World Applied Sciences Journal 18 (6): 737-744, 2012
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2012.18.06.6316

# Fecal Pregnanediol Glucuronide and Serum Progesterone Patterns for Ovarian Function Assessment of Javan Gibbons (*Hylobates moloch)*

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**Abstract:** Pregnanediol glucuronide (PdG) in feces and serum progesterone ($P_4$) were analyzed for determining ovarian cycle as well as to evaluate ovarian function of captive-housed Javan Gibbon (*Hylobates moloch*). A total of four females Javan Gibbons maintained in single-typed cage at Ragunan Zoo (Owa1 and Owa2) and in pairing-typed cage at Taman Margasatwa Taman Sari, Bandung (Owa3 and Owa4) were used in this research. Fecal and serum samples were collected twice a week throughout 3-months period. Following methanolic extraction of lyophilized fecal powder, samples were analyzed using enzyme linked immunosorbent assay (ELISA) for PdG as well as for $P_4$. Results indicated that the two females in pairing-typed cage did not show any regular cycle whereas the other two females in single-typed cage showed distinct pattern reflecting cyclicity. Fecal PdG concentrations were ranged between 0.56-4.95 ug/mg DW while the levels of serum $P_4$ were ranged between 0.09-15.92 ng/ml for all females. The length of the cycle obtained was ranged between 24 and 29 days which comprised of 14-15 days of follicular phase and 10-14 days of luteal phase. The time lag between serum $P_4$ and PdG excretion was estimated about 6 days.

**Key words:** Javan Gibbon · Fecal Pdg · Serum Progesterone · Ovarian Cycle

## INTRODUCTION

The Javan Gibbon [1] *Hylobates moloch* AUDEBERT 1797 is one of the primate species native to the tropical rainforests of Java, Indonesia. This species ranks among the most threatened primates and listed as Endangered in IUCN Red List of Threatened Species [2], with the population appearing more stable than in a 2004 assessment of the species being Critically Endangered [3].

Javan Gibbon is mostly confined to Java's western provinces (Banten and West Java) [4] and is also present in central Java as far east as the Dieng Mountains [5]. Their existing habitat in Java consists of a number of small, isolated patches, which are scattered over the western half of the island, West Java (Gunung Gede-Pangrango, Gunung Halimun and Ujung Kulon) and Mid Java (Gunung Slamet and Gunung Prahu) and only 4% of its original habitat remains [6].

Despite the recent reduction in population numbers due to habitat loss as a result of expanding human population and fragmentation, as well as the trapping of young animals to be kept as pets, many factors continue to threaten the long-term survival of this species in the wild because of its biological characteristics [7]. The population of the Javan Gibbons in the wild is estimated to be 2,000-4,000 gibbons, then more recently, it was reported that the total number of wild gibbons in Java to be in order of 4,000-4,500 individuals [5]. Exact figures for the captive population are difficult to obtain. Not surprisingly, knowledge of the reproductive

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physiology of the Javan Gibbon is limited even though data obtained in the same species [8, 9] and some reproductive hormone profiles in female white-handed gibbon [10,11] have given such valuable supports in conducting research in Javan Gibbon.

In recent years, measurement of steroid metabolites from both urine and feces have been applied successfully to a number of primate species (UTI). From our previous study, we found that fecal steroid pattern of female Javan Gibbon maintained in individual cage [12] and in pairs [13] reflecting high individual variation. To provide feasible practical and accurately information on reproductive events using steroid metabolite measurement, we need to find out the time course, route and recovery of excreted metabolites. Although radio metabolism study is not applied to this research, we tried to figure out the predicted time shifted between hormones detected in the blood and those of secreted in feces. So that the objective of this study was to evaluate the profile of the steroid metabolite by measuring fecal pregnanediol glucuronide (PdG) and serum progesterone of the female Javan Gibbon kept in both single-typed and pairing-typed cage, as well as to assess its ovarian function combining with observation of genital swelling and menstruation blood. The result obtained is hoped to be valuable for further research in Javan Gibbon for improving management system of these species particularly when using steroid metabolite profile for monitoring its reproductive status.

## MATERIAL AND METHODS

**Animals:** Four sexually adult female Javan Gibbons, non pregnant, 7-12 years of age and 6-7 kg of body weight with no history of breeding were used in this study over the period of three months. The animals were maintained at Ragunan Zoo, Jakarta (Owa 1 and Owa 2) in a single-typed cage and at Taman Sari Zoo, Bandung (Owa 3 and Owa 4), in pairs with their partners. A mixture of chopped fruits and vegetables were given twice a day and access to water was *ad libitum*.

**Housing:** The animals maintained at Ragunan Zoo, Jakarta were caged individually in a 4.5 m (L) x 1.5 m (W) x 2.0 m (H) cage size, whereas the animals maintained at Taman Sari Zoo, Bandung were caged in pairs with their partners in a cage with approximately 4.5 m (L) x 4.0 m (W) x 4.0 m (H) in size. The cages of both locations were made of iron mesh including the roof for facilitating the brachiation behavior and with water and food containers, as well as place for resting and sleeping. Temperature ranged between 25-35°C and 23-33°C in Ragunan Zoo and Taman Sari Zoo respectively, with relative humidity ranged from 70%-80%.

### Sample Collection

**Feces Sampling:** A 5-10 g of fecal sample was collected in a plastic tube between 07.0h and 08.0h, 5-7 days per week over 3-months period and following collection, samples were immediately stored at -20°C without adding of preservatives until assayed. Daily records of menstruation were monitored and visual inspections of the genital swelling were also carried out at the same time as sample collections. The degree of genital swelling was scored as 0) no swelling; 1) partial swelling, no colour change and no discharge; 2) relative increase in swelling, reddish but no discharge; 3) maximum swelling with discharge and red in color [14].

**Blood Sampling:** Blood samples were drawn twice a week at the same period of fecal sampling. Collection of blood samples were started when the swelling of external genitalia was at score 1 [15]. Blood samples (5 ml) were collected from a femoral vein after Ketamine HCl (10 mg/kg b.wt, i.m) sedation of each gibbon. Samples were kept cold at 4°C for 1.5-2 hrs to allow the serum to separate from the blood cells and after centrifugation the serum was stored at -20°C until analysis.

### Hormones Analysis

**Feces:** Fecal samples were measured for pregnanediol glucuronide (PdG) after methanolic extraction [16]. A total amount of fecal sample collected was lyophilized and the resulting dried pellets were pulverized. A sample of the resulting powder representing 0.05-0.10 g dry weight was then extracted with 3 ml of 80% methanol in water by vortexing for 10 minutes followed by centrifugation at 2200 x g for 10 minutes. Supernatant was decanted into a clean glass tube and after appropriate dilution in assay buffer (5.96 g $Na_2HPO_4$, 8.50 g NaCl and 1 g BSA Fr. V in 1 L $H_2O$, pH 7.2) was taken directly to assay. Individual extraction efficiencies were monitored by the recovery of [$^3$H]-progesterone (35,000 cpm; NEN Du Pont, Bad Homburg, Germany), which was added to the fecal powder randomly before extraction.

Microtitre plate EIAs were then used to determine immunoreactive PdG in feces [17]. The samples were diluted in assay buffer with a certain dilution depending on the reproductive status. Parallelism test was also performed to the samples in replicate dilutions using PdG assay to validate the assay used.

**Serum:** Serum samples were analyzed directly for progesterone ($P_4$) by microtitre plate enzyme-immunoassays using commercial KIT (DRG Progesterone Enzyme Immunoassay Kit).

**Data Analysis:** The patterns of serum and hormone metabolite were used to describe the ovarian function of this species and to estimate the lengths of the ovarian cycles [18]. The stages of ovarian cycle and the lengths of its component phases were determined on the basis of a defined rise in either fecal PdG or serum $P_4$ concentration. An increase above a threshold value of 2SD above the mean of the preceding inter-luteal phase values (statistically significant with $P < 0.05$) was taken to indicate the first day of the luteal phase of the ovarian cycle [19]. The length of the ovarian cycle was determined as the interval between the onset of two successive luteal phases. Estimation of time lag between serum and metabolite hormone excretion was also described.

## RESULTS AND DISCUSSION

Great efforts have been undertaken in developing and establishing captive breeding programs for preserving rare and endangered animal species. Monitoring reproductive status is therefore one of the most important prerequisite for any work designed to enhance captive breeding [17, 33, 35]. An effective method for monitoring reproductive events in non-human primates is measurement of the serum concentration of hormones. However, concerning to the nature of wild animals that are very sensitive to any distraction, the most likely methodology to be applied for Javan Gibbon is non-invasive approach [20].

Available information on reproductive biology of the Javan Gibbon is sparse, but the data taken from white-handed reproductive hormones profile [11, 21] and recent reports [8, 9] as well as some data of female white-handed gibbon [10, 11, 21] can be adopt for initiating research in Javan Gibbon in the area of reproductive biology.

In our study, we tried to assess ovarian function of female Javan Gibbon by applying both invasive and non-invasive approaches. The patterns of fecal PdG and serum $P_4$ in Owa 1, Owa 2, Owa 3 and Owa 4 over the period of 3-months sample collection along with its serum progesterone patterns are shown in Figure 1, 2, 3 and 4, respectively. The results showed that three of the four females (Owa 1, Owa 2) which are maintained at the Ragunan Zoo, Jakarta and Owa 4 which is kept in Taman Sari Zoo, Bandung, revealed distinct $P_4$ peaks while Owa 3 which is kept in Taman Sari Zoo, Bandung showed irregular fluctuation during the period of sample collection.

Based on the rise in the level of fecal PdG, only the profiles of two females (Owa 1 and Owa 2) could be used to estimate the length of the ovarian cycle including the length of its components. As seen in Figure 1, the profile of fecal PdG of Owa 1 represented one complete cycle, which have two luteal phases of 10-14 days and the follicular phase of 14 days, resulting in the length of the ovarian cycle of 24 and 28 days. Despite of the ovarian cycle determined for Owa 1, in Figure 2 which showed fecal PdG profiles of Owa 2 could also be used to determine the lengths of its ovarian cycle. After the rise of fecal PdG, the length of the luteal phase was obtained to be 10-14 days with the follicular phase of 14 days, resulting in the length of the cycle of 24-28 days.

As for the serum $P_4$ profiles, Owa 1 and 2 also showed distinct fluctuation of $P_4$ and could be used to estimate the length of the cycle that were 22-26 days with the length of luteal phase 10-14 days and the length of follicular phase 12 days. Of the other two females (Owa 3 and Owa 4), fecal PdG profiles obtained had highly fluctuation but did not show any complete cycle, so that it is difficult to interpret and to determine the length of the

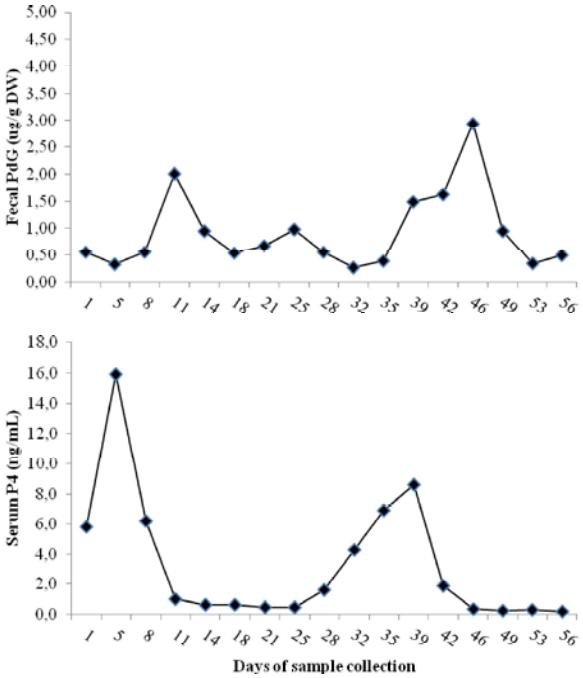


Fig. 1: Fecal PdG and serum $P_4$ patterns in Owa1 during the period of 3-months sample collection

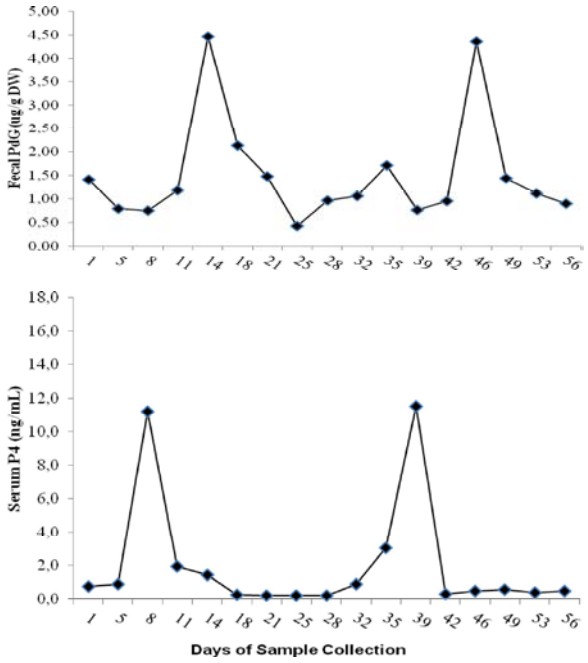


Fig. 2: Fecal PdG and serum $P_4$ patterns in Owa 2 during the period of 3-months sample collection

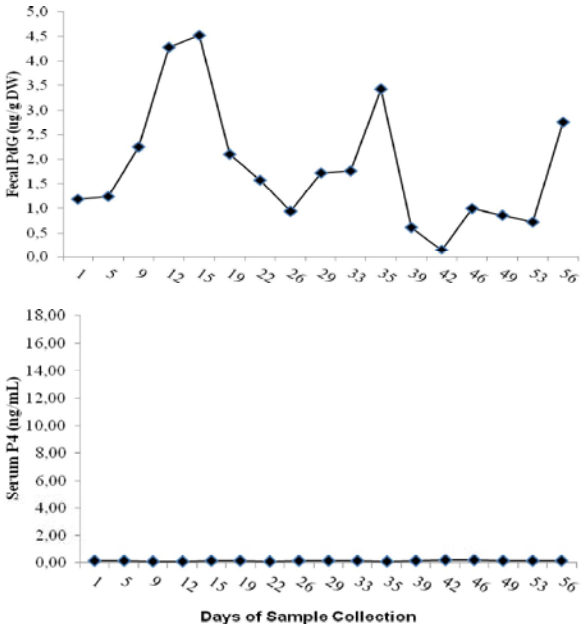


Fig. 3: Fecal PdG and serum $P_4$ patterns in Owa 3 during the per 3-months sample collection

ovarian cycle. However, it can be seen in Owa 4 (Figure 4), one of the luteal phase can be described with the length of 10 days. In general, cycle lengths determined by the interval between successive rises in PdG of the two cyclic females were 24-28 days, with 10-14 days of luteal phase

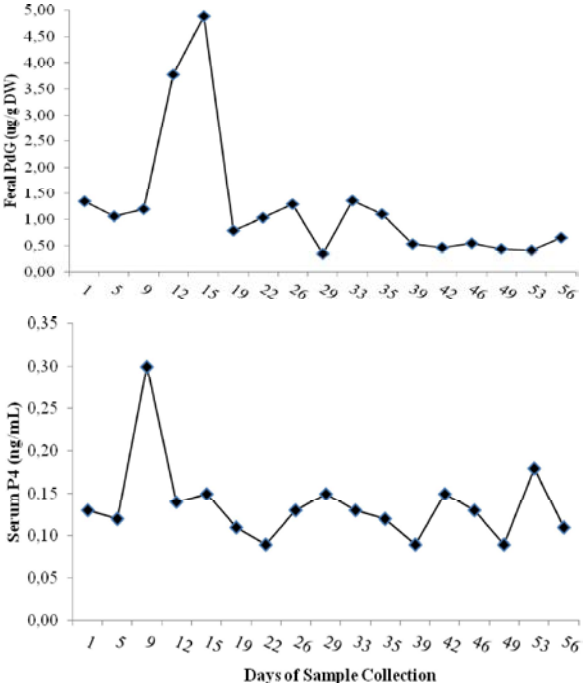


Fig. 4: Fecal PdG and serum $P_4$ patterns in Owa 4 during the period of 3-months sample collection

and 14 days of follicular phase. Nevertheless, individual variation should be taken in consideration. This pattern was also the same as the profile obtained from serum $P_4$.

As for concentration of metabolite progestin, it was vary amongst individuals and so at different stages of ovarian cycle. As seen in Table 1, fecal PdG concentrations were ranged from 0.26-2.93 µg/g DW for Owa 1, 0.43-4.48 µg /g DW for Owa 2, 0.16-4.95 µg /g DW for Owa 3 and 0.35-4.89 µg /g DW for Owa 4 during the period of observation.

Same as metabolite concentration, serum $P_4$ concentrations were also varied from one cycle to another as well as amongst individuals. The concentrations of serum progesterone were ranged between 0.20-15.93 ng/mL for Owa 1 and 0.22-11.54 ng/mL for Owa 2. These values were higher than values in Owa 3 and Owa 4 that found to be 0.10-0.35 ng/mL and 0.09-0.30 ng/mL, respectively (Table 2).

Representative fecal PdG profiles as compare to the serum $P_4$ at the same time of sample collection showed similar pattern in all females, although more stable baseline can be seen in the serum $P_4$ concentration. This result indicated a close correspondence profile between the serum hormone and its metabolite. Despite ini primates, close correlation between serum hormonal profile and its metabolites was also reported in rats using other methodology [31, 32].

Table 1: Range of fecal PdG concentration of four female Javan Gibbons (µg/g DW)

| Phase of Ovarian Cycle | Owa1 | Owa2 | Owa 3 | Owa 4 |
|---|---|---|---|---|
| Follicular phase | 0.26-0.53 | 0.43-0.76 | - | 0.35-0.41 |
| Luteal phase | 0.56-2.93 | 1.19-4.48 | 0.72-4.95 | 1.20-4.89 |

Table 2: Range of serum $P_4$ concentration of four female Javan Gibbons (ng/mL)

| Phase of Ovarian Cycle | Owa1 | Owa2 | Owa 3 | Owa 4 |
|---|---|---|---|---|
| Follicular phase | 0.26-0.66 | 0.22- 0.28 | - | - |
| Luteal phase | 1.59-15.92 | 0.88-11.54 | - | - |

As expected, the peak of serum $P_4$ occurred preceding the peak of fecal PdG in most of the profiles, indicating the metabolite excreted measured in correlation with the serum hormone. It was clearly shown for Owa 1 and Owa 2, but not for Owa 3 and Owa 4 because the concentration of serum $P_4$ was too low.

As seen in Figure 1, a rise in serum $P_4$ concentration was at point 2 and 12 for Owa 1 and at point 3 and 12 for Owa 2 (Figure 2), whereas a rise in fecal PdG was reached at point 4 and 14 for Owa 1 and at point 5 and 14 for Owa 2. The time shifted between follicular and luteal phases of the cycle resulted from the time lag of metabolite excreted in feces as well in the urine. The time lag of the excreted hormone is actually determined accurately by using radiometabolism study as reported in some primate species [18, 22-24].

Although radiometabolism study was not conducted in this research, the profiles of PdG and $P_4$ showed normal ovarian function of those females and excretion process of the hormone as well. Profiles obtained from serum progesterone as well as fecal PdG reflected the ovarian cycling of the females (Owa 1 and 2) which kept in Ragunan Zoo, in single-typed cage during the sampling period.

As we did not have Luteinizing Hormone (LH) measurements to accurately detect the ovulation day, we assumed that the approach of ovulation is signaled by an estradiol peak, followed by the fall of estradiol and the rise of progesterone. However, the subsequent rise in serum $P_4$ provides indirect evidence that ovulation has occurred and the ruptured follicle has become luteinized.

Measurements of estradiol and progesterone in serum were used to assess endocrine changes during the menstrual cycle since these hormones are known to accurately reflect changes in corresponding circulating hormones in other species [11, 25-27]. The circulating levels of most major reproductive hormones have been shown to be fluctuated. This accounts for the large variations shown by individual cycles. The concentration of serum $P_4$ obtained was quite similar compared to maximum concentration of plasma progesterone reported previously [28] in captive-housed Japanese macaques (*Macaca fuscata*). For a given hormone, pulse frequency may vary between species and within each species, pulse patterns usually change with the endocrine milieu [29].

The length of the cycles obtained from this research were longer than the length of the cycle in *Hylobates lar* [11] that was found to be 19 to 22 days, with follicular phase ranged from 7 to 11 days and luteal phase ranged from 8 to 15 days. However, this result is almost similar with the result reported previously [24].

Unfortunately, samples from two other females from Taman Sari Zoo, Bandung showed no clear pattern of $P_4$ profiles during sampling period. From these data, we could not predict the time of ovulation, (furthermore ovulation might not occur), confirm the length of menstrual cycle and distinguish phases in their menstrual cycle. It seemed that the menstrual cycles are abnormal. The condition of two gibbons from Bandung might be related to the environment that influences their reproduction. The environment could be the external and the internal factors and can be affected not only reproduction aspects of the animals but also productivity [36]. The external factors such as cage condition, mating pair, social interaction and may be visitors [34]. On the other hand, internal factors depend on external factors that influence the central nervous system, leading to hormonal regulation.

There are some possibilities to describe between the difference condition of gibbons from Jakarta and from Bandung. Although gibbons in Jakarta were cage individually, still they can contact visually and auditory with other primates surround them, especially with *Presbytis comata*, which lives sympatrically with them in the wild. Caged in Quarantine, gibbons from Jakarta faced fewer visitors (only keepers and Vets or sometimes researchers) than gibbons from Bandung, which are caged in displays, they must contact with a lot of visitors especially on holidays. Contact with a lot of people could make them frightened, worried or disturbed.

Another possible reason is gibbons at Bandung might not receive the males as their partners. Reproduction is only one of a host of activities in which an individual may engage [30]. Since we know that mating system of this species is monogamous, partners would influence the time of estrus. Unwilling partners would make the female does not come into estrus, so that would affect their menstrual patterns.

In the primate, physical or psychological stress may produce chronic amenorrhea. There have several hypotheses as to the mechanism or chain of events whereby stressful stimuli decrease pulsatile LH secretion. Stress, probably through catecholamines, activates hypothalamic-pituitary-adrenal axis and hypercortisolism is associated with cyclic irregularities or anovulation [29]. Based on the profile of fecal PdG and serum $P_4$, it is concluded that the two females of Javan Gibbons from Ragunan Zoo, Jakarta (Owa 1 and Owa2) displayed cyclic pattern that reflected ovarian function while the other two showed no clear pattern. The lengths of the ovarian cycle were ranged between 22 and 28 days with 10 to 14 days of luteal phases and 12-14 days of follicular phases. The range of fecal PdG concentration was 0.26-4.95 µg /g DW and 0.22-15.92 ng/mL for serum $P_4$.

## ACKNOWLEDGEMENTS

This research was supported, in part, by Research Grants from DP2M and the University of Indonesia. Monitoring and samplings were made possible by permission of Director of Biodiversity Conservation, Directorate General of PHKA, Department of Forestry RI, Director of Ragunan Zoo, Jakarta, staffs and keepers, Director of Taman Sari Zoo, Bandung, staffs and keepers. Special thank goes to Prof. K. Hodges and Dr. M. Heistermann for providing reagents for EIA, Head of the Laboratory of Reproduction, LIPI, Indonesia and the technicians at the Laboratorium of Physiology, Faculty of Veterinary Medicine, IPB.

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