (which is defined as both DHEA and DHEAS collectively) is in the sulfate form (Cutler et al. 1978; Thomas et al. 1994), indicating that DHEAS measurements are reliable for assessing adrenal function (Smail et al. 1982; Muehlenbein et al. 2003; Nasrallah and Arafah 2003). In humans, DHEAS levels increase with the expression of the zona reticularis in adrenal glands in the pre-pubertal period (Smith et al. 1975; Hopper and Yen 1975; De Peretti and Forest 1976, 1978). This increase is called adrenarche, and the human-like patterns of increasing over several decades are also seen in bonobos and chimpanzees (Behringer et al. 2012; Sabbi et al. 2020). Recent findings have shown adrenarche to be associated with brain development, which illustrates the importance of studying DHEA/S in great apes and other primates (Campbell, 2020). In humans, DHEA/S concentrations decline extremely after 20-30 years of age, and the physical effects of decreasing DHEA/S in old age have been investigated (Migeon et al. 1957; Labrie et al. 2017). For example, all estrogen in postmenopausal women is made from DHEA/S in peripheral tissues, and DHEA/S supplementation has been shown to increase circulating estrogen (Labrie et al. 1998). The role of DHEA/S in human aging has attracted attention as a direct intracrine converter to replace deficient sex hormones (Labrie et al. 1991, 2015, 2017; Pomari et al. 2015). Therefore, studies of non-human primates as human models are gaining importance; however, they are still limited (Blevins et al. 2013; Edes, 2017).

Most previous studies on DHEA/S and the adrenal glands of macaques have been conducted by histochemical analysis or longitudinal frequent circulating DHEA/S

analysis (Ducsay et al. 1991; Nguyen et al. 2008; Pattison et al. 2009; Conley et al. 2011). Conley et al. (2011) pointed out the importance of longitudinal and frequent sample collection to elucidate the adrenarche period, because adrenal change may appear in a narrow developmental window in infants. However, the frequent collection of circulating DHEAS may cause stress, and samplings from infants raise a large concern particularly. Maninger et al. (2010) showed that DHEAS concentrations in rhesus monkeys are elevated by acute and chronic stress from chair restraint. This is likely because DHEA/S also has a similar secretory mechanism in response to corticotropin-releasing hormones such as found with cortisol (Goncharova and Lapin 2002). In addition, studies of DHEA/S therapy in macaques using 24-hour remote blood collection systems, have shown diurnal variation in circulating DHEAS (Urbanski, 2011; Sorwell et al. 2012; Urbanski et al. 2014). On the contrary, the measurement of DHEAS in non-invasive excrete samples allows frequent, non-stressful sampling and moderates the effects of diurnal fluctuations. However, although it is known that DHEAS is largely excreted in an unmodified form in human urine (Schiffer et al. 2019), it has not been elucidated how blood DHEA/S is excreted in the urine and feces of Japanese macaques. Importantly, we would be able to use excreta alternatives to blood samples if the relative patterns from excreta DHEA/S were synchronized to those of blood DHEA/S (Lasley and Kirkpatrick 1991).

Therefore, in the present study, I first developed and validated an enzyme immunoassay (EIA) for DHEAS in the blood, urine, and feces of Japanese macaques. After DHEAS was administered to the study subjects, I then evaluated the relationship between the variability of DHEAS in each matrix over time. In addition, estrone conjugate (E_1C) and pregnanediol glucuronide (PdG) concentrations in excreta were measured to determine the effects of administered DHEAS.

3.3 Materials and Methods

Animals

Three adult female Japanese macaques (*Macaca fuscata*) were studied (age: 14, 15, and 15 years old, weight: 8.7, 8.4, and 7.7 kg, respectively). The experiment was conducted outside of their breeding season. Animals were housed individually in indoor cages at the Primate Research Institute, Kyoto University, Japan. They were provisioned monkey chow daily, supplemented with fruits and sweet potatoes, and had free access to water. The use of all subjects adhered to *The Guide for the Care and Use of Laboratory Primates of the Primate Research Institute, Kyoto University*.

DHEAS administration experiments and sample collections

Three Japanese macaques were injected subcutaneously with 100 mg/kg of dehydroepiandrosterone 3-sulfate sodium salt dehydrate (DHEAS) (Sigma-Aldrich St. Louis MO USA) dissolved in 30% ethanol at 9:30 am. After the administration, their health conditions were carefully monitored. Two to 3 mL of blood samples were cooperatively collected from the cubital vein. One blood sample was collected 10 days prior to DHEAS administration, and 6 samples were collected 6 hr and 1, 2, 4, 5, and 7 days post-injection. Samples were coagulated at room temperature, then the supernatants were stored at -30℃. Urine and fecal samples were collected at 9:30 am from mesh-covered pans placed below each respective cage on the previous evening. All feces excreted on the mesh net were collected and the pooled urine was mixed and the supernatant was aspirated. The mesh net prevented contamination from feces and monkey chow. Two urine and fecal samples were collected 10 days and 1 day before DHEAS

administration and 6 urine and fecal samples were collected 1, 2, 3, 4, 5, and 7 days post-injection, then stored at -30℃. In preliminary experiments, pools of urine and fecal samples excreted at night (5 p.m. to 10 a.m.) were not observed to be affected by diurnal variation or storage at room temperature (Supplementary file 3.1).

Sample preparation

I investigated DHEAS concentrations as an indicator of adrenal functions in this study. DHEAS is a water-soluble conjugate and is not extracted into the organic ether layer (Behringer et al. 2012). Therefore, I chose the extraction method of each matrix so that DHEAS is extracted effectively. Serum samples were separated using ethanol to coagulate inhibitors of enzyme assays (the results of other preliminary extraction methods were shown in Supplementary file 3.2). Urine samples were directly used for assays without extraction. Fecal DHEAS was extracted by a simple method validated for water-soluble steroid conjugates (Shimizu and Mouri 2018).

Serum: One hundred μL of serum sample was suspended in 700 μL of 100% ethanol and mixed. The solution stayed for 30 min at 4℃, then centrifuged and the residues were precipitated. Six hundred μL of supernatants were transferred to a microtube and dried in a water bath. The residues were reconstituted in 1 mL of assay buffer and measured.

Urine: Ten μL of urine samples were diluted in the appropriate amount of deionized water and measured directly. The results were compensated by creatinine concentrations.

Feces: Fecal samples were extracted in the same manner as described in Shimizu and Mouri (2018). Briefly, fecal samples were dried in an oven. The dried fecal powder was weighed (0.25 g) and extracted with 2.5 ml of 0.1 M phosphate buffer (pH 7.0, 0.1% BSA, 0.05% Tween 20) into a 14 ml polypropylene test tube rotating on a test tube rotator for

24 hr at room temperature. Following centrifugation, the supernatants were diluted with assay buffer and assayed. The concentrations were expressed per dry fecal gram. Using the above extraction methods, serum extract and urine will contain both DHEA/S. However, the fecal extract using the simple water-soluble extraction method may contain less DHEA.

DHEA/S EIA

DHEA/S in all matrices was determined by EIA using heterological DHEA derivatives conjugated with horse-radish peroxide (HRP). The assay uses a polyclonal antibody against dehydroepiandrosterone-3-succinate-BSA raised in a rabbit (FKA110E) by Kambegawa Laboratory, Tokyo, Japan. The cross-reactivities of steroids were DHEA 320%, 4-Androntene-3,17-dione 5.4%, 5-Androntene-3β,17β-diol 4.1%, dihydrotestosterone 7.1%, testosterone 1.6%, pregnenolone, progesterone, estradiol, and cortisol <0.1%, when the DHEAS was set as standard. DHEA-3-glutarate-HRP was synthesized and used as a heterological sensitive conjugate. (The details of synthesis and the results of pretests are in Supplementary file 3.3. Table S 3.1) Plate preparation, reagents, and buffers followed the protocols for E_1C and PdG EIA developed by Shimizu and Mouri (2018). For partial modifications, anti-DHEA-3-succinate BSA rabbit IgG was diluted 1:100,000 with assay buffer and incubated with plates coated by goat anti-rabbit IgG antibody overnight at room temperature. The plate was washed, and DHEAS (Sigma-Aldrich St. Louis, MO, USA) standards (0.03–300 ng/mL), controls, and appropriately diluted samples were added to each well. Correspondingly, DHEA-3-HRP conjugates were added and incubated overnight. The optical density developed by the substrate solution was read. The concentrations of each sample were calculated by fitting the

absorbance for a standard curve using a four-parameter logistic model. The sensitivity of the assay, defined as the minimum detectable dose by mean −2 SD of the optical densities from the zero concentration of the standard, was 0.03 ng/mL (1.5 pg/well) and the hormone concentration at 50% binding was 7.2 ng/mL Intra-assay CVs for low and high controls were 17.1% and 5.5%, and inter-assay CVs were 17.5% and 5.8%, respectively. Serum extracts, urine, and fecal extracts were diluted to 1:10–200, 1:20–50,000, and 1:1–200, respectively. Urine samples were compensated for creatinine concentrations, which were measured by the method of the Jaffe reaction (Taussky, 1954).

Assay validations for DHEAS EIA in each matrix of Japanese macaques

Serum extracts, urine, and fecal extracts were serially diluted to demonstrate parallelism. The slopes of optical density curves against log concentrations of serum extracts, urine, and fecal extracts generated by serial dilutions were not significantly different from the slope of the standards (ANOVA, range of P-value was from 0.141 to 0.554, P>0.05) (Fig. 3.1, data was shown in Table S3.2). For recovery, the known amount of spiked DHEAS dose (1.5, 5, 15, and 50 ng/mL) response tests generated a regression line with a slope of 1.14 (r2 = 0.98) for serum, a slope of 1.01 (r2 = 0.99) for urine, and a slope of 0.82 (r2 = 0.96) for fecal samples (Fig. 3.2). Mean DHEAS ± SEM recoveries of serum, urine, and fecal samples were 94.8 ± 4.2% (range: 73.2–111.6%, n = 9), 96.7 ± 2.6% (range: 80.6–115.7%, n = 14), and 92.8 ± 4.4% (range: 72.5–119.7%, n = 11), respectively.

The changes in concentrations of DHEAS, E_1C, and PdG by DHEAS administration

The timing of metabolism was investigated for DHEAS in serum, urine, and feces, and the concentrations in excretory samples were compared to the respective serum samples.

I also examined E_1C and PdG levels in urine and fecal samples using a previously validated E_1C and PdG EIA for urine and fecal samples of Japanese macaques (Shimizu and Mouri 2018). Intra- and inter-assay CVs for low and high E_1C controls were 13.5% and 10.4%, and 14.3% and 12.3%, respectively. Intra- and inter-assay CVs for low and high PdG controls were 11.3% and 1.8%, and 10.2% and 15.7%, respectively.

Statistical analysis

I used the base functions of R software (v 3.6.1; R Core Team 2019) to perform statistical testing. The relationships between serum DHEAS levels and those in corresponding urine and fecal samples were analyzed using Spearman's rank correlation.

3.4 Results

The changes in concentrations of DHEAS, E1C, and PdG by DHEAS administration

Relationship between urine, fecal, and serum DHEAS levels

The basal DHEAS levels in the three adult female Japanese macaques were 0.333–1.251 μg/mL for serum, 0.620–2.178 μg/creatinine mg for urine, and 0.130–1.225 μg/dry feces grams. Serum DHEAS peaked at 6 hours, and urine and fecal DHEAS peaked at 24 hours in samples after injection. The peaks in serum, urine, and fecal samples were 15 to 62-fold, 105 to 312-fold, and 23 to 60-fold higher than basal levels respectively. Serum peaks declined to basal levels 2 to 3 days, whereas urine samples took 7 days and fecal samples took 4 to 7 days after injection (Fig. 3.3). These were statistically significant both in the correlation between the pair of serum and urine DHEAS values and the correlation between serum and fecal DHEAS values, excluding the peak sample (Spearman's correlation coefficient between serum and urine: rho = 0.64, p = 0.004, corresponding

regression equation y = 2. 227 + 2.069×, and serum and fecal Spearman's correlation coefficient: rho = 0.83, $p < 0.001$, corresponding regression equation y = −0.427 + 1.538×) (Fig. 3.4). Since basal DHEAS levels that depended on the sample matrix among the three individuals were correlated, it was found that serum DHEAS levels accurately reflected DHEAS levels found in respective urinary and fecal samples (Fig. 3.5).

E_1C and PdG levels in urine and fecal samples by DHEAS administration

The basal E_1C levels in urine and fecal samples from the three females before DHEAS treatment were 7.4–24.8 ng/mg creatinine in urine and 1.34–3.30 ng/dry feces grams feces and the basal PdG levels were 0.098–0.265 μg/mg creatinine in urine and 0.48–1.30 μg/dry feces grams in feces. Whereas urine samples showed peaks of E_1C and PdG 24 hours after injection, those declined to baseline the following day. Thereafter, urinary E_1C levels in two of the three females increased sharply 7 days later. Unlike the results of the urine samples, no apparent fluctuations were observed in feces E_1C and PdG (Fig. 6). The peaks of E_1C and PdG in urine were 2.7 to 6.8 and 4.4 to 17.8 times the basal values, respectively. The peak levels of E_1C and PdG were equivalent to the peak levels in the ovulatory cycle of the Japanese macaque in my previous study (Shimizu and Mouri 2018), but the shape of the peaks was sharp and abrupt.

3.5 Discussion

My DHEA/S EIA system using heterological HRP conjugates was able to detect DHEA/S in serum, urine, and feces of Japanese macaques with high sensitivity.

The serum DHEA/S levels in the three adult female Japanese macaques determined using my EIA were almost equal to the previously reported levels of rhesus macaques

and pig-tailed macaques (Smail et al. 1982). Whereas the plasma DHEAS levels in middle-aged female rhesus macaques, which were measured via radioimmunoassay using an antibody against DHEA-17-(*O*-carboxymethyl) oxime-BSA with high specificity for DHEAS (Sorwell et al. 2012), were much lower than my data. As my method using an antibody against DHEA-3-succinate-BSA has cross reactions to both DHEA and DHEAS, my data may reflect total DHEA and DHEAS concentrations. Bernstein et al. (2012) report the ratio of DHEA per DHEAS in the Macaca genus is higher than in humans. Therefore, previous data (Sorwell et al. 2012) might have appeared lower in concentration when compared to my results. My fecal DHEAS profiles possibly do not include DHEA due to employing an aqueous solution extraction without any alcohol or ether. Depending on the research purpose, the target hormone can be measured by using different extract solutions.

The variation of DHEAS in urine and feces showed a good correlation with that of serum DHEAS, indicating that excretory DHEAS levels can be used to evaluate serum DHEAS levels. Therefore, as my non-invasive methods allow us to collect samples frequently, it may be possible to determine long-term DHEAS fluctuations accurately. In my previous cross-sectional study in Japanese macaques (Takeshita et al. 2013), we did not find adrenarche of DHEAS peak like rhesus macaque (Conley et al. 2011). However, the frequent and longitudinal collection of excreta samples might find adrenarche of DHEAS peak in neonates or infant Japanese macaques.

In the present study, I showed the metabolic characteristics of DHEAS via subcutaneous administration in Japanese macaques. DHEAS levels in excreta took 4 to 7 days until the peaks of urine and fecal DHEAS levels returned to the basal levels after DHEAS administration. Some levels of administrated DHEAS rapidly metabolized to urine and

feces within 1 or 2 days, but the remaining DHEAS was assumed to have likely continued circulating thereafter and slowly metabolized into urine or feces. The slow metabolic clearance rate of DHEAS has been previously reported by Haning et al. (1989). Furthermore, I measured E_1C and PdG concentrations to determine the effects of administrated DHEAS in Japanese macaques. E_1C profiles in feces and urine showed small rises over a few days suggesting possible gentle effects from the administered DHEAS. In addition to a gradual increase after DHEAS administration, estrogen in the urine of two subjects increased 1 week after administration. Further research is needed to understand these effects and their implications more clearly. The urinary PdG levels showed a steep and sharp peak 24 hours after the administration of DHEAS. One possible reason is that administration of large amounts of exogenous DHEAS may have temporarily suppressed the consumption of pregnenolone upstream of steroid biosynthesis in the Δ^5 pathway, causing more pregnenolone to be converted to progesterone and more PdG to be excreted as its metabolite (Schiffer et al. 2019).

DHEAS studies in non-human primates have gradually revealed the evolutionary importance of DHEA/S and several roles in the adrenal cortex. However, the roles of DHEA/S concerning aging (Lane et al. 1997; Roth et al. 2002, 2011; Bellino and Wise 2003; Muehlenbein et al. 2003; Labrie et al. 2005) have not been clarified. I revealed that DHEAS concentration in the excreta can accurately represent serum DHEAS concentration. Moreover, there was a time lag in the DHEAS metabolism of Japanese macaques. The present study provides an alternative but accurate method to clarify the role of DHEA/S in adrenal function in Japanese macaques, and possibly in those populations living in the wild, in zoos, or where non-invasive measures are necessary.

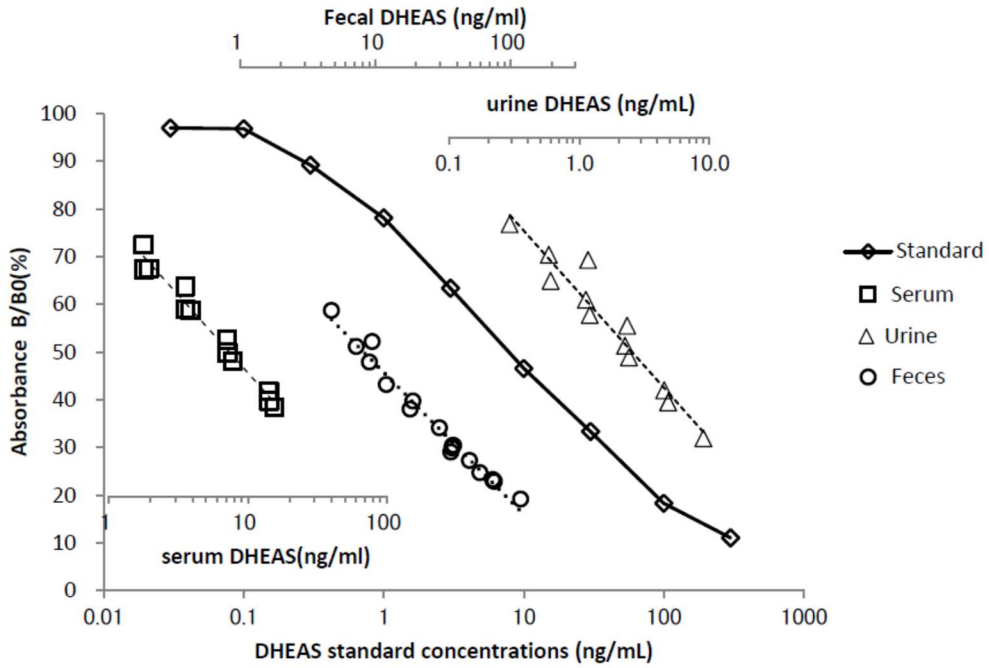


Fig. 3.1 Dilution curves of serum, urine, and fecal samples of Japanese macaques exhibited parallelism with the standard curve.

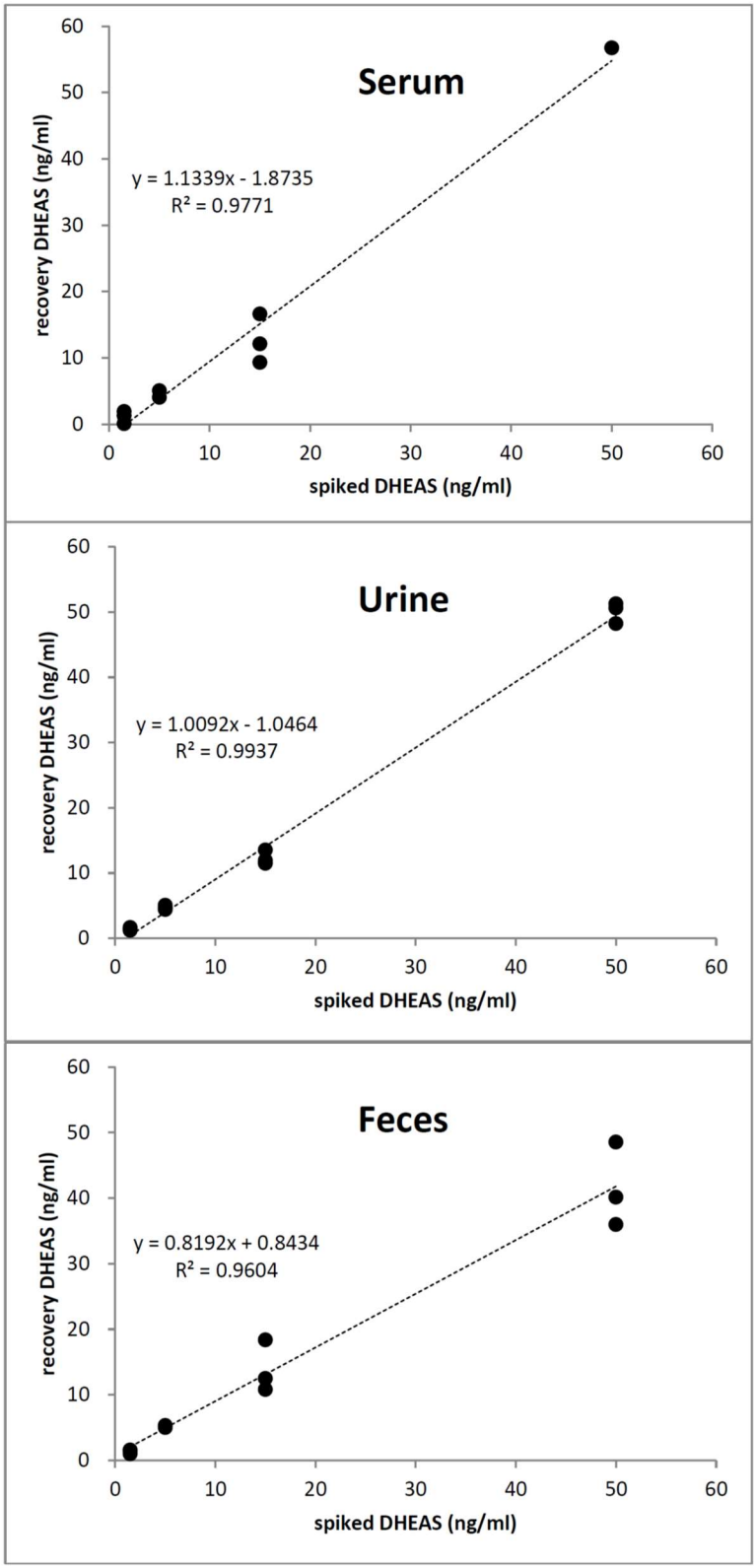


Fig. 3.2 Spiked dose-response curves showing DHEAS recovery for serum, urine, and feces.

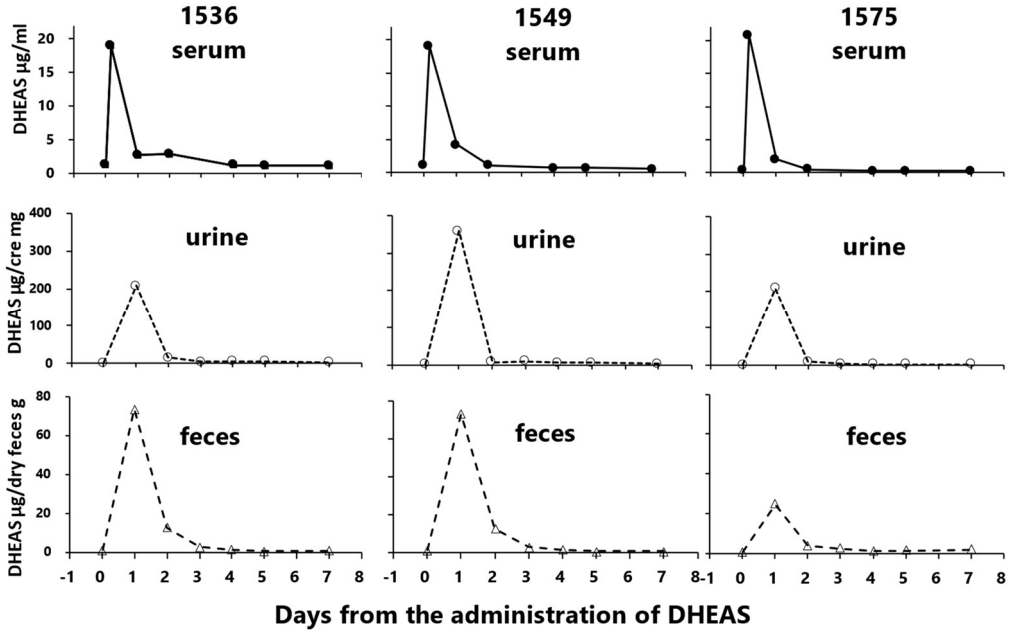


Fig. 3.3 Serum, urine, and fecal measurement of DHEAS in three female Japanese macaques. Day 0 represents the average DHEAS concentrations collected prior to administration.

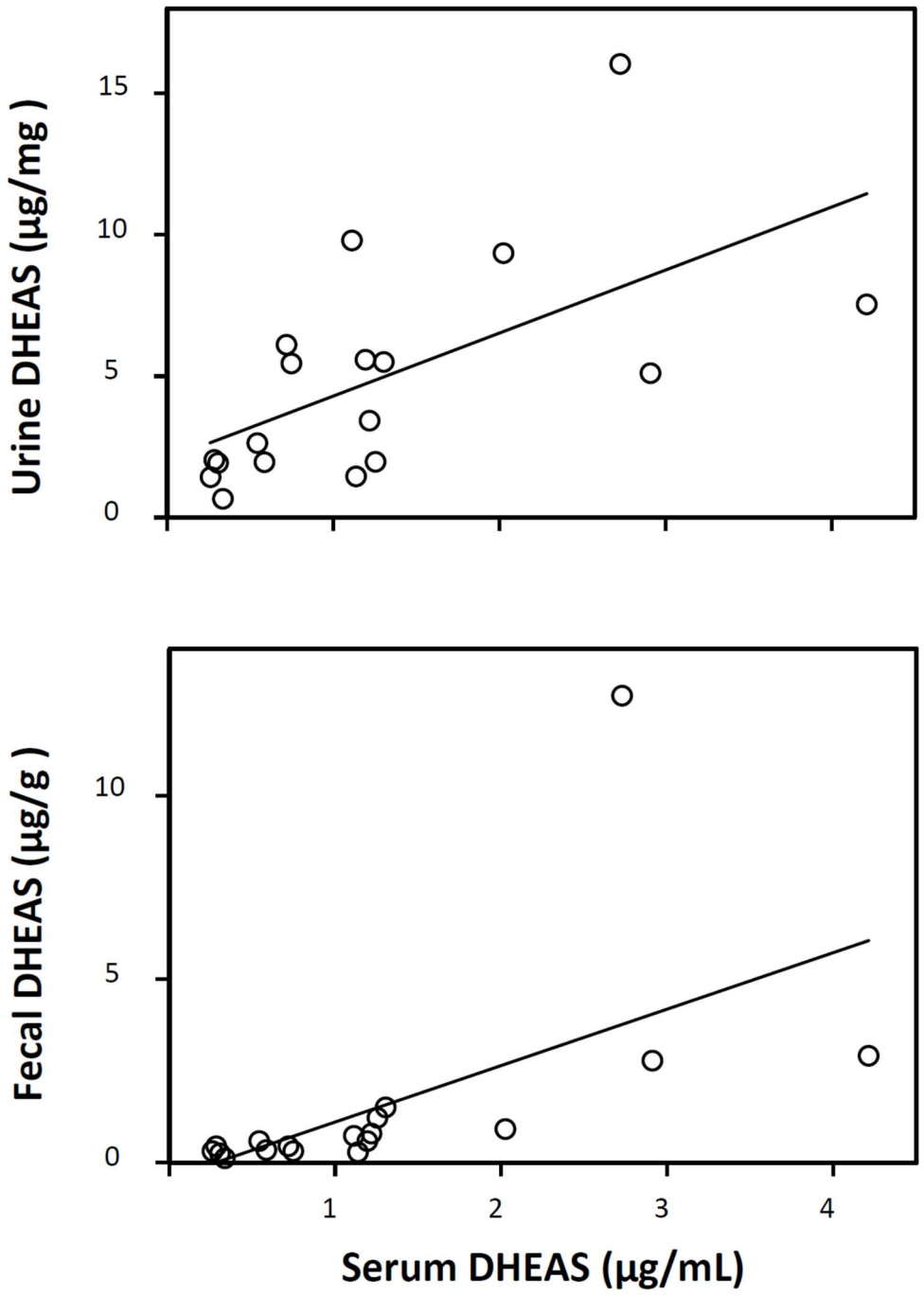


Fig. 3.4 Scatter plot of DHEAS levels in serum (µg/mL) and urine (µg/mg creatinine) samples and in serum (µg/mL) and fecal (µg/g dry feces) samples excluding samples of the peak.

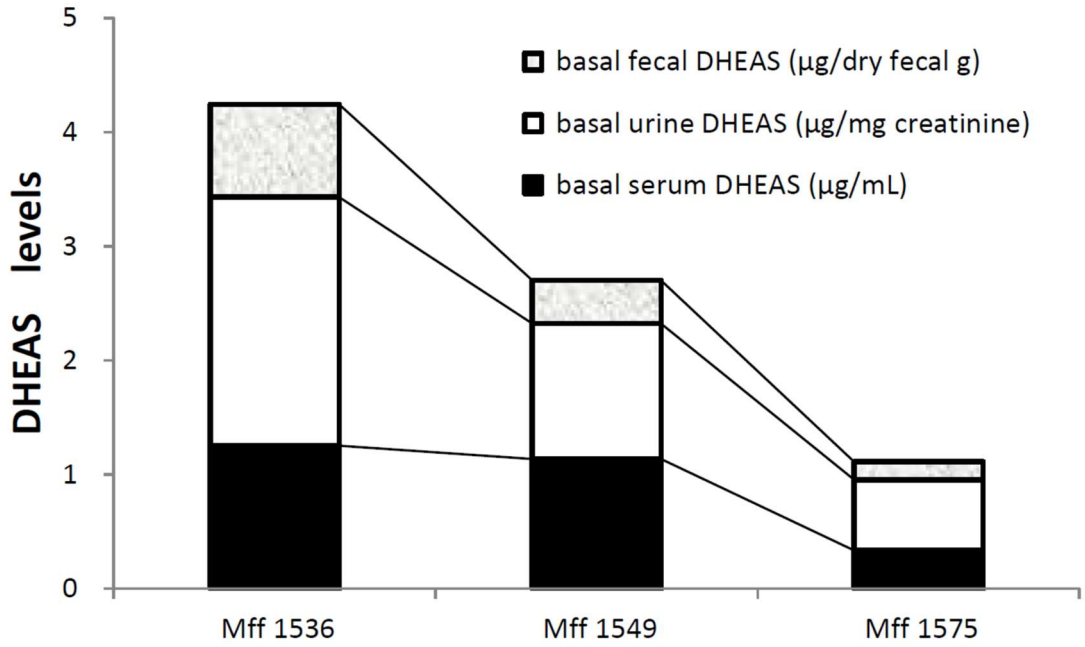


Fig. 3.5 The comparison of basal DHEAS levels in sample materials between three individuals.

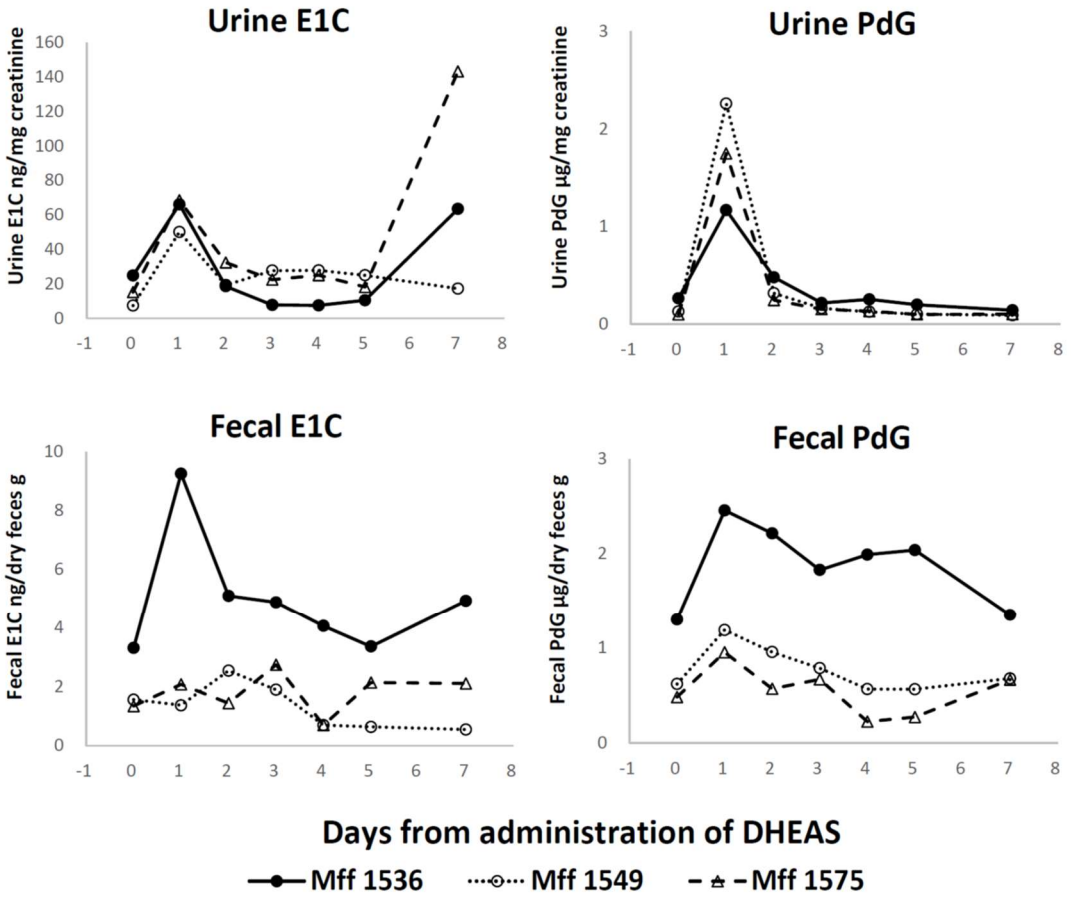


Fig. 3.6 Urine and fecal measurement of E_1C and PdG in three female Japanese macaques. Day 0 represents the average E_1C and PdG concentrations collected prior to administration.

Supplementary file 3.1

I collected all urine and fecal samples excreted from three Japanese macaques for three days and measured DHEAS, E_1C, and PdG concentrations in each sample to investigate the diurnal variations of steroid metabolites. A subset of the fresh fecal samples was stored at room temperature to determine changes in hormone concentrations over time.

Supplementary Methods

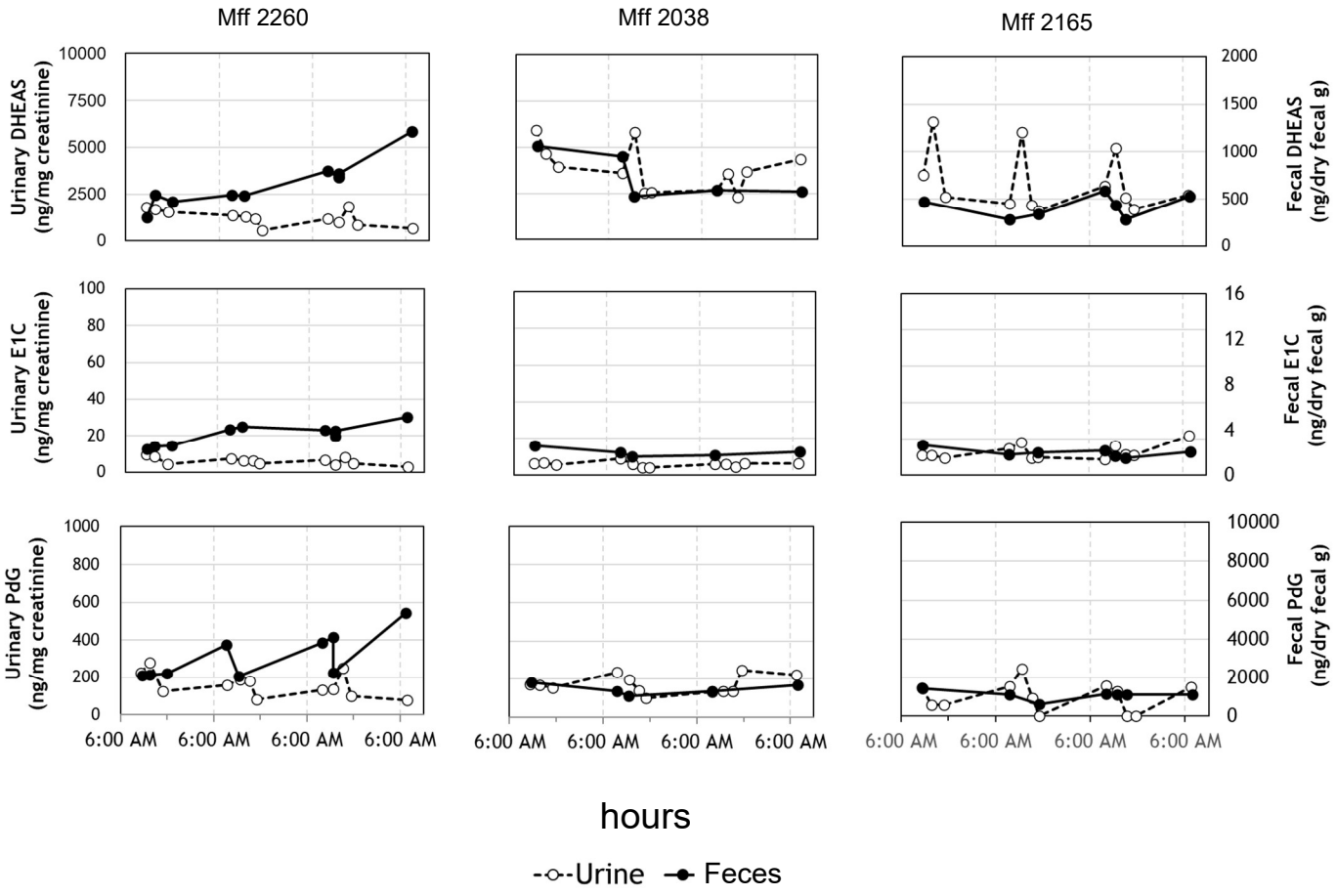
The collection time and volume of urine and fecal samples excreted over three days in three Japanese macaques.

urine	Sample collection start time	collection time	volume(ml)	feces	Sample collection start time	collection time	dry weight(g)
2260U-01	8:45 AM	11:15 AM	58.5	2260F-01	8:45 AM	11:30 AM	7.87
2260U-02	11:15 AM	1:30 PM	44.5	2260F-02	11:30 AM	1:30 PM	2.29
2260U-03	1:30 PM	5:00 PM	189.0	2260F-03	1:30 PM	6:00 PM	7.38
2260U-04	5:00 PM	9:30 AM	299.5	2260F-04	6:00 PM	9:15 AM	24.28
2260U-05	9:30 AM	12:45 PM	23.5	2260F-05**	9:15 AM	12:30 PM	3.98
2260U-06	12:45 PM	3:15 PM	133.5				
2260U-07	3:15 PM	5:00 PM	106.5				
2260U-08	5:00 PM	10:00 AM	181.5	2260F-06	5:00 PM	10:00 AM	8.18
2260U-09	10:00 AM	12:45 PM	20.5	2260F-07*	10:00 AM	12:45 PM	4.69
2260U-10	12:45 PM	3:15 PM	51.0				
2260U-11	3:15 PM	5:30 PM	90.0	2260F-08*	12:45 PM	5:30 PM	4.05
2260U-12	5:30 PM	7:45 AM	100.0	2260F-09	5:30 PM	7:30 AM	2.64
2038U-01	8:45 AM	11:15 AM	98.0	2038F-01	8:45 AM	11:30 AM	5.35
2038U-02	11:15 AM	1:45 PM	23.5				
2038U-03	1:45 PM	5:00 PM	81.5				
2038U-04	5:00 PM	9:30 AM	198.5	2038F-02	5:00 PM	9:30 AM	7.7
2038U-05	9:30 AM	12:45 PM	38.5	2038F-03	9:30 AM	12:30 PM	3.7
2038U-06	12:45 PM	3:15 PM	48.5				
2038U-07	3:15 PM	5:00 PM	44.5				
2038U-08	5:00 PM	10:00 AM	99.5	2038F-04	5:00 PM	10:00 AM	12.03
2038U-09	10:00 AM	12:45 PM	17.5				
2038U-10	12:45 PM	3:15 PM	68.5				
2038U-11	3:15 PM	5:30 PM	91.5				
2038U-12	5:30 PM	7:30 AM	178.5	2038F-05	10:00 AM	7:50 AM	1.88
2165U-01	8:45 AM	11:30 AM	214.5	2165F-01	8:45 AM	11:30 AM	3.91
2165U-02	11:30 AM	1:50 PM	289.0				
2165U-03	1:50 PM	5:00 PM	316.5				
2165U-04	5:00 PM	9:30 AM	302.5	2165F-02	5:00 PM	9:30 AM	11.74
2165U-05	9:30 AM	1:00 PM	218.5				
2165U-06	1:00 PM	3:15 PM	173.5				
2165U-07	3:15 PM	5:00 PM	249.0	2165F-03*	3:15 PM	5:00 PM	5.67
2165U-08	5:00 PM	10:00 AM	372.5	2165F-04	5:00 PM	10:00 AM	8.98
2165U-09	10:00 AM	12:45 PM	210.0	2165F-05	10:00 AM	12:45 PM	2.03
2165U-10	12:45 PM	3:15 PM	196.5	2165F-06	12:45 PM	3:15 PM	1.5
2165U-11	3:15 PM	5:00 PM	208.5				
2165U-12	5:00 PM	7:30 AM	110.5	2165F-07	5:00 PM	7:52 AM	3.84

*: These fresh fecal samples were used in experiments to investigate the effects of long-term storage at room temperature on hormone levels.

Supplementary Results

Diurnal variation of DHEAS, E_1C, PdG concentrations in the urine and feces of Japanese macaques.

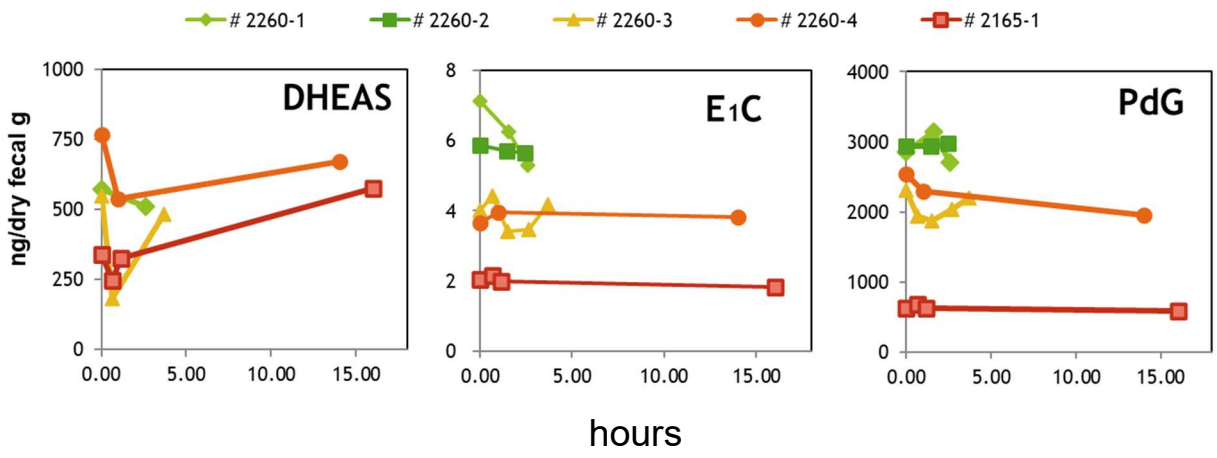


Peaks were observed in urinary PdG and DHEAS for one subject (Mff 2165) collected around noon. No significant diurnal variation was observed in fecal samples.

Effects of storing fresh fecal samples at room temperature (approx. 24°C) on steroid hormones

sample ID	Time left at room temp. (hr)	EtC (ng/dry fecal g)	PdG (ng/dry fecal g)	DHEAS (ng/dry fecal g)
# 2260-1	0.0	7.12	2860	570
	1.6	6.26	3150	no data*
	2.6	5.29	2700	510
# 2260-2	0.0	5.87	2940	no data*
	1.5	5.70	2940	no data*
	2.5	5.65	2980	no data*
# 2260-3	0.0	4.02	2310	549
	0.7	4.41	1940	183
	1.5	3.41	1870	no data*
	2.7	3.46	2040	no data*
	3.7	4.18	2200	483
# 2260-4	0.0	3.66	2540	768
	1.0	3.96	2300	537
	14.0	3.81	1950	669
# 2165-1	0.0	2.05	620	339
	0.7	2.16	680	246
	1.2	1.98	630	324
	16.0	1.83	580	576

no data*: Unable to measure due to insufficient sample.



No significant effects were observed with fecal hormones stored at room temperature.

Supplementary file 3.2

Preparation of blood samples for DHEAS EIA
Linearity test non-extracted samples

Supplementary Methods

I serially diluted non-extracted serum samples with assay buffer and deionized water.

Supplementary Results

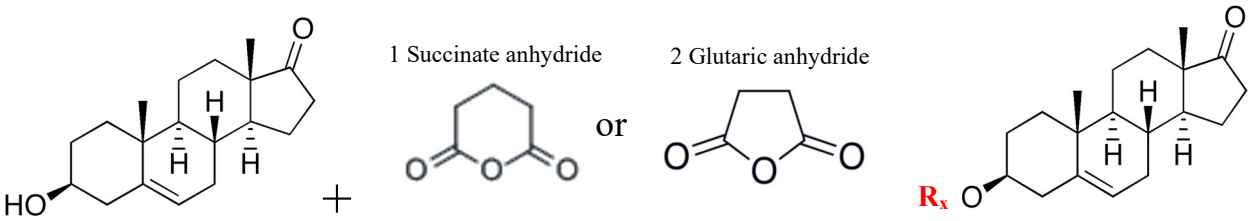
Samples	Dilution rate	diluted by assay buffer		diluted by deionized water	
		DHEAS concentrations (ng/mL)	Adjusted concentrations (μg/mL) with dilution rates	DHEAS concentrations (ng/mL)	Adjusted concentrations (μg/mL) with dilution rates
Sample 1	3	561.993	1.685	671.771	2.015
	9	480.237	4.322	585.924	5.273
	27	540.653	14.598	489.908	13.228
	81	467.673	37.881	415.836	33.683
Sample 2	3	77.912	0.234	146.081	0.438
	9	41.776	0.376	112.396	1.011
	27	76.308	2.060	106.213	2.868
	81	79.635	6.450	89.416	7.243

DHEAS EIA using direct serum samples did not show linearity.

Supplementary file 3.3

Synthesis of succinate and glutarate Spacer

Five mg of DHEA was added to 5 mg of succinate anhydride (or glutaric anhydride) in the glass test tube with a small magnetic stirrer. One hundred µl of pyridine was added to the test tube and mixed with a cap at 60°C for 3 h of well-mixed DHEA.



R_1 DHEA-3-succinate DHEA-3-CO-$CH_2CH_2CH_2COOH$

R_2 DHEA-3-glutarate DHEA-3-CO-CH_2CH_2COOH

After incubation, 1 ml of deionized water was added to the test tube and the reactant was acidified with 100 µl of 2N hydrochloric acid. The precipitated crystals were recrystallized by ethyl acetate to obtain DHEA-3-succinate (or -glutarate).

NHS esterification of the carboxylic acid of the spacer to react with HRP.

Supplementary Table 3.1

	DHEA EIA with DHEA-3-succinate-HRP	DHEA EIA with DHEA-3-glutarate-HRP
Antibody dilution rate	1:10000	1:100,000
HRP conjugated DHEAS dilution rate	1:3000	1:50,000
1/2 B0 concentration	77.5 ng/mL	7.2 ng/mL (mean of 8 assays)

Supplementary Table 3.2

Data from the linearity test for each matrix

Serum extract

Samples	Dilution rate	diluted by assay buffer	
		Observed DHEAS concentrations (ng/mL)	Adjusted concentrations (μg/mL) with dilution rates
Sample 1	100	14.197	1.420
	200	7.444	1.489
	400	3.820	1.528
	800	2.145	1.716
Sample 2	100	14.287	1.429
	200	7.675	1.535
	400	4.460	1.784
	800	2.606	2.085
Sample 3	100	15.563	1.556
	200	8.547	1.709
	400	4.519	1.808
	800	2.561	2.049

Urine

Samples	Dilution rate	diluted by deionized water	
		Observed DHEAS concentrations (ng/mL)	Adjusted concentrations (μg/mL) with dilution rates
Sample 1	50000	9.004	450.2
	100000	4.600	460.0
	200000	2.434	486.8
	400000	1.135	454.0
Sample 2	50000	2.333	116.7
	100000	0.677	67.7
	200000	0.587	117.4
	400000	0.241	96.4
Sample 3	50000	4.819	241.0
	100000	2.234	223.4
	200000	0.987	197.4
	400000	0.453	181.2

Fecal extract

Samples	Dilution rate	diluted by assay water	
		Observed DHEAS concentrations (ng/mL)	Adjusted concentrations (μg/mL) with dilution rates
Sample 1*	100	73.499	7.350
	200	38.944	7.789
Sample 1*	200	36.115	7.223
	400	16.908	6.763
	600	11.177	6.706
	800	7.790	6.232
	1000	6.008	6.008
Sample 2	10	75.588	0.756
	20	38.340	0.767
	40	19.023	0.761
	80	8.211	0.657
	160	5.294	0.847
Sample 3	8	74.330	0.595
	12	50.219	0.603
	16	38.340	0.613
	20	28.542	0.571
Sample 4	10	118.276	1.183
	20	63.669	1.273

*Sample 1 was tested twice on different days.

Chapter 4

Stability of chimpanzee (*Pan troglodytes*) urinary reproductive hormones preserved long-term on filter paper

4.1 Abstract

Urine contains multiple water-soluble hormones, which are valuable non-invasive biomarkers for the monitoring of reproductive status and health. An effective method for drying urine on filter paper was previously developed to preserve wildlife urine samples where electrical equipment was not available for this; however, the stability of samples preserved in this way remains to be verified. Here, I developed and validated a method to elute multiple water-soluble reproductive hormones from filter paper that had been stored for an extended period of time. Aliquots of urine from chimpanzees were adsorbed on filter papers, air-dried, and stored for 1 year at room temperature. Estrone-3-conjugate (E_1C), pregnanediol-3-glucuronide (PdG), estriol-3-glucuronide (E_3G), and chorionic gonadotropin (CG) were eluted into deionized water from the filter papers and measured using enzyme immunoassays (EIAs). The mean recoveries of E_1C, PdG, and creatinine from filter papers stored for 1 year were 69.5%, 128.7%, and 83.8%, respectively. The profiles of E_1C and PdG from preserved filter papers significantly correlated with those derived from a direct analysis of the frozen urine of menstruating chimpanzees. I detected E_3G and CG from 1-year-old filter papers for urine collected during early pregnancy, but the recovery of E_3G was low and CG profiles did not correlate with those of the original frozen urine samples. The method proposed here for the elution and measurement of reproductive hormones in urine preserved for a long time on filter paper provides a

practical and simple way to monitor the reproductive status of chimpanzees. I propose that this method can also be utilized in field studies of other wild nonhuman primates.

4.2 Introduction

Measurements of urinary or fecal hormones have been widely used as non-invasive alternatives to the measurement of circulating hormones to monitor the reproductive status of great apes (Graham et al. 1972; Czekala et al. 1983, 1988ab; Dahl et al. 1991; Heistermann et al. 1996; Jurke et al. 2000; Shimizu et al. 2003a). Urine is a particularly informative byproduct as it contains steroid hormone metabolites as well as peptide hormones such as gonadotropin (McArthur et al. 1981; Steinetz et al. 1992; Shimizu et al. 2003b). Moreover, variations in the concentrations of hormones and hormone metabolites in urine can be compensated for by measuring creatinine to determine more accurately urinary hormone profiles. However, studies of urinary hormones in wildlife inhabiting remote areas where researchers do not have access to electricity require the use of special materials such as liquid nitrogen and/or dry ice to properly preserve and transfer collected samples (Douglas et al. 2016).

The filter paper was validated as an effective medium from which to screen the blood of newborns (Kraiem et al. 1980; Bassett et al. 1986) and has since been used as a collection and storage device for liquid biological samples in the field (Campbell, 1994; Shideler et al. 1995; Knott, 1997, 2005). Shideler et al. (1995) measured steroid metabolites from urine-soaked small filter papers with a non-instrumental enzyme immunoassay (NEIA) method using high-binding tubes. Knott (2005) later developed a method to elute the urine contents of an entire filter paper with methanol and measure them via radioimmunoassay (RIA). Elution with methanol allowed multiple hormone

assays to be carried out and avoided the problem of uneven absorption of urine across the filter paper by using the entire urine contents that had been eluted. However, RIAs have limitations as they require special equipment and produce radioactive waste; furthermore, elution with organic solvents precludes the detection of peptide hormones in urine.

In the present study, I developed and validated a simple method for eluting the urine contents of filter paper with deionized water. I determined the concentrations of estrone-3-conjugate (E_1C), pregnanediol-3-glucuronide (PdG), and estriol-3-glucuronide (E_3G), as well as chorionic gonadotropin (CG), in the eluents using a microplate EIA. I compared the results obtained using urine-adsorbed filter papers stored for an extended period (1 year) with those obtained from the direct analysis of the corresponding original (frozen and thawed) urine samples to validate the use of filter paper as a viable long-term storage option. This method should facilitate the easy collection, preservation, and transportation of urine samples from wild primates for the long-term monitoring of their reproductive status under basic field conditions.

4.3 Materials and Methods

Collection and preparation of urine samples

I used urine samples collected from three female chimpanzees (*Pan troglodytes verus*; A, B, and C) housed at the Great Ape Research Institute (GARI), Hayashibara Biochemical Laboratories, Okayama, Japan. The staff of GARI recorded the date and time of sample collection, as well as observations of vaginal bleeding and the physical condition of the chimpanzees. Urine contaminated with blood was not collected. Samples were stored in the freezer at GARI and transported to Okayama University of Science for later analysis.

Information about the reproductive status of all three subjects during sample collection is summarized in Table 1. To validate the reliability of filter paper as a storage medium, I used a total of 131 samples (E_1C and PdG, n = 122; E_3G, n = 45; CG, n = 15) collected during the menstrual cycle and both early and mid-pregnancy for the three chimpanzees. For evaluation of the stability of these hormones during 1 year of storage, I used a total of 78 samples (E_1C and PdG, n = 78; E_3G and CG, n = 15) collected during the menstrual cycle in chimpanzees A and B, and during early pregnancy in chimpanzees A, B, and C. Creatinine measurements were conducted for all samples.

One hundred and fifty microliters of thawed urine sample were absorbed onto a halved filter paper (55.0-mm-diameter Whatman no. 1 analytical grade filter paper, 1001-055; GE Healthcare Life Sciences, UK). The urine-adsorbed filter papers were dried on a wire mesh at room temperature overnight. Thereafter, the completely dried filter papers were stored individually in labeled plastic bags in the dark at room temperature until elution. The remaining volumes of thawed urine samples (hereafter "original urine samples") were used for hormonal measurements for comparison with the levels in the filter paper eluents.

Hormonal analyses by microplate EIA

I measured levels of E_1C and PdG as the major urinary metabolites of circulating estradiol (E_2) and progesterone (P_4). In addition, I measured E_3G and CG as biomarkers of pregnancy in chimpanzees. I used the E_1C, PdG, and E_3G EIA methods previously validated for Japanese macaque urine (Shimizu and Mouri 2018); the cross-reactivities of antibodies are described therein. Concentrations of CG were determined via EIA as previously described for chimpanzee urine (Shimizu et al. 2003b). Sensitivities of the

EIAs were 6.6 pg/ml for E_1C, 2.1 ng/ml for PdG, 0.35 ng/ml for E_3G, and 0.023 ng/ml for CG.

In this study, I first conducted linearity and recovery tests to assess the applicability of EIA methods to E_1C, PdG, and E_3G in frozen and thawed chimpanzee original urine samples (Supplementary file 1). Linearity and recovery tests of the CG assay were previously validated for chimpanzee urine (Shimizu et al. 2003b).

The inter-assay coefficients of variation (CVs) of high and low internal controls were 5.9% and 2.9% for E_1C, 7.2% and 19.5% for PdG, 8.5% and 26.7% for E_3G, and 8.5% and 25.4% for CG, respectively. The values of the low internal controls for E_3G and CG were set to extremely low levels for this assay system, which led to a high variance in the CVs for E_3G and CG. In contrast, the high internal controls for E_3G and CG were stable and indicated low CVs. The intra-assay CVs were 4.19% for E_1C, 8.2% for PdG, 19.3% for E_3G, and 3.5% for CG.

Creatinine concentrations were measured on a microplate using the Jaffe reaction (Taussky, 1954). Samples were diluted with deionized water to appropriate concentrations and analyzed.

Elution procedure for filter papers

Preliminary tests were performed to determine the appropriate elution conditions via the microplate EIA method described above using urine-adsorbed filter papers from the chimpanzees (Supplementary file 2). Recoveries of E_1C, PdG, and creatinine from filter paper eluents were compared under different shaking methods and intensities. The final elution conditions were determined as follows: the dried filter papers were folded in four to approximately 1 cm^2, then placed into 16 × 100-mm 14-ml disposable polypropylene

tubes containing 1.5 ml of deionized water. The tubes were shaken at 150 rpm on a reciprocal shaker (NR-3; TAITEC, Saitama, Japan) for 2 h at room temperature. The tubes were then centrifuged at 1500 g for 10 min, and the eluents were aspirated and transferred to microtubes.

Assay validation of eluents from urine-adsorbed filter paper

Linearity

I serially diluted three filter paper eluents of three different chimpanzee subjects with deionized water. The slopes generated from the absorbances of the serial dilutions of the filter paper eluents were compared with the slopes of the standard curves for E_1C, PdG, and E_3G EIA. Linearity in the CG assay for the filter paper eluents could not be determined because the volume of eluent was insufficient.

Recovery

I added zero, low, medium, and high doses of hormone standards to three pooled filter paper eluents from three chimpanzees. The results were expressed as the percentage of the actual added amount that was recovered by the assay. The recovery of CG from the filter paper eluents could not be determined because the volumes of eluent were insufficient for this.

Recoveries and equivalences of filter paper eluents and original urine samples

To validate the reliability of filter paper as a storage medium for urine, urine-adsorbed filter papers were dried at room temperature overnight. On the following day, the urine contents of the dried filter papers were eluted using the determined elution procedure, and E_1C, PdG, E_3G, CG, and creatinine concentrations of the filter paper eluents measured to

test whether the components of the filter paper had any effect on the hormonal analyses.

For the evaluation of stability during long-term storage, I stored urine-adsorbed filter papers for samples obtained during menstrual cycles and early pregnancy in plastic bags at room temperature for 1 year. After 1 year of storage, I eluted the urine contents from the filter papers and measured the levels of E_1C, PdG, E_3G, CG, and creatinine in the eluents.

The recoveries of hormone and creatinine concentrations from the filter papers stored for 1 day and for 1 year were expressed as percentages (mean ± SD) of those measured for the corresponding original urine samples. To assess equivalence, filter paper eluents were compared to original urine samples for E_1C/creatinine, PdG/creatinine, E_3G/creatinine, and CG/creatinine.

Endocrine changes during the menstrual cycle and early pregnancy detected in eluents from 1-year-old urine-adsorbed filter paper

I also compared the hormonal profiles obtained from the 1-year-old filter paper eluents to those from the original urine samples collected during the menstrual cycle and early pregnancy, to confirm whether the long-term storage of urine on filter papers is suitable for the monitoring of ovulation and early pregnancy.

4.4 Results

Assay validation of eluents from urine-adsorbed filter paper

Linearity

Figure 4.1 depicts the results of the linearity of the three steroid metabolites in the filter paper eluents. The slopes of the displacement curve were not significantly different from those of the standard curve for E_1C, PdG, and E_3G.

Recovery

The mean ± SD recovery of known added amounts of steroid hormones to the eluents from filter papers were 102.8 ± 36.1%, 106.0 ± 20.2%, and 123.2 ± 37.0% for E_1C, PdG, and E_3G, respectively.

Recoveries and equivalences of filter paper eluents and original urine samples

In Table 4.2, I show recoveries of E_1C, PdG, E_3G, and creatinine from filter papers for two storage periods, 1 day and 1 year. I do not show the recovery of CG because of a lack of correlation between the CG levels of the original urine samples and those of the filter paper eluents. Recoveries of E_1C and creatinine from 1-year-old filter papers slightly decreased in comparison with the recoveries from 1-day-old filter papers. On the other hand, the recovery of PdG from 1-year-old filter papers increased. The recovery of E_3G from 1-year-old filter paper for urine collected during early pregnancy was lower than that for 1-day-old samples.

Figure 4.2 illustrates the relationships between levels of E_1C, PdG, E_3G, and CG compensated for by corresponding creatinine levels in the filter paper eluents and the original urine samples. The E_1C/creatinine and PdG/creatinine of the eluents from both 1-day-old and 1-year-old filter papers were significantly correlated with those in the original urine samples (Fig. 4.2a, b). E_3G/creatinine from 1-day-old filter papers for both early- and mid-pregnancy samples were significantly correlated with those in the original urine samples, whereas those from 1-year-old filter papers for samples collected during early pregnancy showed a weak correlation with those in the original urine samples (Fig. 4.2c). CG/creatinine showed no correlation for either 1-day-old or 1-year-old filter paper eluents and original urine samples collected during early pregnancy (Fig. 4.2d).

Endocrine changes during the menstrual cycle and early pregnancy detected in 1-year-old filter paper eluents

Figure 4.3 illustrates the hormonal profiles of chimpanzee A during its menstrual cycle and early pregnancy. The patterns of E_1C and PdG obtained from the eluents of the 1-year-old filter papers (Fig. 4.3b left) suggest two menstrual cycles, the first of which is illustrated by an E_1C peak followed by a rise in PdG and another mid-cycle rise in E_1C, which is repeated during the subsequent menstrual cycle. These profiles from the eluents of 1-year-old filter papers suggest that the indicated timing of ovulation was consistent with that estimated from the profiles of the original urine samples. PdG levels in some filter paper eluents for urine collected during the follicular phase were extremely low and at times undetectable. However, even though PdG was at times undetectable for urine collected during the follicular phase, PdG profiles from the 1-year-old filter paper exhibited similar patterns to those of the original urine samples.

The hormonal profiles of E_1C and PdG from the eluents of 1-year-old filter papers for urine samples collected during early pregnancy were also similar to those of the original urine samples (Fig. 4.3b center). Following the E_1C peak preceding conception, PdG levels were approximately double their peak levels in the normal luteal phase. E_1C levels increased and reached five times their preceding peak levels at gestational day 30. In contrast, although CG in the original urine samples increased after approximately 14 days post the E_1C peak, CG concentrations in eluents of 1-year-old filter papers were relatively low (Fig. 4.3b right). There was no direct correlation between these CG profiles; however, CG concentrations were detectable from approximately 14 days after the last E_1C peak. The levels of E_3G in the eluents of the 1-year-old filter paper were low but gradually

increased from gestational day 30.

The patterns of E_1C and PdG for chimpanzee B (not shown) suggested one menstrual cycle, evidenced by an E_1C peak followed by a rise in PdG and another mid-cycle rise in E_1C. As also seen in chimpanzee A, the indicated timing of ovulation in chimpanzee B was consistent in the original and filter paper samples. The patterns and extremely high levels of E_1C and PdG for chimpanzee C (not shown) suggested pregnancy. E_1C and PdG profiles of 1-year-old filter paper eluents and original urine samples were highly correlated for each of the three chimpanzees. CG and E_3G levels were below the limit of detection in eluents of 1-year-old filter papers for chimpanzees B and C (CG was not detected in the original urine samples of chimpanzee B) because these samples were collected during very early pregnancy.

4.5 Discussion

I validated a method to measure E_1C, PdG, E_3G, and creatinine in urine samples preserved for up to 1 year on filter paper at room temperature. Although the method is simple, recoveries of the steroid metabolites were nonetheless high. While I was less successful in accurately quantifying concentrations of CG, the qualitative results suggest that my method, which importantly uses only non-volatile solvents, can detect CG during early pregnancy. The extremely high levels of E_1C and PdG, which were measured from the urine-adsorbed filter paper that had been stored for 1 year, can indicate implantation in chimpanzees. I, therefore, believe that this method is useful for researchers and conservationists aiming to monitor physiological processes such as reproduction in wild primates, particularly in remote locations where access to appropriate facilities and/or electricity may be scarce or periodic.

I failed to measure PdG concentrations in approximately 10% of the eluents from the 78 one-year-old filter papers for urine collected during the menstrual cycle and early pregnancy; 66% of the 15 one-year-old filter papers also failed to yield measurable E_3G concentrations for urine collected during early pregnancy. The levels of PdG in the original urine samples collected during the follicular phase, as well as those of E_3G collected before gestational day 30 during early pregnancy, were also low. Moreover, the filter paper eluents had already been diluted tenfold compared to the original urine samples during the extraction process. Therefore, PdG and E_3G in these cases were likely undetectable because of the sensitivities of their respective EIA. To deal with such low concentrations, I concentrated some urine eluents using a centrifugal vacuum concentrator before measurements. However, I needed a larger volume of eluents to achieve sufficiently high concentrations for multiple hormone measurements. It is important to note that, even in the case of undetectable levels of PdG in urine collected during the follicular phase, I could still estimate ovulation by integrating the remaining PdG results with the E_1C peaks during the follicular phase and the PdG rise during the luteal phase. To assess early pregnancy, I could also estimate pregnancy before gestational day 30 by integrating extremely high levels of E_1C and PdG.

Concerning recoveries, I found that the concentrations of PdG increased in 1-year-old filter paper-preserved urine samples with respect to the original samples, whereas those of E_1C and creatinine slightly decreased. These results are in line with those of a previous study, which reported that E_13G (E_1C) and creatinine are less stable when stored at 25 and 37 ℃, whereas Pd3G (PdG) recoveries were only minimally affected by the storage temperature (Kesner et al. 1995). Although I thought that the high PdG/creatinine may have been artificially inflated by decreased creatinine levels, comparison of actual levels

of PdG from 1-year-old filter papers with those of the original urine samples showed high statistical significance ($p = 0.0066$, $df = 30$). I am unsure as to why PdG levels increased in 1-year-old filter papers.

Unlike the case for the other products examined, 1-day and 1-year storage of filter papers proved unsuitable for the accurate measurement of CG concentrations in the urine of the chimpanzees tested. Sadler and Lynskey (1979) reported a successful method for the RIA of thyroid-stimulating hormone from dried blood stored on filter paper for long periods of time. I conducted a preliminary test for the recovery of CG using a gentler elution method with deionized water (Supplementary file 4.2). However, I failed to detect the hormone in many of the samples, and the results showed a very low recovery. Campbell (1994) reported 92 ± 3% recoveries of protein and peptides from dried-urine filter paper samples when using a method of dissolving and removing the filter paper from the sample. In the present study, for the qualitative assessment of pregnancy in wild primates, I aimed to simplify the elution procedure using deionized water. Concerning the questionable results obtained for CG, another explanation might be the duration of the drying process. Emery Thompson and Knott (2008) speculated that a loss of C-peptide in urine occurred during the initial drying process rather than during extraction. Moreover, Birken et al. (2001) reported that the CG of urine collected from pregnant chimpanzees was significantly affected by protease activity. Further examination of how the process of rapid drying of urine on filter paper and its subsequent elution might damage peptide hormones is thus warranted. However, although only qualitative, my results for CG are consistent with those of a previous study that showed that CG is detectable in urine approximately 14 days after the E_1C peak in chimpanzees (Shimizu et al. 2003b). This remained true even for my 1-year-old samples, so my method may still apply to the

qualitative assessment of CG as a biomarker of early pregnancy.

In conclusion, my elution method demonstrated the reliability of filter paper as a medium for the stable long-term preservation of urinary reproductive hormones, which could prove useful for monitoring ovulation and pregnancy. This method should facilitate the collection, preservation, and transportation of urine samples for monitoring the reproductive status of wild primates under basic field conditions and over extended periods of time.

Table 4.1 Number of samples and reproductive status of the three female chimpanzees (A, B, and C) for each sample collection

	Menstrual cycle (number of cycles)	Early pregnancy	Mid pregnancy
Chimpanzee A	24 (two menstrual cycles)	41 (until GD 59)	10 (GD 71 – 164)
Chimpanzee B	8 (one menstrual cycle)	12 (until GD 20)	10 (GD 70 – 162)
Chimpanzee C	9 (one menstrual cycle)	16 (until GD 40)	10 (GD 85 – 182)

GD Gestational day

Table 4.2 Recoveries (%) of dried urinary hormones and creatinine from filter papers using a microplate assay (mean ± SD)

	E_1C (n)	PdG (n)	E_3G (n)	Creatinine (n)
One-day storage	78.1 ± 23.2 % (122)	56.0 ± 31.9 % (117)	104.5 ± 22.4 % (39)	85.1 ± 28.1 % (131)
One-year storage	69.5 ± 19.4 % (78)	128.7 ± 47.9 % (70)	29.5 ± 20.1 % (5)	83.8 ± 11.0 % (78)

E_1C Estrone-3-conjugate, *PdG* pregnanediol-3-glucuronide, *E_3G* estriol-3-glucuronide

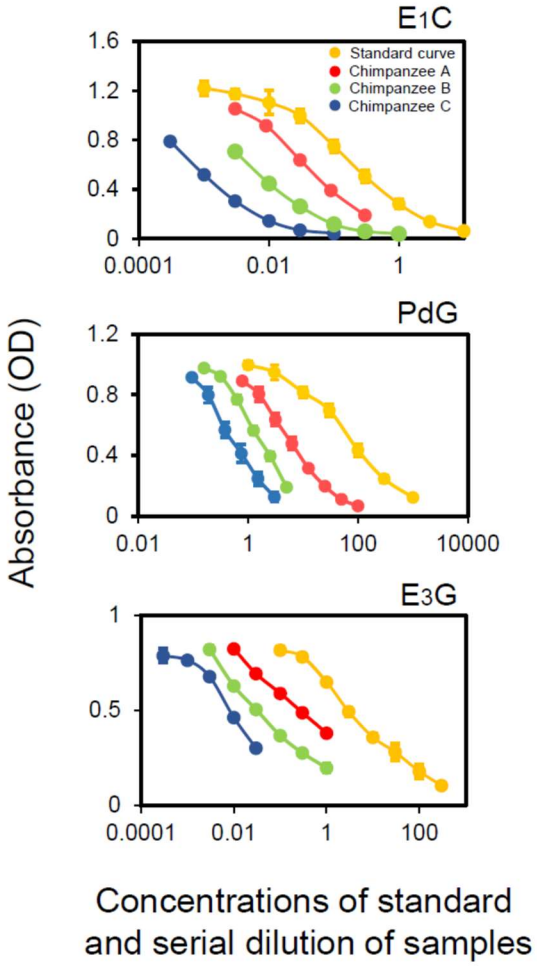


Fig. 4.1 Linearity of three steroid metabolites [estrone-3-conjugate (E_1C), pregnanediol-3-glucuronide (PdG), and estriol-3-glucuronide (E_3G)] in filter paper eluents. The slopes generated for the metabolites from the serially diluted filter paper eluents [three individuals (A, B, and C) for each] were not significantly different from those of the standard curves (analysis of covariance, range of P-values 0.110–0.150). OD Optical density

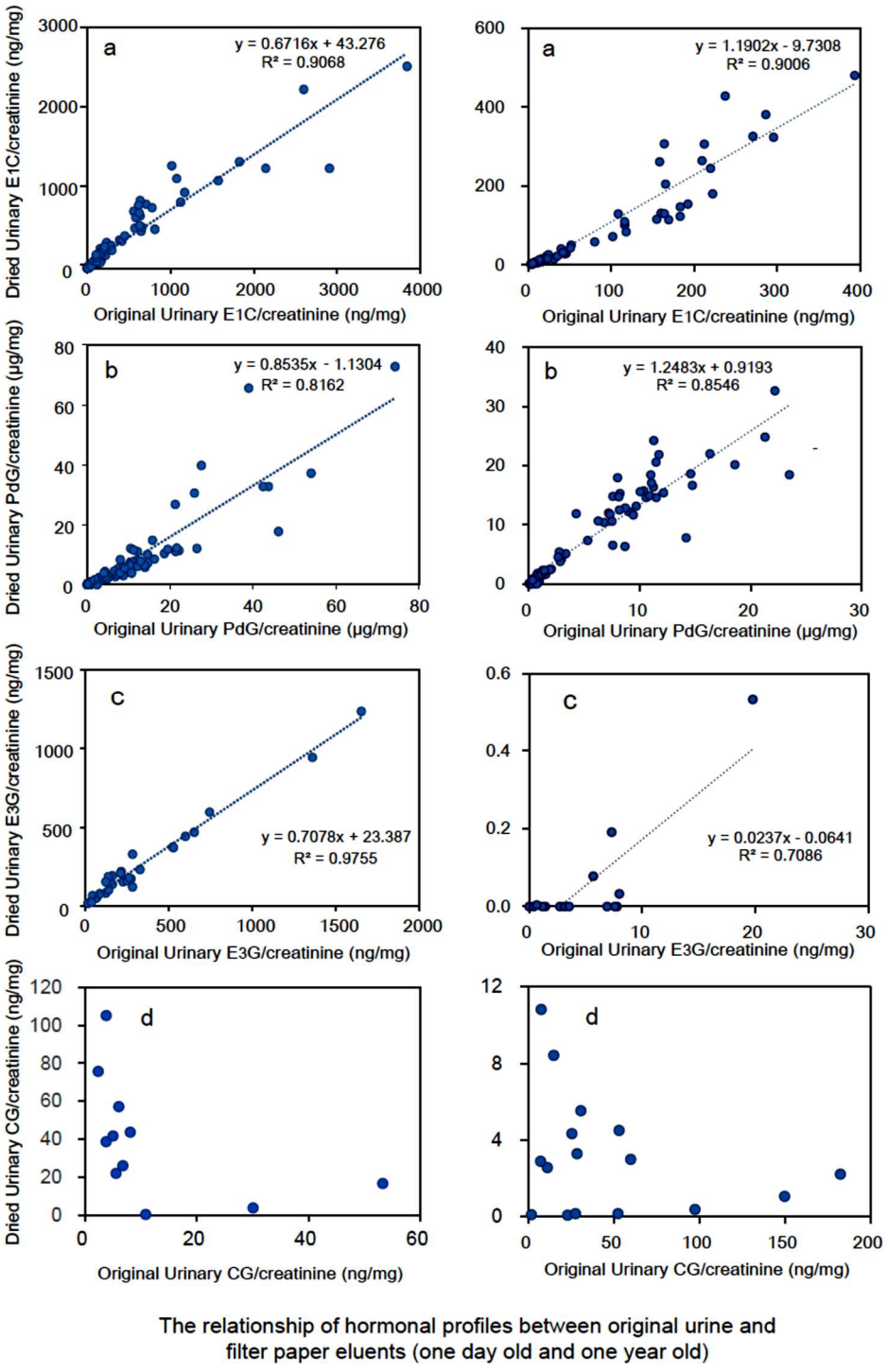


Fig. 4.2 Measurements of a: E_1C/creatinine, b: PdG/creatinine, c: E_3G/creatinine, and d: CG/creatinine in filter paper-dried urine samples compared to those of the original (frozen and thawed) urine samples. Left figure parts show comparisons between the contents of eluents of 1-day-old filter paper and original urine collected during the menstrual cycle and both early and mid-pregnancy. Right figure parts show comparisons between the contents of eluents of 1-year-old filter papers and original urine samples collected during the menstrual cycle and early pregnancy. For abbreviations, see Fig. 4.1

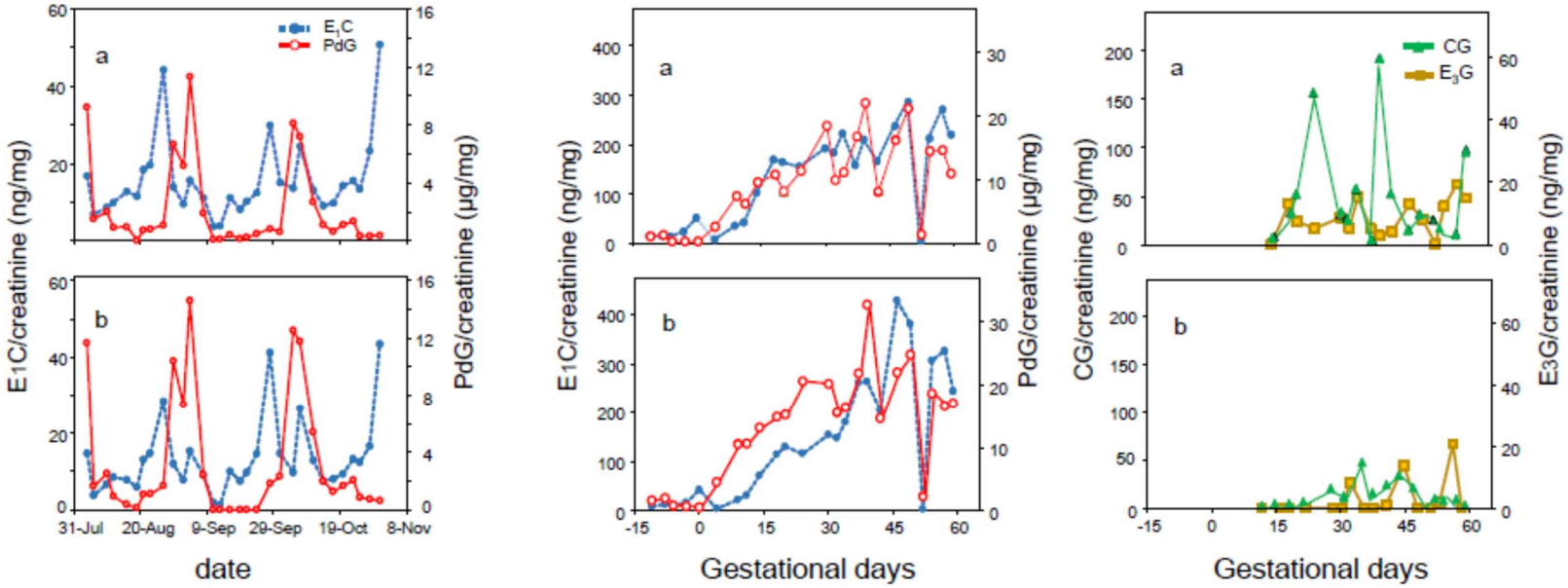


Fig. 4.3 Hormone profiles of the urine of chimpanzee A collected during its menstrual cycle (left figure parts) and early pregnancy (center and right figure parts). Measurements reflect analyses of a: original urine samples, and b: dried urine eluted from 1-year-old filter papers. Day 0 E_1C peak preceding conception (center and right figure parts). For abbreviations, see Fig. 4.1

Supplementary file 4.1

Further details of parallelism and recovery tests to assess the applicability of E_1C, PdG, and E_3G EIA for use with chimpanzee original urine samples

Supplementary Methods

Samples

Thawed urine samples from three female chimpanzees.

Linearity

I serially diluted urine samples containing high E_1C, PdG and E_3G concentrations with deionized water. The slopes generated from absorbance of serial dilutions of urine samples were compared with the slope of a standard curve for each EIA.

Recovery

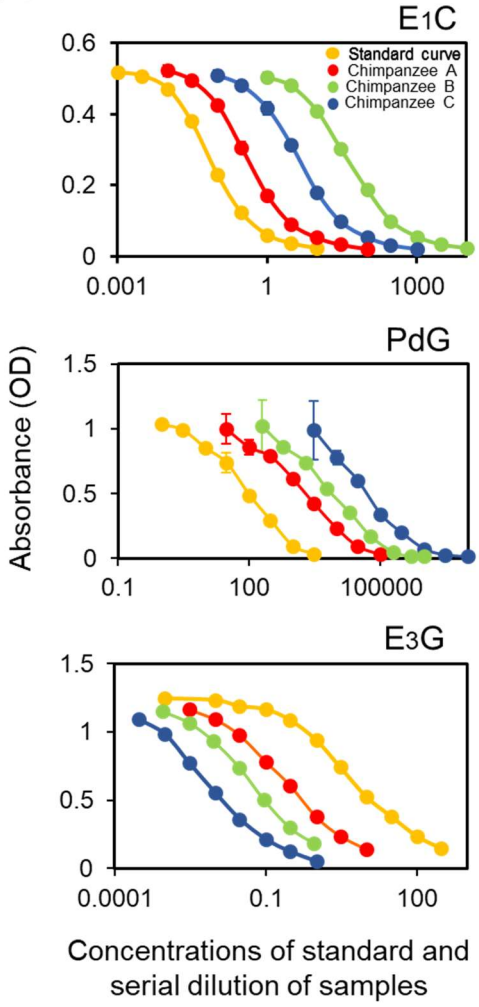
I added zero, low, medium, and high doses of hormone standards to urine samples containing low in endogenous steroids from three chimpanzees. Additions were prepared in deionized water and added as 10% of the specimen volume. The results were expressed as the percentage of the actual added amount that is recovered by the assay.

Supplementary Results

Linearity

Figure S 4.1 depicts the results of the linearity of the three steroid metabolites from the original chimpanzee urine. The slopes generated from serially diluted original urine (three individuals for each) were not significantly different from the standard curve slopes of E_1C, PdG, and E_3G, respectively (ANCOVA, range of *P* value was from 0.539 to 0.956).

Fig. S 4.1



Recovery

The mean ± SD recovery values for E_1C, PdG, and E_3G were 117.5 ± 16.2 %, 99.5 ± 17.0 %, and 110.5 ± 28.0 %, respectively.

Supplementary file 4.2

Further details of preliminary tests to determine the appropriate elution conditions for urine samples stored on filter paper

Supplementary Methods

Urine samples
I used pooled samples across the 3 reproductive stages collected from three female chimpanzees. Reproductive stages include the follicular, luteal, and early pregnancy periods.

The Intensity of the shaking test
To test the influence of shaking intensity on hormone extraction, halved filter papers containing 150 µl of urine were folded and put into a plastic test tube with 1.5 ml of deionized water. I eluted the urine contents under the following 6 conditions.

I. Remain still without shaking for 2 hours
II. Rotate for 2 hours on the rotator
III. Shake at 40 rpm for 2 hours on the reciprocal shaker
IV. Shake at 150 rpm for 2 hours on the reciprocal shaker
V. Shake at 150 rpm for 30 min. on the reciprocal shaker
VI. Shake at 210 rpm for 2 hours on the reciprocal shaker

Supplementary Results

Table S 4.2 details the results of my preliminary analyses to determine the best elution conditions.

Table S 4.2: Comparative elution tests using urine of chimpanzees

	Elution efficiency (mean ± SD %)					
	I	II	III	IV	V	VI
E_1C	91.9 ± 6.1	89.5 ± 9.1	81.9 ± 7.0	85.8 ± 8.7	84.5 ± 11.1	83.1 ± 7.7
PdG	82.7 ± 14.2	94.3 ± 10.7	91.9 ± 21.7	102.5 ± 19.6	93.0 ± 22.3	97.2 ± 16.5
Creatinine	104.1 ± 2.6	109.2 ± 10.5	106.0 ± 10.1	101.8 ± 9.3	98.1 ± 2.1	102.3 ± 5.4

There were no significant differences among the elution tests for E_1C and creatinine recovery. PdG recovery without shaking was slightly lower. Condition VI caused the filter paper fibers to decay. I concluded that the elution condition "shaken on the reciprocal shaker at 150 rpm for 2 hours" was adequate for my study.

Further details of preliminary tests to determine the appropriate elution conditions for urine samples stored on filter paper for CG EIA

Supplementary Methods

Urine samples

I used 8 samples during gestational day (GD) 3 through 59 in early pregnancy collected from one female chimpanzee.

Elution procedure

Halved filter papers containing 150 µl of urine were folded four and put into the 2 ml microtube with 1.5 ml of deionized water. The filter paper was submerged completely under deionized water. The tubes stayed at 4°C overnight. On the following day, the tubes were vortexed briefly and the elution was used for the CG EIA.

Supplementary Results

Recovery for the gentler elution method was 3.6 ± 2.6% (mean ± SD) and levels of CG were extremely low and nearly undetectable. As compared to shaking elution methods, this gentler method showed lower recoveries.

Chapter 5

General Discussion

5.1 Metabolites in Excreta as Alternative Indicators of Hormones in Blood

In primate females, the follicle biosynthesizes and secretes estradiol (E_2) into the bloodstream in the process of follicle maturation. Subsequently, when the ovum is released, the follicle cells luteinize and produce progesterone (P_4) for approximately two weeks (Watanabe et al. 1990; Hillier et al. 1994; Christensen and Stouffer 1997; Weinbauer et al. 2008; Shimizu, 2008; Young and McNeilly 2010). Once the ovum is fertilized, the corpus luteum extends the duration of P_4 and E_2 production to support pregnancy. Hormone levels and their fluctuations throughout pregnancy vary among species, but in all primates examined thus far, there is consistent production of the same or higher levels of P_4 as observed during their peak levels in the luteal phase (Chambers and Hearn 1979; Nozaki et al. 1990). Additionally, high levels of estrogen persist until parturition (Tulchinsky and Hobel 1973; Bosu et al. 1973; Reyes et al. 1975; Pepe and Albrecht, 1995; Shimizu et al. 2002). It was anticipated that these distinctive patterns of steroid hormones in the blood during the ovulatory cycle and pregnancy would likewise be reflected in those of the excretory metabolic forms.

Previous studies with macaques have reported that the major metabolites of E_2 were two estrone monoconjugates (E_1C) in urine and unconjugated estrone (E_1) in feces (Shidelar et al. 1989, 1993a, 1993b). Metabolites of P_4 in the urine are monoconjugates

of various 20α-hydroxy C21 compounds (Shidelar et al. 1989, 1993a, 1993b). In feces, it has been reported to be unconjugated pregnanediol (Shidelar et al. 1989, 1993a, 1993b; O'Neil, 2004). Placenta-derived E_3 is excreted in large amounts in feces and urine during human pregnancy (Adlercreutz and Martin 1976). However, in pregnant Japanese macaques, it was not clear whether E_3 is absent or not in the feces and urine (Short and Eckstein 1961; Laumas, 1965) or, when present, in what excretion pattern throughout their pregnancy (Hopper and Tullner 1967).

In Chapter 2, I developed three enzyme immunoassays (EIAs) for E_1C, PdG, and E_3G as urinary and fecal metabolites of E_2, P_4, and E_3 that I expected to be secreted into the blood during the ovulatory cycle and pregnancy in Japanese macaques. It was observed that the antibodies employed in these EIAs exhibited cross-reactivity with both conjugated and unconjugated metabolites in both urinary and fecal samples. Furthermore, in Chapters 2 and 4, these EIAs demonstrated parallelism and reproducibility in the measurement of urine and fecal samples in Japanese macaques, as well as urine samples in chimpanzees.

The fluctuations of E_1C and PdG in the excreta of Japanese macaques and chimpanzees during the ovulatory cycle were examined by the developed EIAs. In both species, E_1C began to rise approximately 1 week from the first day of menstrual bleeding, followed by an increase in PdG when E_1C peaked and subsequently decreased, showing typical ovulatory cycle changes. However, a gradual rise in E_1C during the luteal phase was observed in chimpanzees but this pattern was not recognized in Japanese macaques. The results for E_1C in excreta were consistent with a previous study that reported the absence of a secondary rise in E_2 in the blood during the luteal phase of Japanese macaques (Shimizu, 2008).

The levels of E_1C in both urine and feces of pregnant Japanese macaques gradually increased as the pregnancy progressed until parturition. In both urine and feces, PdG reached its peak during early pregnancy and sustained a gradual increase until parturition. The E_1C and PdG profiles in the urine of chimpanzees during early pregnancy resembled those of Japanese macaques, yet their rates of increase were more pronounced. Around gestational day 30, E_1C was approximately five times higher than the peak level at ovulation, and PdG was approximately twice the luteal phase level. Substantial quantities of E_3G were detected in the urine of pregnant chimpanzees. This suggests that assessing E_3G in excreta is valuable to detect pregnancy in wild chimpanzees. On the other hand, E_3G concentrations in Japanese macaques remained very low until mid-pregnancy. E_3 is biosynthesized in the placenta from DHEAS in fetal adrenal glands. The majority of DHEAS is converted to E_3, leading to a decrease in blood and urine DHEAS during pregnancy in humans (Yanaihara, 1993). Contrary to the typical pattern observed in humans, in our previous study comparing fecal DHEAS concentrations of pregnant Japanese macaques that gave birth to healthy and dead infants, we found DHEAS concentrations in females that gave birth to healthy infants, were elevated in late pregnancy (Takeshita et al. 2016). Considering low levels of excretory E_3 in early- and mid-pregnancy and a rise of fecal DHEAS in late pregnancy in Japanese macaques, this indicates that the main metabolic forms of fetal adrenal-derived DHEAS for them are different from that of humans and chimpanzees.

In addition, an EIA for DHEA/S in the blood, urine, and feces of Japanese macaques was established in Chapter 3. DHEAS, synthesized in the adrenal glands, serves as a reliable indicator for assessing adrenal function (Nasrallah and Arafah 2003). Moreover, DHEA/S functions as a precursor for intracrine synthesis of estrogen in the peripheral

tissues expressing aromatase (Labrie et al. 2005). It can be inferred that comprehending the dynamics of DHEA/S will not only help to evaluate adrenal function but also to understand the mechanisms of adrenarche onset and aging, given its role as a precursor for estrogen synthesis. Nevertheless, the relationship between the fluctuations of DHEAS in blood and excreta in Japanese macaques had not been clarified previously. Using this developed assay system, I quantified DHEAS in blood, urine, and feces of female Japanese macaques administered with DHEAS. My results revealed a strong correlation between DHEAS levels in blood and excreta. Based on this, I concluded that the determination of DHEAS levels in the excreta of Japanese macaques can be a good indicator of DHEAS levels in blood and adrenal gland status. To further investigate the effect of administered DHEAS on estrogen and progesterone biosynthesis, I measured E_1C and PdG concentrations in excreta. The results revealed pronounced peaks of E_1C and PdG in urine immediately after the administration of DHEAS, independent of ovarian function. This observed rise in estrogen metabolites suggests that DHEAS functions as a precursor in the steroid biosynthesis pathway. Moreover, one possible reason for the observed steep and sharp peak of urinary PdG is that the administration of large amounts of exogenous DHEAS might have temporarily suppressed the consumption of pregnenolone upstream of steroid biosynthesis in the Δ5 pathway. This could lead to an increased conversion of pregnenolone to progesterone and subsequently more excretion of PdG as its metabolite (Schiffer et al. 2019).

5.2 Technical Issues of EIAs for Metabolite Measurement

In Chapters 2 and 3, I established EIAs for steroid hormones using samples from Japanese macaques. For E_1C, PdG, E_3G, and DHEA/S EIAs, I employed polyclonal antibodies immunized with estrone-3-glucuronide-BSA, pregnanediol-3-glucuronide-BSA, estriol-6-carboxymethyloxime-BSA, and DHEA-3-succinate-BSA, respectively. These antibodies exhibit group-specific reactivity to various metabolites of each hormone, making them versatile EIAs capable of measuring metabolites in both urine and feces. In assay systems except for PdG EIA, corresponding enzyme-labeled steroids were employed, i.e. estrone-3-carboxymethylether-HRP for E_1C EIA; estriol-3-carboxymethylether-HRP for E_3G EIA; DHEA-3-glutarate-HRP for DHEA/S EIA, to increase sensitivity. In general, there are many ways to increase sensitivity. When the spacer is short, the affinity between the antibody and the enzyme-labeled steroid decreases, making it more likely for even small amounts of the measured hormone to react with the antibody due to competitive effects. Hence, in these studies, favorable sensitivity was achieved in the measurement systems for E_1C, E_3G, and DHEA/S.

5.3 The Collection, Storage, and Transportation of Excreta in Remote Field Sites

In Chapter 4, I demonstrated that E_1C, PdG, E_3G, CG, and creatinine could be measured in chimpanzee urine that was adsorbed onto filter paper, dried, and stored at room temperature for an extended period of time without the need for freezing or refrigeration facilities. The fluctuations in the hormonal changes showed no difference compared to those measured from frozen urine, even after long-term storage at room temperature. By using this method, it may be possible to estimate more detailed information such as

approximate ovulation date, miscarriage, etc., from urine adsorbed onto filter paper. The use of filter paper as a storage medium has the advantage of simplifying the storage of urine samples during extended field studies in remote areas. Moreover, it offers the ease and safety (without the need for organic solvents or dry ice) of transporting a large number of samples back to the laboratory at the end of a field season.

Collecting urine samples can be challenging for certain animal species. In such cases, to obtain a more accurate picture of long-term, cumulative hormone fluctuations, it might be preferable to utilize fecal samples for the analysis of hormones. I compared and verified the hormone levels extracted from two methods of storing fecal samples: (1) immediate freezing after collection and subsequent drying in an oven just before analysis, and (2) drying at room temperature in tubes containing silica gel (Fig. 5.1). The recovery of E_1C and PdG values measured from feces dried on silica gel was 131.3% and 112.7%, respectively, relative to values measured from frozen feces. These values demonstrated a strong correlation with the corresponding values from matched frozen feces (E_1C: r = 0.97, PdG: $r = 0.97$, $p < 0.05$) (Fig. 5.2) (Mouri et al. 2015 abstract).

5.4 Applications for Field Endocrinological Research of Wild Primates

The methods described in Chapters 2 and 4 were utilized to monitor reproductive status noninvasively and to reveal behavioral and endocrine interactions of wild primates in remote study sites.

In collaboration with Rigaill, I determined the reproductive status corresponding to behavioral and physical changes in wild female Japanese macaques on Koshima Island in Miyazaki Prefecture (Rigaill et al. 2015). Progesterone and estrogen metabolites were

quantified in 138 fecal samples from seven females to investigate their reproductive status and hormonal changes. Fecal samples were preserved using the previously mentioned silica gel method and E_1C and PdG levels were determined using the simple extraction method and the assay system described in Chapter 2. Presumed days of ovulation and conception were defined based on fecal PdG profiles. The first day of the luteal phase was identified by the day exhibiting a fecal PdG concentration at least two standard deviations above the mean PdG concentration for the preceding 3 to 4 days. The day of fertilization was defined as the last day of the fertile phase. The most probable day of fertilization for each focal female was determined using the hormonal data in conjunction with the day of delivery. In this way, the periods of high fertilization potential, one month and two months into pregnancy were each successfully determined from fecal extracts. The hormonal data corresponding to each period were then compared with the behavior of the females and the brightness-redness of their facial color. The concentration of PdG during pregnancy clearly decreased in the second month compared to the first month of pregnancy, while the concentration of E_1C remained constant. The hormonal fluctuations observed in wild Japanese macaques mirrored the trends identified in the six captive pregnant females described in Chapter 2 (Fig. 5.3). Furthermore, we found that Japanese macaque facial redness in Koshima rose slightly after conception and then distinctly decreased in the second month (Fig. 5.4; Rigaill et al. 2015). Facial redness is generally thought to be linked to estrogen and is considered a signal of estrus. However, we did not find a relationship between facial redness and hormonal changes or fertile timing (Rigaill et al. 2015, 2017).

Using the urine preservation method developed in Chapter 4, the hormonal changes of wild female bonobos were investigated. These field studies of wild bonobos were

conducted at Wamba in the northern sector of the Luo Scientific Reserve, Democratic Republic of the Congo (Hashimoto et al. 2022; Toda et al. 2022). As mentioned previously, urine samples, rather than fecal samples, were the optimal medium for monitoring the reproductive status of wild bonobos. Stable long-term storage of urine samples at Wamba, requiring the installation of electrical equipment and bringing in liquid nitrogen is costly. To overcome this challenge, the urine samples were dried and stored on filter paper. Subsequently, we extracted the urinary components from the filter paper transported from Wamba back to the laboratory using the methods outlined in Chapter 4 and analyzed the hormones using the developed EIAs as detailed in Chapter 2.

In a study with Hashimoto et al. (2022), I investigated hormonal fluctuations measured from urine samples of the subject during postpartum periods. The urinary profiles of E_1C and PdG from the samples dried on filter paper allowed the detection of 14 ovulatory cycles in five females. Figure 5.5 (Hashimoto et al. 2022) illustrates a composite profile of the 11 ovulatory cycles that did not result in pregnancy, based on the estimated ovulation day. Figure 5.6 (Hashimoto et al. 2022) shows the changes in E_1C and PdG concentrations during pregnancies in live birth and miscarriage. The variations in E_1C and PdG in those ovulatory cycles aligned with the general pattern reported in studies of hormone fluctuations measured from frozen stored urine samples in captive and wild bonobos (Heistermann et al. 1996; Douglas et al. 2016). This demonstrated the effectiveness of our methods for the storage of urine samples and analyses of reproductive hormones in monitoring the changes in concentration of these hormones in wild bonobos. In addition, the results of measuring urinary E_1C concentrations at the time of maximum sexual skin swelling (MS) revealed that E_1C concentrations at MS increased with time

after delivery and reached the highest point in the cycle that resulted in pregnancy (Fig. 5.7; Hashimoto et al. 2022).

In a study with Toda et al. (2022), I investigated hormonal changes in young female bonobos before and after dispersal. Given the low concentrations of reproductive hormones in young females, I adjusted the antibody dose at the solid phase to further enhance the sensitivity of the assay method described in Chapter 2. By employing these adjustments, we were able to estimate the timing of the onset of puberty and the first pregnancy of bonobos in the wild. Our finding indicated that ovulation and pregnancy signs are not detected until 1-2 years after a female disperses from her natal group (Fig. 5.8; Toda et al. 2022). Furthermore, we observed a difference in the rate of increase in urinary E_1C before and after dispersal. E_1C and copulation rates began to increase slightly before dispersal and increased significantly after dispersal (Fig. 5.9; Toda et al. 2022).

5.5 Contributions to Field Endocrinology and Future Directions

The present thesis developed and validated EIA systems using excreta, an extraction method without the use of volatile organic solvents, and established a sample preservation method that does not involve freezing for non-invasively analyzing endocrine levels in wild primates. These developed EIAs could monitor typical endocrine changes in ovulatory cycles and pregnancies from excreta in captive female Japanese macaques. In addition, changes in excretory DHEAS were consistent with those observed in blood. Metabolites of estrogen and progesterone, as well as CG, were quantifiable from urine stored for long periods on filter paper. Furthermore, the biosynthesis, metabolism, and excretion mechanisms of reproductive hormones, both within specific primate species

and across genera (i.e. *Macaca* and *Pan*) showed considerable diversity. As discussed in the previous sections, these included the gradual rise of estrogen during the luteal phase in chimpanzees, the difference in the rate of estrogen and progesterone increase in early pregnancy between Japanese macaques and chimpanzees, and the low synthesis rate of E_3G in the placenta of Japanese macaques. Notably, all of these variations were also detected in the excretory analysis.

In the history of field endocrinology, advances in the development of methods for extracting steroids from urine and feces have increased the options available to field workers, who can now select the most appropriate methodology for their study subjects, field conditions, and specific research questions (Strier and Ziegler 2005). Field endocrinology can be regarded as a non-invasive methodology for examining the interaction between behavior and endocrinology in primates living in natural conditions and social settings (Hodges and Heistermann 2011). In other words, it should not be forgotten that field endocrinology is made possible by advances in methodological research. The developed endocrine measures that reflect species-specific dynamic variations, and the use of long-term storage methods for samples in the field, have provided new insights, offering more refined interpretations of behavioral data in studies involving wild primates. In this sense, these lab-developed and validated methods contribute greatly to the study of primate behavior and endocrinology in the wild, by providing an effective means of revealing accurate reproductive status and long-term endocrine dynamics in field sites without electrical facilities.

In the future, I want to validate methodologies for which established reliable methods are currently not available for measuring other steroid hormones associated with sexual behavior and develop long-term storage techniques for peptide and protein hormones.

This pursuit will provide an approach for elucidating diverse aspects of wild primate behavior. In conclusion, collaborative endeavors with laboratory-based techniques are essential to comprehensively understand animals in their natural habitat. An integrated approach with new methodological advancements in non-invasive endocrine analysis will greatly contribute to the elucidation of behavioral endocrine interactions in wild primates.

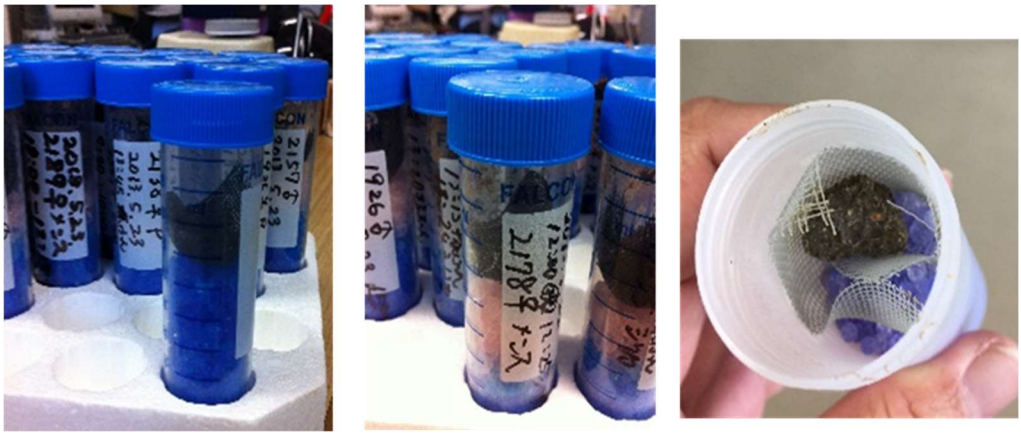


Fig. 5.1 Fecal samples were stored in tubes containing 35 mL of silica gel (left). After approximately one week of storage, the fecal samples dried completely, and the silica gel changed to pink color (center). Dried fecal sample in the tube (right)

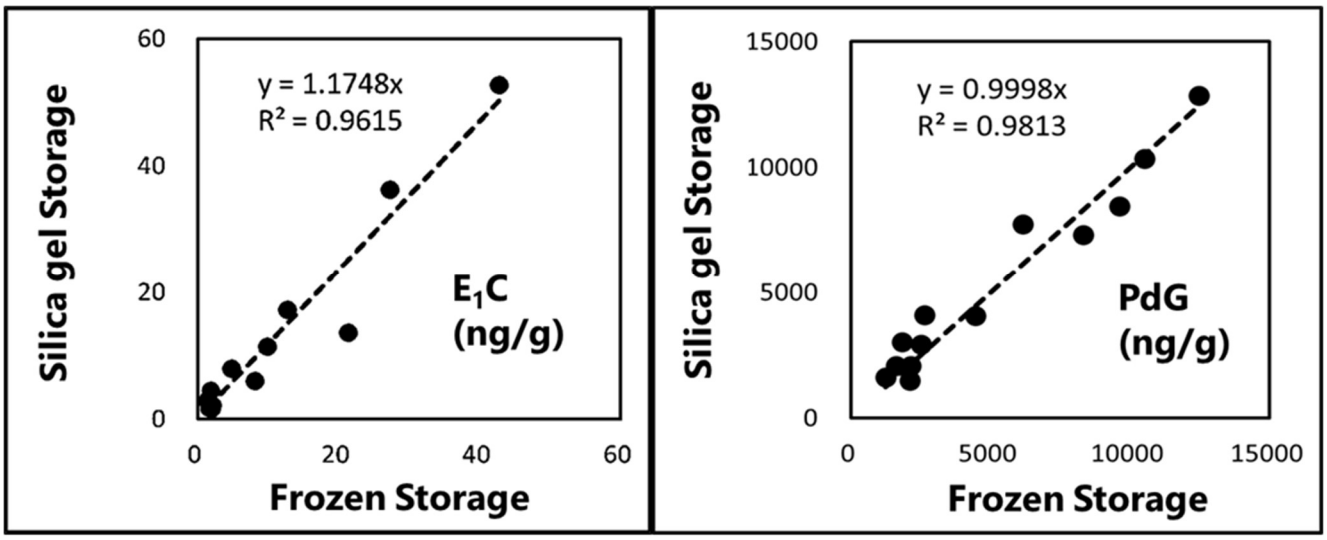


Fig. 5.2 The relationship between the amount of hormone extracted after freezing feces immediately after collection and drying in an oven (horizontal axis) and the amount of hormone extracted after drying in silica gel tubes (vertical axis) (left panel: E_1C, right panel: PdG)

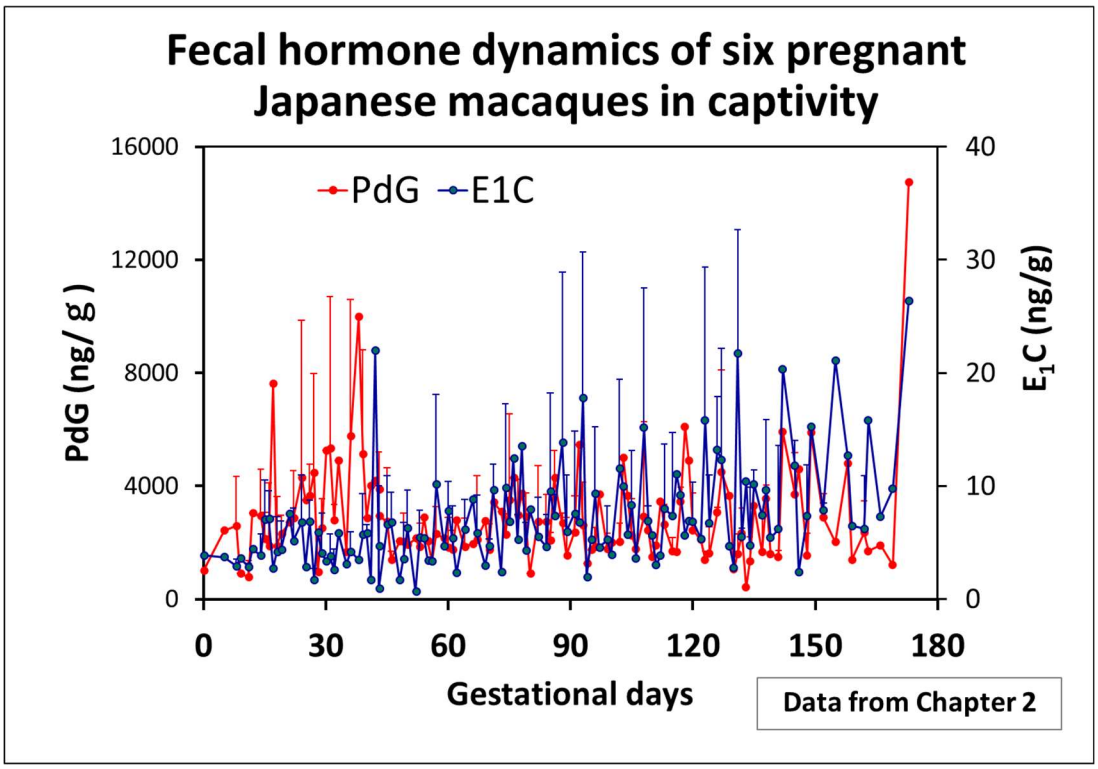


Fig. 5.3 Composite fecal E_1C and PdG concentrations of six captive pregnant Japanese macaques based on estimated conception days. Data from Chapter 2.

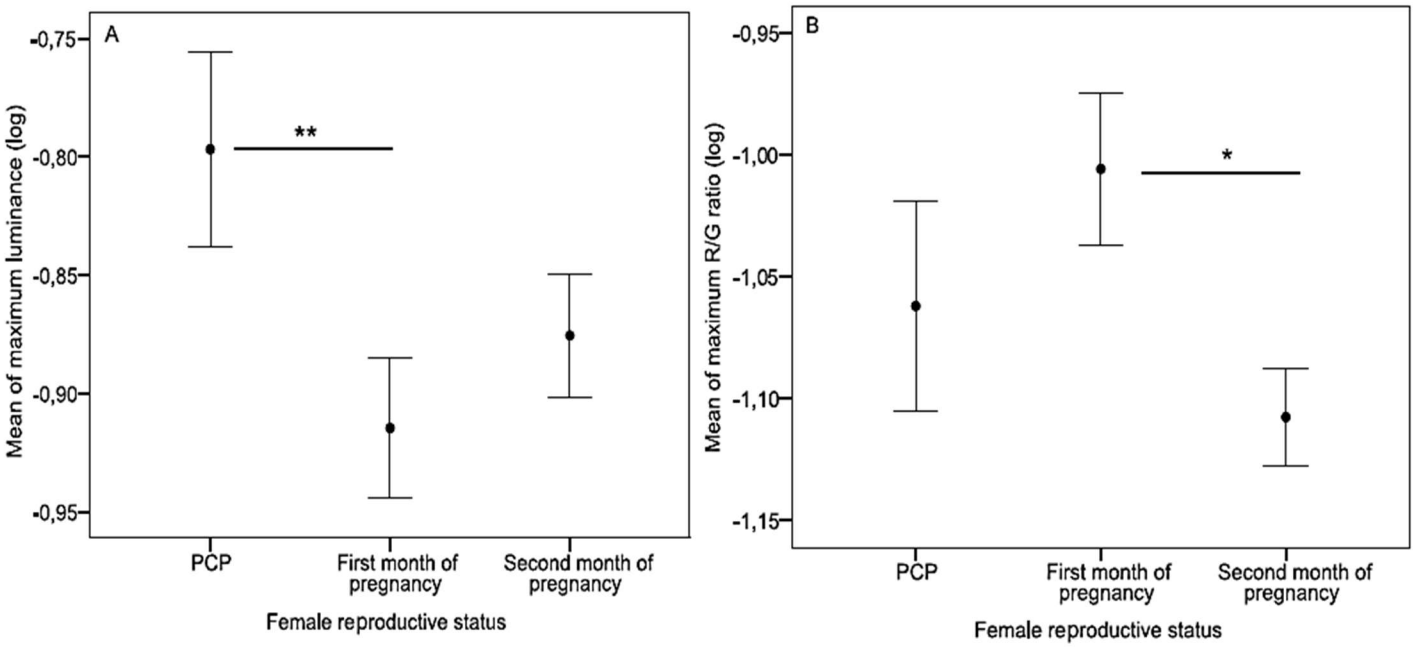


Fig. 5.4 Female facial color features between pre- and post-conceptive phases. Values represent the mean (± standard error of the mean) of maximum luminance (A) and R/G ratio (B) (* $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$). (Reproduced from Rigaill et al. 2015)

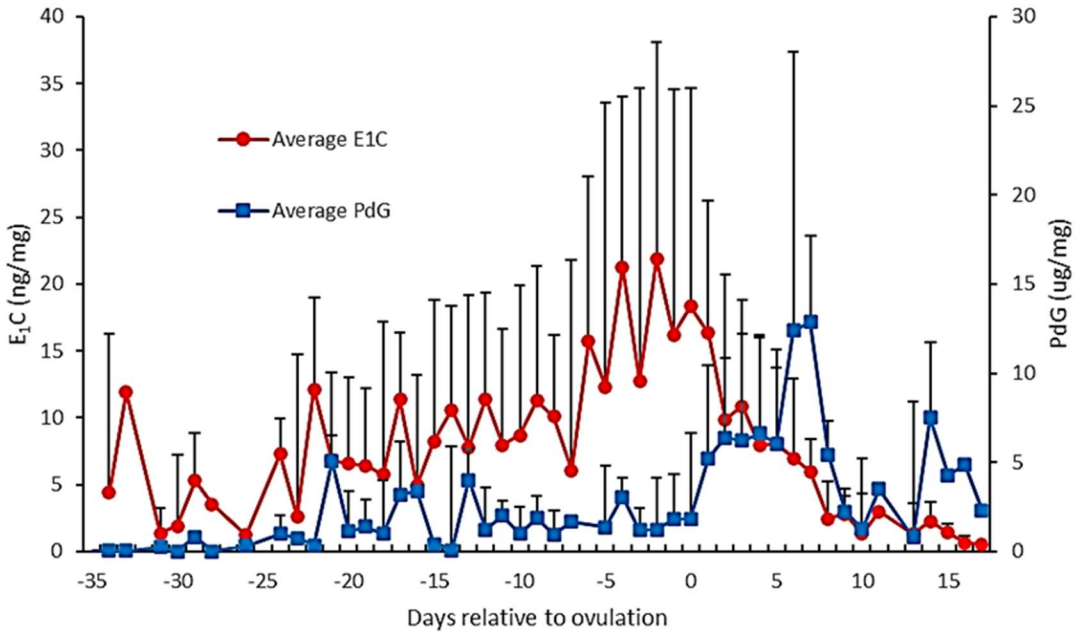


Fig. 5.5 A composite profile of E_1C and PdG concentrations during non-conception cycles relative to the estimated day of ovulation. Error bars indicate standard deviation. Day 0 is the day of ovulation (Reproduced from Hashimoto et al. 2022).

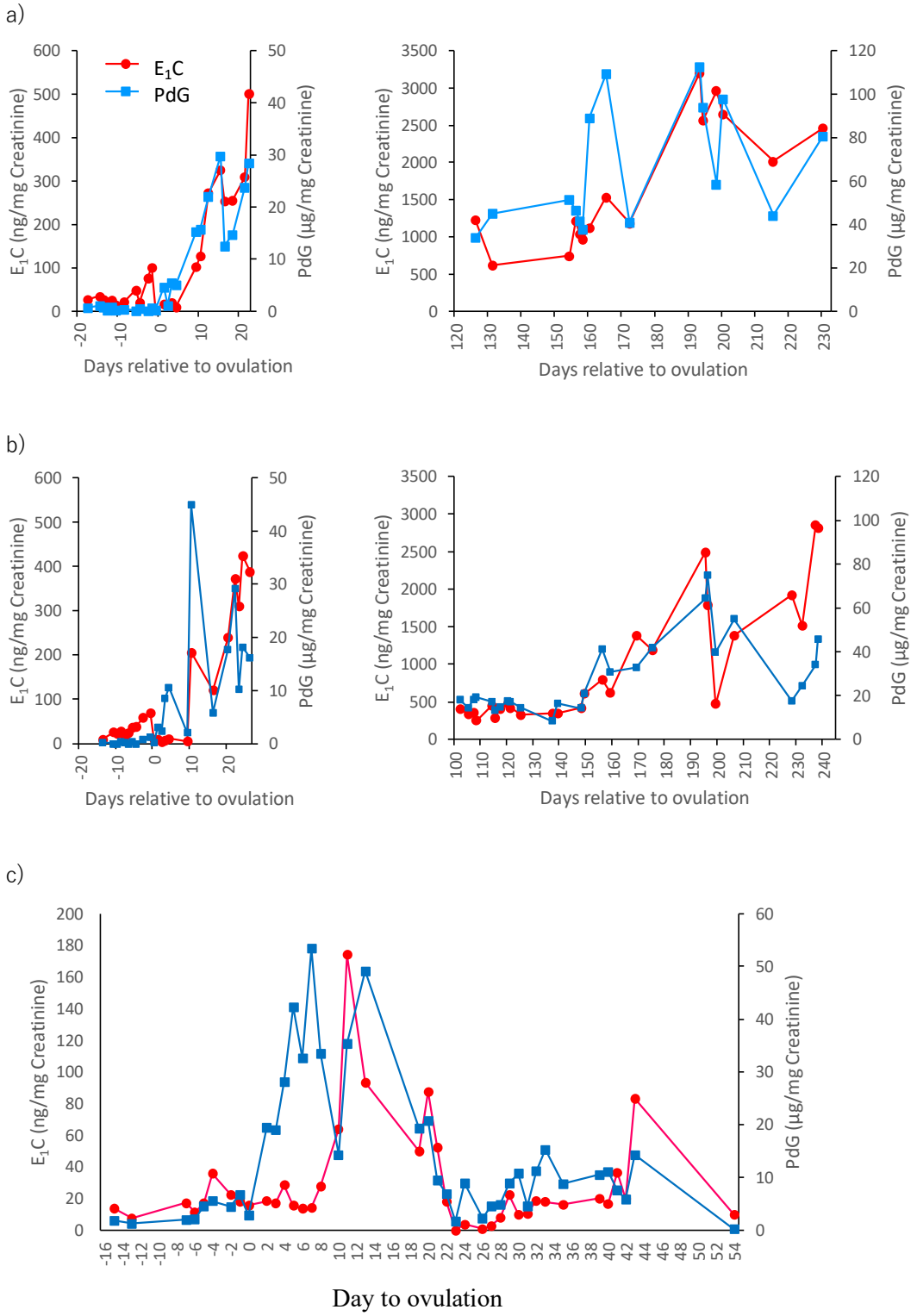


Fig. 5.6 Changes in E_1C and PdG concentrations during a) pregnancy by Otomi in August 2014, b) pregnancy by Nao in August 2014, and c) miscarriage by Nao in January 2014 (Reproduced from Hashimoto et al. 2022).

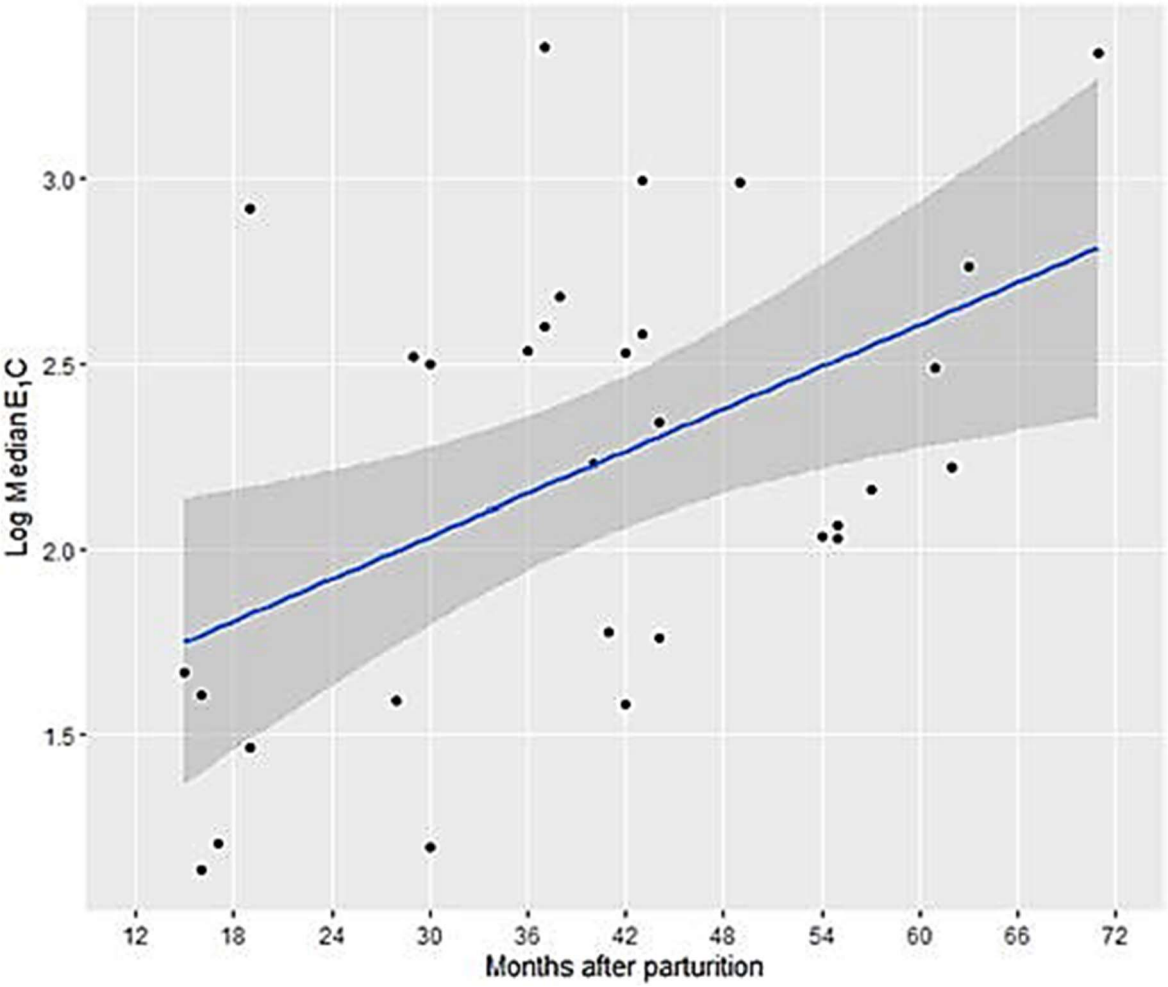


Fig. 5.7 Changes in E_1C concentrations with time since parturition (model 4). The x-axis shows the duration in months from parturition. The y-axis shows the median E_1C value during MS for a given female in a given 1-month period (N = 47 for eight interbirth intervals of eight females). We log-transformed the value to fit the normal distribution. The blue line shows the fit of the model including the linear term of the number of months after parturition, and the grey shading shows the 95% confidence area of the fitted line (Reproduced from Hashimoto et al. 2022)

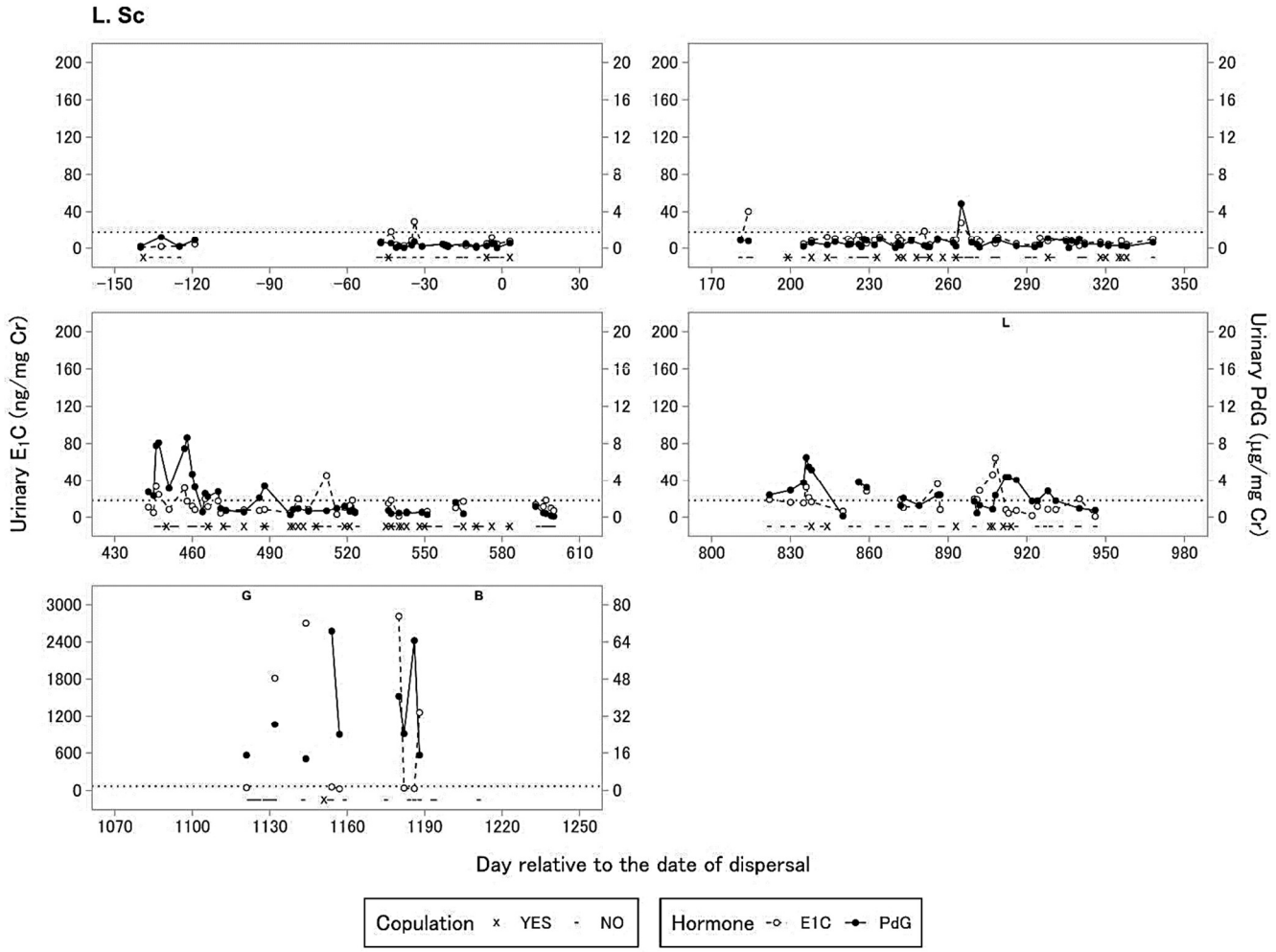


Fig. 5.8 Daily hormonal and behavioral profiles for a representative female bonobo sampled before and after dispersal. Each point shows urinary E_1C (white) and PdG (black) levels, corrected by creatinine (Cr). The lines between neighboring points are connected if they are within 10 days od each other. The horizontal dotted line represents the threshold for PdG levels. The cross mark and horizontal bar at the bottom represent the presence and absence of copulations with mature males on observational days. Capital letters at the top indicates the first day that the luteal phase (L) and gestation (G) were estimated to begin, as well as when newborn infants (B) were confirmed (Reproduced from Toda et al. 2022).

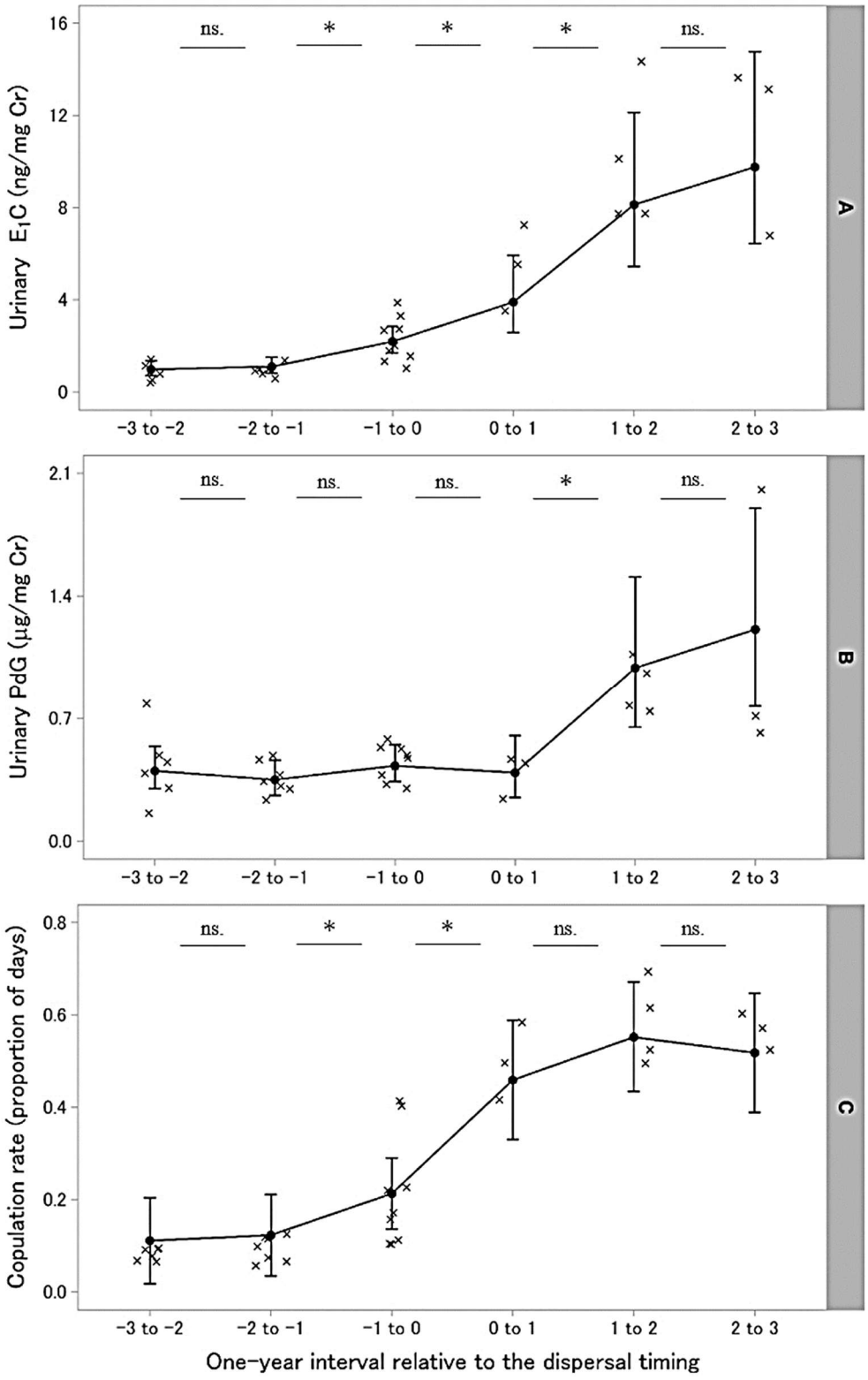


Fig. 5.9 The geometric mean of urinary E_1C (A) and PdG (B) concentrations corrected by creatinine (Cr), and (C) the arcsine-square-root transformed proportion of copulation rates for each female bonobo, aggregated for one-year intervals based on dispersal timing (Reproduced from Toda et al. 2022).

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Chapter 3

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- 第 2 章　JVMS 掲載論文のリポジトリについて
  Blue (査読後論文)、出版社版利用可能　公開場所　機関リポジトリ可　出典表示を行うこと

  著者：Shimizu, K, Mouri, K.
  タイトル：Enzyme immunoassays for water-soluble steroid metabolites in the urine and feces of Japanese macaques (Macaca fuscata) using a simple elution method
  掲載雑誌：The Journal of Veterinary Medical Science
  番号：80(7): 1138–1145, 2018
  doi: 10.1292/jvms.17-0507

- 第 3 章　GCE (Elsevier)
  DOI を明示すること

  著者：Mouri, K., Shimizu, K.
  タイトル：Dehydroepiandrosterone sulfate (DHEAS) in excreta is a good indicator of serum DHEAS in Japanese macaques (Macaca fuscata)
  掲載雑誌：General and Comparative Endocrinology
  番号：338 (2023) 114277
  doi.org/10.1016/j.ygcen.2023.114277

- 第 4 章　Primates　著者の再利用
  著者は、自分の論文の記録バージョンの全部または一部を自分の論文で再利用する権利を有します。さらに、授与学術機関の要求に応じて、シュプリンガー・ネイチャーの内容を含む論文を複製し、利用可能にすることができます。著者は、現在の引用基準に従って、論文の中で出版された論文を適切に引用する必要があります。
  「著者、タイトル、雑誌タイトル、出版年 [年]、[出版社 - 著作権ページに表示されているもの]」

  著者：Mouri, K., Shimizu, K.
  タイトル：Stability of chimpanzee (Pan troglodytes) urinary reproductive hormones during long-term preservation on filter paper.
  掲載雑誌：Primates 62, 289–296
  出版年：2021.
  doi.org/10.1007/s10329-020-00864-9

- 第 5 章　Pros one PLOS 記事コンテンツの再利用

PLoS ONE
Title: Multimodal Advertisement of Pregnancy in Free-Ranging Female Japanese Macaques (Macaca fuscata).
Author: Rigaill L, MacIntosh AJJ, Higham JP, Winters S, Shimizu K, Mouri K, et al.
Publication: PLoS ONE 10(8): e0135 (2015)
https://doi.org/10.1371/journal.pone.0135127

Primates
Title: Testing for links between face color and age, dominance status, parity, weight, and intestinal nematode infection in a sample of female Japanese macaques.
Author: Rigaill, L., MacIntosh, A.J.J., Higham, J.P. et al.
Publication: Primates 58, 83–91 (2017).
https://doi.org/10.1007/s10329-016-0575-6

Title: Physical, behavioral, and hormonal changes in the resumption of sexual receptivity during postpartum infertility in female bonobos at Wamba.
Author: Hashimoto, C., Ryu, H., Mouri, K. et al.
Publication: Primates 63, 109–121 (2022).
https://doi.org/10.1007/s10329-021-00968-w

Hormones and Behavior
Title: Do female bonobos (Pan paniscus) disperse at the onset of puberty? Hormonal and behavioral changes related to their dispersal timing
Author: Kazuya Toda, Keiko Mouri, Heungjin Ryu, Tetsuya Sakamaki, Nahoko Tokuyama, Takumasa Yokoyama, Shohei Shibata, Marie-Laure Poiret, Keiko Shimizu, Chie Hashimoto, Takeshi Furuichi
Publication: Hormones and Behavior
Publisher: Elsevier
Date: June 2022
https://doi.org/10.1016/j.yhbeh.2022.105159