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Physiological Cycle of Adult Females and the Sex Identification of Juveniles in Hainan Gibbons: A Preliminary Study

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***Nomascus hainanus*, Fresh feces, Sex hormone, Physiological cycle, Sex determination**

ABSTRACT

In this study, feces samples were collected from 7 individuals in Hainan gibbon (*Nomascus hainanus*) family group C by through non-invasive methods, and the levels of progesterone and testosterone were analyzed by high-performance liquid chromatography mass spectrometry. The results showed that the menstrual cycle of adult females in Hainan gibbon group C was 32±3.6 days and the duration of the menstrual period was 10±2.4 days. There was no significant difference in progesterone levels between individuals of different age and sex classes, no significant periodicity in the change of testosterone in the feces samples of Hainan gibbon group C, and no significant difference in the level of testosterone between individuals of the same sex and age. However, there was a significant difference in testosterone levels between adult males and adult females. By comparing the levels of testosterone in different individuals, it could be hypothesized that both juveniles in group C may be males. The results show that the circadian cycle of adult females and the sex identification of juveniles in Hainan gibbons may be inferred from differences in fecal progesterone and testosterone levels.

INTRODUCTION

Hainan gibbon (*Nomascus hainanus*), the most endangered ape in the world, belongs to the family Hylobatidae and the genus *Nomascus* (i.e., crested gibbons) (Chan, 2017). The Hainan gibbon is classified as critically endangered by the IUCN Red List and is regarded as a first-class protected animal in China (Chan, 2017; Geissmann and Bleisch, 2020). The Hainan gibbon is endemic to China, and the remaining population inhabits the Bawangling part of the Hainan Tropical Rainforest National Park (HTRNP), covering an area of about 16 square kilometers (Guo *et al*., 2021). Currently, there are only 36 Hainan gibbons, of which 31 inhabit 5 family groups and 5 are solitary (The People's Government of Hainan Province, 2022).

At present, there is very little work done on endocrine and physiological health monitoring of Hainan gibbons, and only a few studies on gut microbial diversity of some individuals of the Hainan gibbons (Li *et al*., 2022; Yang *et al*., 2022). Studies have estimated the gestation period of Hainan gibbons to be 136-173 days, and females have given birth every two years (Zhou *et al*., 2008). The first reproductive age of Hainan gibbons is late (9-10 years old) compared to other primates, and the reproductive rate is relatively slow (Inter-birth interval= 24-42 months) (Fan *et al*., 2021; Reichard and Barelli, 2008; Zhou *et al*., 2008). However, the monitoring of sex hormone levels in Hainan gibbons is still absent from literature. Monitoring the reproduction and stress physiology of endangered wild animals living in natural habitats can be achieved by collecting feces and other samples to detect steroid

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metabolites through non-invasive methods (Hodges and Heistermann, 2003). Fecal matter is a source of DNA in field samples and has a promising application in the study of rare and endangered wild animals with high vigilance and small population numbers. The feces of most vertebrates contain metabolized forms of major steroid hormones (e.g., progesterone, testosterone, etc.), which are secreted into the intestine via bile (Touma *et al*., 2005; Heistermann *et al*., 2006; Higham *et al*., 2012), these metabolites can be measured by hormone assay techniques.

Monitoring the levels of progesterone and testosterone in the feces of Hainan gibbons is of great significance. Progesterone is an essential substance for reproductive function in female organs such as the uterus, ovary, and mammary gland. It also plays an important role in non-reproductive tissues such as the brain, cardiovascular system, bone, and central nervous system. Additionally, progesterone plays an important role in the metabolism of steroid hormones. Progesterone is a C-21 steroid hormone involved in the female menstrual cycle, gestation (promoting pregnancy), and embryonic development in humans and other animal species (Wishart *et al*., 2007). Testosterone is the major sex hormone that affects male vertebrate development, including growth, maturation, reproduction, immunity, and aging (Dixson, 1998; Heyland *et al*., 2005; Reed *et al*., 2006). Testosterone also affects male behavior, promoting territorial aggression (Wingfield *et al*., 1990), as well as courtship and sexual behavior (Wiley and Goldizen, 2003). The reproductive behavior and outcome of non-human primates are mostly determined by the female's estrus status. The reproductive strategy of non-human primates can be understood by monitoring the female's sex hormone levels. Previous research has been conducted in other non-human primates, for example, Japanese macaques (*Macaca fuscata fuscaca*) (Aso *et al*., 1977), François langurs (*Trachypithecus françoisi*) (Wang *et al*., 2006), and Yunnan snub-nose monkeys (*Rhinopithecus bieti*) (Xia *et al*., 2016). Yet, the research on the determination of sex hormones in Hainan gibbon feces is still understudied.

A previous study about estradiol (E2) and progesterone (P4) from the feces of primates showed a correlation between feces progesterone levels and serum progesterone levels in yellow baboons (*Papio cynocephalus*) (Wasser *et al*., 1988). Another study found that there is also a correlation between the concentration of testosterone in plasma and feces in male Japanese monkeys (*Macaca fuscata*) (Barrett *et al*., 2002). These indicate that the measurable changes in sex hormone in feces can reflect the changes of sex hormone in plasma in primates.

Liquid chromatography-mass spectrometry (LC-MS) has been validated as a method for studying steroid metabolites in primate urine and feces (Hauser *et al*., 2008; Weltring *et al*., 2012). The advantage of this method is that simultaneous measurements of different compounds in the same sample can be performed with high specificity (Murtagh *et al*., 2013). Therefore, conducting experiments in this way can save scarce samples like the feces of Hainan gibbons, thus ensuring the successful completion of the experiment. This is the first study to analyze the sex hormone levels in the feces of Hainan gibbons by non-invasive sampling. The aim of this study was to provide a methodological basis for feces-endocrine monitoring to facilitate further research on this species. We analyzed testosterone and progesterone, which provided the basis for the reproductive biology of Hainan gibbons.

MATERIALS AND METHODS

Study site

Bawangling National Nature Reserve (BNNR), the core area of HTRNP, is located in Hainan Province, China. The geographical coordinates are 18°57 'N- 19°11' N and 109°03 'E - 109°17'. The reserve has a total area of 29,998 hectares and an altitude of 350 to 1438 meters above sea level, with a tropical monsoon climate (Deng and Zhou, 2018).

Target animals

Seven Hainan gibbons from family group C in the core area of HTRNP were involved in this study, including adult females CF01 and CF02, adult males CM01, CM02, and CM03, and juveniles CJ01 and CJ02. Among them, adult females CF01 and CF02 were female gibbons with reproductive experience, and adult male CM01 was the male leader of the family group (Fig. 1).

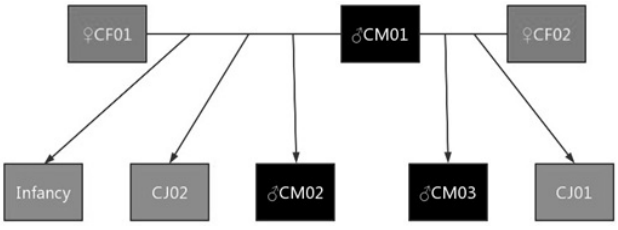

Fig. 1. Social structure of Hainan gibbon group C

Reagents and instruments

The analytically pure formic acid used in this experiment was purchased from Sigma (St. Louis, MO, USA). Ms. Pure methanol, acetonitrile, and ethyl acetate were purchased from thermo-fisher scientific (FairLawn, NJ, USA). Target hormone standards were purchased from Sigma. Ultrapure water was prepared by a Millipore

Reference ultrapure water system (Billerica, MA, USA) equipped with a 0.22μm filter head for LC-MS. The hormone standards were accurately weighed and dissolved in ethanol to form a master batch at a concentration of 5.0 mg/ml and subsequently diluted with 20% methanolic water to obtain a standard curve at a range of concentrations from 0.01, 0.05, 0.1, 0.5, 1, 5, 15, 40, 80, and 200 nmol/L. In this project, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (ACQUITY UPLC-XEVO TQS, Waters Corp., Milford, MA, USA) was used to detect hormones.

Sample collection and processing

Sample collection was carried out from late May to mid-November 2021. During sample collection, we followed the gibbons closely and identify individuals. Fresh feces were collected in sterile petri dishes at the moment of defecation by the gibbons. After removing the contaminated part with a sterile scalpel, the remaining uncontaminated part was put into a labled sterile tube and stored in liquid nitrogen.

The lyophilized stool (10 mg) was hemogenized in a centrifuge tube for 3 min (BB24, Next Advance, Inc., Averill Park, NY, USA) with 10 grinding beads and 50 μL deionized water, which was followed by addition of 200 μL deionized water and homogenization for 3 min, 50 μL methanolic solutions containing the internal standard to the standard curve working solution, the feces sample and 750 μL methyl tert-butyl ether was added. The extract was shaken and the mixture was centrifuged at 2000 rpm for 3 min at room temperature (MSC-100, Hangzhou Allsheng Instrument Co., China) at 18,000g for 10min at 10°C (Microfuge20R, Beckman Coulter, Inc., Indianapolis, IN, USA), 650 μL supernatant was aspirated into a 96-well plate and blow dried with nitrogen. The extract was resolubilize in 80 μl 50% methanol water and shaken at 1450 rpm for 20 min at 10°C, Centrifuged at 4000g for 20min (Allegra X-15R, Beckman Coulter, Inc., Indianapolis, IN, USA). Tested on the machine.

Sample thawing was done on an ice bath to avoid changes in metabolite composition and concentration caused by the activation of metabolic enzymes after the sample has sharply returned to room temperature. The reagents used for extraction were pre-frozen and stored at -20°C to avoid the degradation of small molecular metabolites in biological samples due to the exothermic process of adding organic solvents to precipitation proteins.

Data processing

The raw data files generated by UPLC-MS/MS were processed using MassLynx software (version 4.1, Waters, Milford, MA, USA) to integrate, calibrate, and quantify peaks for each metabolite. The data were processed by Excel and SPSS 13.0 software, and the correlation analysis was used to test the correlation of feces sex hormone levels and calculate the sexual cycle time. The significant level was set at $P < 0.05$.

RESULTS

Data analysis of progesterone

The trimmed mean was calculated after measurement and removal of the highest and lowest values in each individual data, which may deviate from the normal value. It was found that the variation range of adult female CF01 progesterone was 2.32-133.88 pmol/g (per gram of dry feces) (Table I), and the trimmed mean was 20.64± 19.89 pmol/g; the range of adult female CF02 progesterone ranged from 1.85 to 43.69pmol/g, and the trimmed mean was 13.07±11.21 (Table I). According to Tables II and III, the menstrual cycle of adult female CF01 was 30± 3 days, and the duration of the menstrual period was 10±2.6 days (Fig. 2A); the menstrual cycle of adult female CF02 was 35±2.5 days, and the menstrual duration was 10±1.0 days (Fig. 2B).

Table I. The progesterone level variation range in Hainan gibbon group C.

Individual	Trimmed mean (pmol/g)
CF01	20.64±19.89 (2.32-133.88)
CF02	13.07±11.21 (1.85-43.69)
CM01	16.15±8.74 (2.37-45.44)
CM02	14.21±10.79 (2.44-65.65)
CM03	9.13±6.61 (1.90-34.73)
CJ01	11.38±9.00 (0.85-33.76)
CJ02	14.20±11.69 (2.75-49.66)

Table II. The menstrual cycle of adult female in Hainan gibbon group C according to the progesterone level.

Individual	Date of progesterone peak to next peak	Menstrual cycle (d)
CF01	6.04-7.07	33
CF01	7.07-8.03	27
CF02	6.28-8.04	37
CF02	8.04-9.05	32
Maximum value (d)		27
Minimum value (d)		37
Average value (d)		32±3.6

Table III. Length of menstruation in adult female

Hainan gibbon group C based on progesterone projections.

Individual	Date of progester-one peak	Progesterone peaks appear to the end time	Duration of menstrua-tion (d)
CF01	6.04	5.30-6.13	14
CF01	7.07	7.03-7.12	9
CF01	8.03	8.01-8.09	8
CF02	6.28	6.24-7.03	9
CF02	8.04	7.29-8.09	11
Maximum value (d)			14
Minimum value (d)			8
Average value (d)			10±2.4

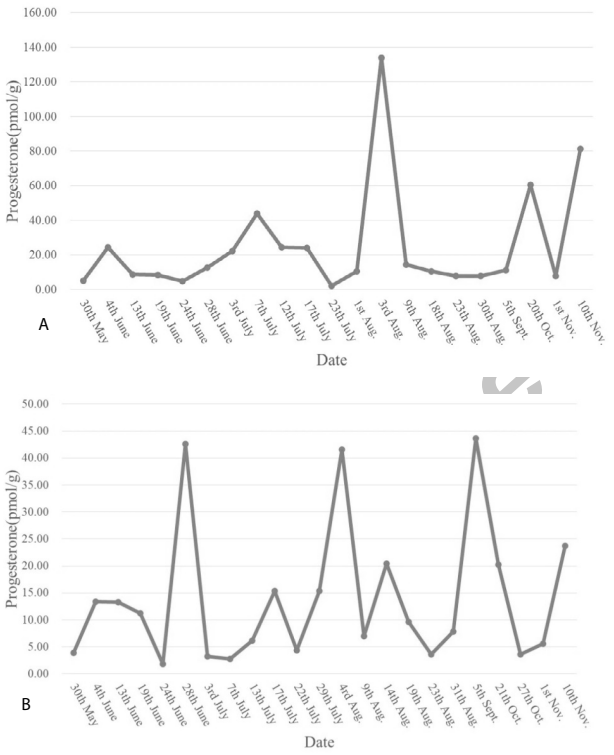

Fig. 2. The progesterone change trend of adult females CF01 (A) and CF02 (B) in Hainan gibbon group C.

We compared the data of progesterone change between males and juveniles in Hainan gibbon group C and found no significant periodicity in the change of progesterone. Also, there was no significant difference in progesterone levels between individuals of different age and sex classes in group C (Fig. 3A). In other words, there was no significant difference in feces progesterone levels between young and adult individuals and no significant difference between adult males and adult females.

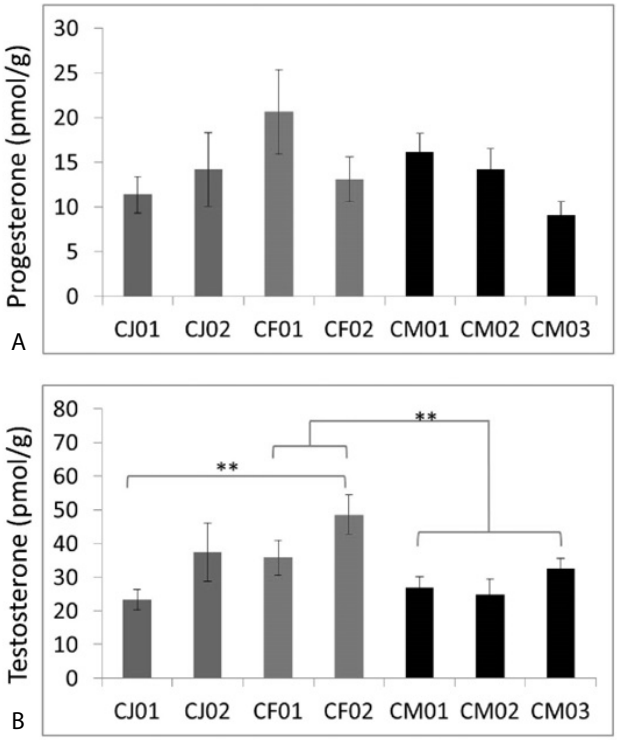

Fig. 3. Comparison of progesterone (A) and testosterone (B) levels in the feces of group C members **0.001<*P*<0.01.

Data analysis of testosterone

The trimmed mean testosterone of adult female CF01 was 35.76±21.62 pmol/g. The trimmed mean testosterone of adult female CF02 was 48.51±26.08 pmol/g. The trimmed mean testosterone of adult male CM01 was 26.90±13.32 pmol/g. The trimmed mean testosterone of adult male CM02 was 24.83±21.20 pmol/g; the trimmed mean testosterone of adult male CM03 was 32.49±13.10 pmol/g. The trimmed mean testosterone of juvenile CJ01 was 23.23±13.46 pmol/g. The trimmed mean testosterone of juvenile CJ02 was 37.48±24.35 pmol/g (Table IV).

We compared the testosterone changes between males and females in Hainan gibbon group C and found no significant periodicity in the change of testosterone. There was no significant difference in the level of testosterone between juveniles and adult individuals among individuals of different ages (Fig. 3B). However, the testosterone levels of adult males and females were significantly different, indicating that the feces testosterone levels of Hainan gibbon group C could discriminate different sexes. The testosterone levels of juvenile CJ01 and the adult female individual CF02 are significantly different. There was no significant difference in testosterone levels between

the two juveniles of unknown sex, so it was concluded that CJ01 and CJ02 might be the same sex. Therefore, according to the results of this study, it can be inferred that juveniles CJ01 and CJ02 of Hainan gibbon group C are both male individuals (Fig. 3B).

Table IV. The testosterone level variation range in Hainan gibbon group C.

Individual	Trimmed mean (pmol/g)
CF01	35.76±21.62 (11.88-130.74)
CF02	48.51±26.08 (3.59-181.19)
CM01	26.90±13.32 (3.54-57.77)
CM02	24.83±21.20 (7.61-119.25)
CM03	32.49±13.10 (12.41-109.54)
CJ01	23.23±13.46 (1.39-74.53)
CJ02	37.48±24.35 (11.97-221.55)

DISCUSSION

In primates such as the Sichuan golden snub-nosed monkey (*Rhinopithecus roxellana*) and François langur (*Trachypithecus françoisi*), progesterone did not change significantly in the first trimester of pregnancy (Yan *et al*., 2003; Wang *et al*., 2006). After 40 to 45 days following fertilization, significant changes in hormones can be reliably detected. In all female individuals, the level of progesterone in the feces rises rapidly compared to the first trimester, after which its level gradually rises, increasing by a factor of 15 to 35 in a short period of time before childbirth to reach a maximum. In this study, there were no significant differences in progesterone levels among individuals of different age and sex classes, suggesting that the adult females in group C were not pregnant or only in early pregnancy during the sampling period.

A study by Ariadna *et al*. (2014) found significant differences in testosterone levels between males and females in feces hormones in black howler monkeys (*Alouatta pigra*), which is consistent with our findings. Additionally, the authors also found significant differences in progesterone levels between pregnant and non-pregnant females (Ariadna *et al*., 2014). There was no significant difference in feces progesterone levels between adult females in our study, which indicates that the two adult female Hainan gibbons were likely not pregnant. Because neither Hainan gibbon female was in gestation during the sampling period, more studies are needed to gain knowledge of sex hormones during physiological cycles.

In another hypothesis of this research, both juveniles may be males, and this result supports the male-biased offspring sex ratio of Hainan gibbons (Bryant, 2014) and western black-crested gibbon (*Nomascus concolor*) (Huang *et al*., 2013). This is highly detrimental to the population rebuilding of Hainan gibbon group C. Physiological information gathered through the use of feces for endocrine monitoring is currently lacking in Hainan gibbons. Therefore, this study is important for a comprehensive assessment of the physiological health status of Hainan gibbon.

Gibbons have high alertness and are difficult to track for a long time in the wild. Rainfall leads to the loss of gibbons' physical strength and the weakening of light intensity, thus delaying the onset time of song and shortening the duration of song (Fei *et al*., 2010). This made it difficult for the field sampling work of this study, therefore the sample collection in this study was not completely coherent. There are five Hainan gibbon groups, but in this study, we only collected samples from group C. First, this is because Hainan gibbons of group C have become accustomed to the existence of the researchers; therefore, they are the easiest family group to track. Other family groups are very difficult to track due to their excessive alertness and relative unfamiliarity with human activities. Secondly, group C has a relatively complete population structure and age structure, and it has the largest number of individuals among all family groups.

In this study, we found that the menstrual cycle of adult females in group C was 32±3.6 days, so the subsequent research on the adult sexual physiological cycle of Hainan gibbons can be further conducted within this time range. Thus, the fieldwork time span of this research direction can be shortened to a certain extent. We would collect more groups' feces samples to determine the sex of juveniles by measuring testosterone levels in order to reduce experimental error and improve the integrity of the results in the future.

CONCLUSIONS

This study shows that the menstrual cycle of adult females in Hainan gibbon group C is 32±3.6 days, and the menstrual duration is 10±2.4 days. There is no significant difference in progesterone levels between different age and sex classes. There is no significant periodicity in the change of testosterone in the feces samples of Hainan gibbon group C, and we found no significant periodicity in the changes of progesterone between males and juveniles. Additionally, there was no significant difference in the level of testosterone between individuals of the same

sex and age in group C. However, there was a significant difference in testosterone levels between adult males and adult females in group C. There was no significant difference in the testosterone levels of the two juveniles CJ01 and CJ02, so it was concluded that CJ01 and CJ02 may be the same sex. The testosterone levels of the juvenile CJ01 and adult female CF02 are significantly different. Therefore, it can be inferred that juveniles CJ01 and CJ02 of group C are both male individuals. The results showed that the physiological cycle of adult females and the sex of juveniles could be identified by the difference in levels of the sex hormones progesterone and testosterone. This research aims to provide a new method of monitoring wild gibbons' reproductive physiology, which contributes to population recovery and protection of Hainan gibbons.

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IRB approval

This paper was approved by Guizhou Normal University and Hainan Institute of National Park of China.

Ethical statement

Ethical review and approval were not required for this animal study because of the non-invasive sampling method.

Field study permissions

These field experiments were approved by the State Forestry Administration of China and the Hainan Institute of National Park.

Statement of conflict interest

The authors have declared no conflict of interests.

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