

Communication

Seasonal Pattern in Serum Estradiol, Progesterone, and Prolactin Concentrations in Rescued Wild Female Taiwanese Pangolin (*Manis pentadactyla pentadactyla*)

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Abstract: Pangolins are under severe threat of surging poaching rates globally; therefore, there is a demand to ascertain reproductive measures to ensure captive breeding and management strategies. Due to the absence of substantial information on the pangolin, endocrinology and reproductive physiology studies around the globe are merely based on captive observations that have failed to report the chronographs and hormonal cyclicity of the reproductive events. This study attempts to evaluate the annual pattern of reproductive steroids (estradiol-17 β and progesterone) and prolactin in 16 wild female Taiwanese pangolins rehabilitated by Pingtung Rescue Center of Endangered Wild Animals, Taiwan. Novel immunoassays, i.e., chemiluminometric assays, have been used to quantify the serum reproductive steroids and contribute to a better understanding of the endocrine correlates of function in the Taiwanese pangolin. The hematological findings were characterized by monthly median concentration. The circulating reproductive hormones demonstrated seasonal reproductive activity by confirming a peak in serum estradiol concentrations in December and considerably higher progesterone concentrations in November/December, and March/April. The rise in prolactin in December and peak values in April suggest participation in the ovulatory process and mating. Collectively, these findings can help maximize the reproductive efficiency of pangolin species in captivity, i.e., by timely pairing and prioritizing the care of the breeding pairs to optimize breeding efforts and, therefore, effectively support conservation breeding programs and restore the natural population in the ecosystems.

Keywords: conservation physiology; seasonal breeding; Taiwanese pangolin; estradiol; prolactin; progesterone



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1. Introduction

The Taiwanese pangolin (*Manis. pentadactyla. pentadactyla*), a subspecies of the Chinese pangolin (*M. pentadactyla*), is an assumed seasonal breeder with conspecifics only interacting for mating [1], as described for the majority of solitary carnivorous species [2,3]. The behavioral data from various captive studies suggest reproductive seasonality; however, supporting endocrinological findings is lacking [4–6]. Pangolins, in general, are known for a low fecundity rate (i.e., one young per birth and a moderate rate of maternal care) [7,8] and such a comparatively low reproduction rate coupled with overharvesting due to intense illegal international trade [9–13] make pangolins susceptible to extinction. This situation is further driven by more than 70% of all pangolins kept under human care failing to survive their first year in captivity [8,14–16], making rehabilitation and breeding an arduous task [4,8,17]. To be able to provide efficient reproductive management strategies,

detailed knowledge about the reproductive endocrinology and behavior of pangolins, including the Taiwanese pangolin, is needed.

Thus far, a solo report on progestogens (P4) was recorded from a wild rescued Taiwanese pangolin from Taipei Zoo, Taiwan, and quantified P4 levels to identify the pregnancy and parturition period [18]. Arora, et al. [19] quantified fecal reproductive steroid metabolites and demonstrated that for free-ranging non-pregnant Taiwanese pangolins that subadult (1.5–3 kg) and adult individuals (>3 kg) have comparable fecal estrogen metabolite levels and classified both groups as actively reproducing. Despite having some suitable tools at hand, information on the endocrine correlates of reproductive seasonality is lacking for pangolins, limiting the options for increasing reproductive success for conservation breeding programs. In addition to missing gonadal steroid annual cyclicity, the information on proteolytic hormone prolactin (PRL) has also not been documented yet, although the nursing period in the Taiwanese pangolin has been established through wild observations.

Lim [17] and Sun, et al. [20] demonstrate that the nursing period in Sunda (*M. javanica*) and Taiwanese pangolins ranges between 120 and 150 days, starting late September to early January and late December to early May, respectively. Husbandry records showed that Sunda pangolins have a lactation period of approximately four months [21,22], but Payne and Francis [23] indicated a period of approximately three months without any seasonal pattern. For the Taiwanese pangolin, a six-month nursing period was reported for individuals at Taipei Zoo [17,24], which was approximately two weeks longer than the reported findings of the field observations by Sun, Sompud and Pei [20]. However, the period indicated by Wang [24] might be susceptible to error, as field observations were based on ad hoc sightings. This pilot study aims to investigate the reproductive window of free-ranging rescued female Taiwanese pangolins by describing annual patterns of reproductive endocrine correlates, i.e., estradiol, progesterone, and prolactin.

2. Materials and Methods

2.1. Study Animals

Between 2012 and 2018, 31 wild rescued females were received for rehabilitation by the Pingtung Rescue Center for Endangered Wild Animals (PTRC), Taiwan. Fifteen female pangolins among the total were diagnosed with disease or anomalies based on evidence of infective agents (inflammatory cell aggregates, suppuration, and mucopurulent discharge). Furthermore, the results from the diagnostic tests such as complete blood count, serum chemistry, and total protein assays to determine emaciation classified these 15 female individuals as unhealthy and were excluded from the study. Only 16 healthy wild female Taiwanese pangolins participated in the study (Table 1). The rehabilitated, healthy female Taiwanese pangolins were sexually mature (weighing 1.3–4.6 kg), characterized by the established bodyweight criteria [18,21].

Upon arrival, each rescued female received a complete physical examination for any obvious physical injuries on the body. Along with morphometric observations, ultrasonic examinations were performed to determine the rehabilitated females' reproductive status (pregnant/non-pregnant). Upon complete examination, all the rescued females were identified as non-pregnant except 1041115P01, and 1051205P01 (Table 1).

Each pangolin had an individual indoor housing space, 1.2 m(L) × 1.2 m(W) × 2.4 m(H), made of cement with a stainless-steel mesh cover. A wooden hide box and dirt and wood shavings were placed inside the chamber. The animals were kept at 26–28 °C without controlling the photoperiodism and humidity. Food was provided based on published data [25], and water was available *ad libitum*. All individuals were returned into the wild within two weeks of arrival, except 1030226P01 and 1060327P01, which were released after three and two months, respectively (Table 1).

Table 1. Morphometric (bodyweight) and serological (month, year, and the number of collected samples) details of rescued female pangolins by PTRC.

Individual ID	Month/Year of Rescue	Body Weight (kg)	Number of Blood Samples Collected	Month/Year of Blood Collection
1010313P01	March, 2012	3.78	1	March, 2012
1010327P01	March, 2012	3.78	1	March 2012
1050323P01	March, 2016	3.43	1	March, 2016
1060413P01	April, 2017	3.48	2	April 2017
1060327P01	March, 2017	1.3	1	May, 2017
1050527P01	May, 2016	1.70	1	June 2016
1030226P01	February, 2014	2.06	1	June, 2014
1060703P01	July, 2017	1.36	2	July, 2017
1030821P01	August, 2014	1.33	1	August, 2014
1070917P01	September, 2018	2.58	1	September, 2018
1041115P01	November, 2015	4.40	1	November, 2015
1051103P01	November, 2016	2.1	1	November, 2016
1041213P01	December, 2015	2.08	2	December, 2015
1051205P01	December, 2016	4.6	2	December, 2016
1051225P01	December, 2016	2.59	2	December, 2016
1061225P01	December, 2017	3.94	3	December, 2017

2.2. Blood Collection and Processing

The serological sampling took place within a week of arrival except for 1030226P01, where blood was only collected after three months (Table 1). Five of the females were sampled twice and one animal three times, all within 5–6 days prior to release (Table 1). Due to the absence of rescue cases, no sampling took place in January, February, and October. Anesthetization, serum collection, and processing followed the protocol established by Khatri-Chhetri, et al. [26]. The processed samples were stored at -20°C until hormone analysis.

2.3. Hormone Analysis

Serum samples were analyzed for quantifying progesterone (P4), estradiol (E2), and prolactin (PRL) at the Union Clinical Laboratory in Taipei, Taiwan, using ADVIA Centaur XPT chemiluminometric assays (Siemens Diagnostics) following manufacturer protocols. The ADVIA Centaur XPT is an automated high throughput system using a two-site sandwich immunoassay technique using direct chemiluminometric technology with a fixed amount of antibodies. The manufacturer module provided the antibody cross reactivities for all the concerned hormones in the study.

The P4 assay required 20 μL of serum for the analysis with an analytical range of 0.21–60 ng/mL. The intra-assay coefficient of variation (CV) was 11%, and the total analytic error (TAE) was 31%. Recoveries ranged from 97.9% to 118%, with a mean recovery of 114%.

The E2 assay required 80 μL of serum for the analysis with an analytical range of 11.3–3000 pg/mL. The intra-assay CV was 6.1% and the TAE was 35%. Recoveries ranged from 82.6% to 115.8%, with a mean recovery of 101%.

The PRL assay required 25 μL of serum for the analysis with an analytical range of 0.3–200 ng/mL. The intra-assay CV was 11%, and the TAE was 16%. The recoveries ranged from 88.9% to 107.8%, with a mean recovery of 97.9%.

CLIA assays for P4, E2, and PRL were biologically validated by comparing median hormone values of two pregnant females with overall median hormone values of non-pregnant rescued female Taiwanese pangolins. Median serum concentrations of P4, PRL, and E2 were about 8-fold, 4-fold, and 0.1-fold higher in pregnant females, respectively (Figure 1a–c), indicating the reliability of the utilized CLIA assays for monitoring concentrations of reproductive steroids in Taiwanese pangolin.

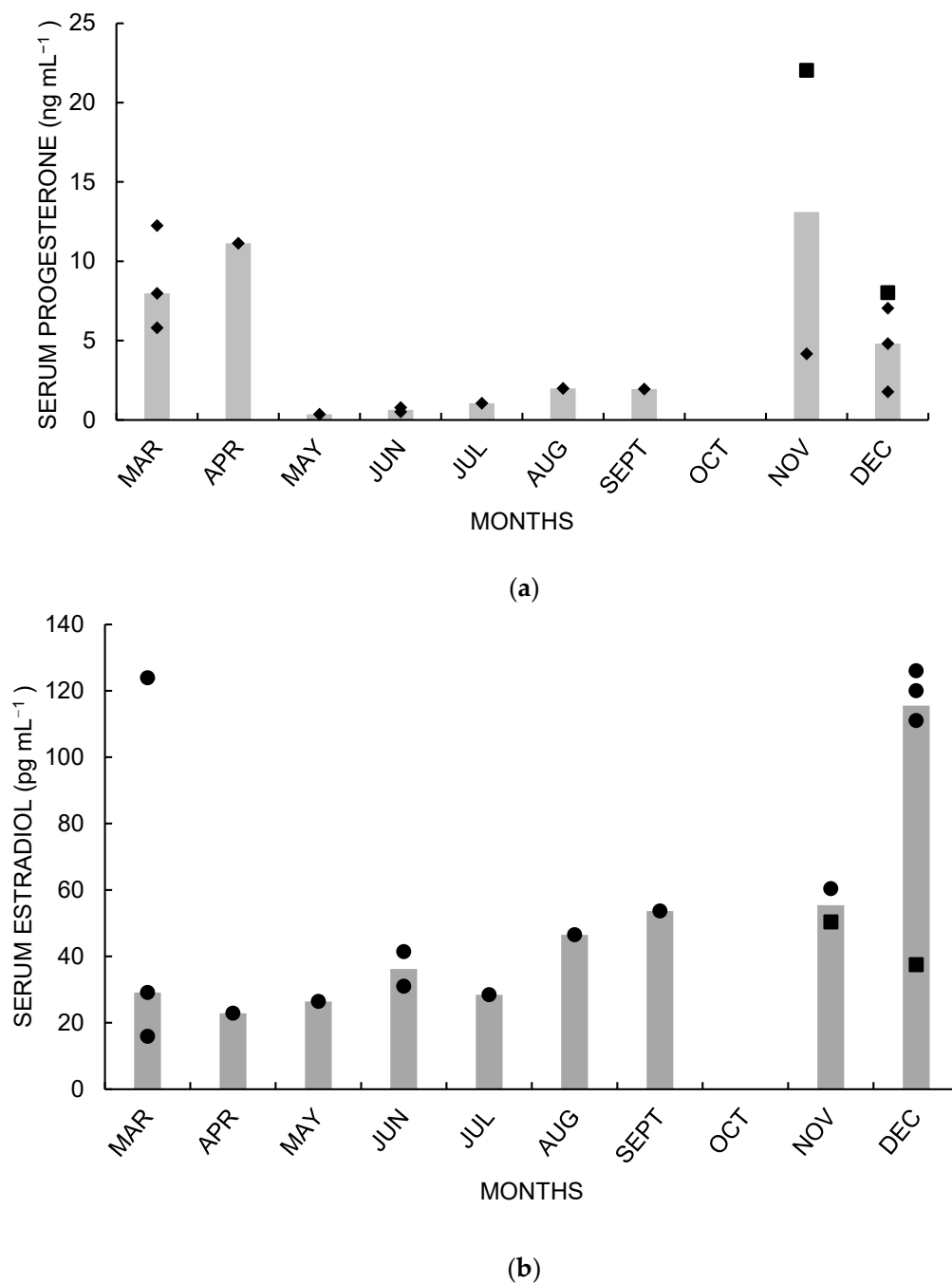
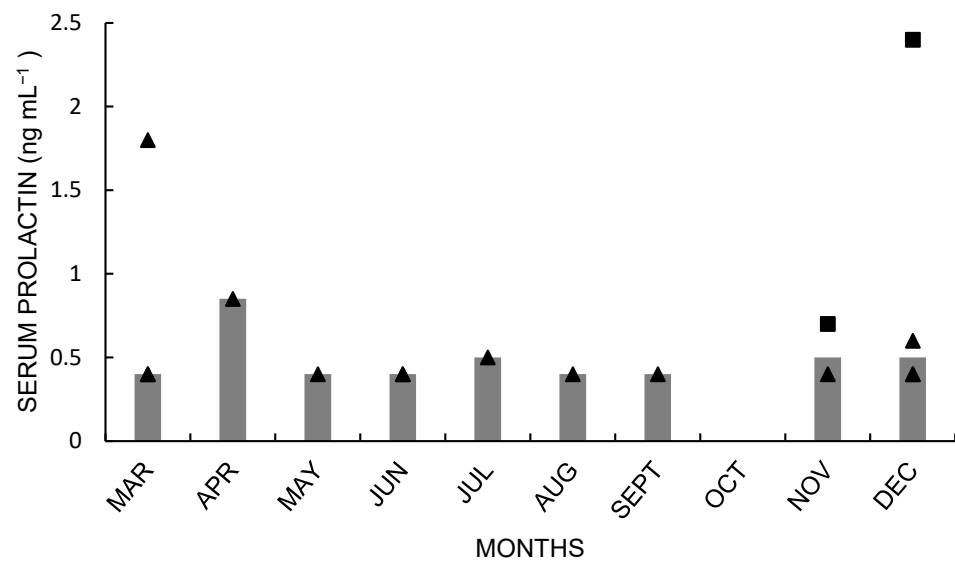


Figure 1. Cont.



(c)

Figure 1. (a) Annual pattern of serum progesterone concentrations obtained from rescued wild female Taiwanese pangolin ($n = 16$). The bar represents the monthly median, and the dots represent the individual values of an animal. The square represents the individual value of pregnant individuals. (b) Annual patterns of serum estradiol concentrations obtained from rescued wild female Taiwanese pangolins ($n = 16$). The bar represents the monthly median, and the dots represent the individual values of an animal. The square represents the individual value of pregnant individuals. (c) Annual pattern of serum prolactin concentrations obtained from rescued wild female Taiwanese pangolins ($n = 16$). The bar represents the monthly median, and the dots represent the individual values of an animal. The square represents the individual value of pregnant individuals.

2.4. Data Analysis

Data were analyzed using Excel 2010 (Microsoft Inc., Redmond, WA, USA) and SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The data distribution was measured using Kolmogorov–Smirnov (K–S) using SPSS. The data were non-normally distributed and were presented as medians. Individual median P4, E2, and PRL concentrations were calculated for the individuals with repetitive blood sampling. Subsequently, monthly median P4, E2, and PRL concentrations were calculated for those months with more than one hormone value available. We compared individual E2, P4, and PRL concentrations of November–December with the remaining hormone concentrations of all other monitored months of the year using the Welch Independent T-test. Because prior field and captive studies, Chin, et al. [18] and Sun, et al. [27] indicated that female reproductive behavior occurs during November and December.

3. Results

Serum P4 concentrations were comparatively high during the beginning of the year (March–April: 7.98 and 11.13 ng/mL, respectively), followed by consistently low values from May to October (0.36–1.99 ng/mL) (Figure 1a). In November, serum P4 concentrations rose again (13.1 ng/mL) and remained high (December 5 ng/mL), clearly above revealed mid-year serum P4 concentrations (Figure 1a), indicating luteal activity between November and April. Serum P4 concentrations did not differ significantly between Mar–Oct and Nov–Dec ($F_{2,2} = 0.23$, $p = 0.65$).

Similar to the pattern of circulating P4 concentrations, serum E2 concentrations began to rise in November (55.4 pg/mL) with the highest levels in December (116 pg/mL) (Figure 1b). Serum E2 concentrations ranged between 29.1 pg/mL–36.2 pg/mL from March to June, with a subsequent rise in September (53.65 pg/mL) (Figure 1b). Serum

E2 concentrations were significantly higher in Nov–Dec compared to the rest of the year ($F_{2,2} = 24.9, p = 0.02$), serving as an indicator of ovulatory behavior coupled later in the year.

Serum PRL concentrations were comparable from May to September (0.4 ng/mL) and rose slightly in December (0.5 ng/mL) before reaching the highest concentration of 1 ng/mL in April (Figure 1c). Serum PRL concentrations for the periods of Mar–Oct vs. Nov–Dec did not statistically differ ($F_{2,2} = 0.5, p = 0.49$).

4. Discussion

The monitoring of serum gonadal steroids over a period of nine months has shed light on the reproductive window of female Taiwanese pangolin, which was thus far concluded by field observations only. The study revealed a peak in serum estradiol levels during December, which overlaps with the behavioral estrus observed in Taiwanese pangolin from November through January. Additionally, a longitudinal study conducted on female Taiwanese pangolin using radio telemetry and camera trapping revealed that postpartum estrus and mating occur between December and May [27]. In line with the field observations, the rise in serum E2 concentrations in December suggests the probable formation of the preovulatory follicle, leading to mating behavior around this period.

The study further demonstrated a concomitant rise in serum progesterone levels from November through April, indicating the activity of luteal or interstitial tissues during ovulation. Physiologically, progesterone plays a critical role in developing and maintaining female reproductive activity by facilitating sexual behavior and controlling ovulation [28]. The revealed pattern again correlates with field observations, thus supporting females' sexual behavior occurring from November to April [27]. Due to the observed rise in progesterone concentrations following estradiol, it is likely that the Taiwanese pangolins ovulate spontaneously irrespective of their physiological state (mated or non-mated) as seen in some carnivores such as pinnipeds [29]. However, the determined endocrine values in the Taiwanese pangolin contradict the observations commenting on Malayan pangolin (*Manis javanica*), representing allegedly induced ovulation; however, the study did not produce any endocrinological evidence to support such an assumption [30]. At the same time, our study suggested that Taiwanese pangolins elicit seasonality, which is incoherent with the aseasonal biological behavior reported by Heath [10], Yan, et al. [30].

A similar pattern of prolactin concentration was found mirroring a subsequent rise, along with gonadal steroids, from December and peaking in April. Therefore, irrespective of the reproductive status of the focal females in this study, we can speculate that prolactin assists in this species' physiological reproductive activity. In phylogenetic relatives, such as mustelids [31], the corpus luteum is the only essential component of the ovary that results in physiological and morphological changes, including cessation and renewed development of the embryo [32,33]. In some carnivores and rodents, prolactin acts on the ovaries in consort with gonadotropins to promote luteal differentiation to stimulate progesterone release from the luteal cells [34,35]. A significant rise in E2 concentrations in December, in conjunction with higher progesterone concentration between November and April, suggests a luteotropic effect of prolactin in this species.

Alternatively, the demonstrated increment in prolactin concentrations in December, March, and April could be as a result of lactation, as field observations reported that Taiwanese pangolins exhibit nursing behavior from late December to early May [20]. However, according to Masui [36], the pectoral mammae of lactating Chinese pangolin (*Manis pentadactyla*) females did not dry up until approximately 86 days of birth. Furthermore, the Chinese pangolin's captive breeding and pregnancy records also unveiled that mammary glands' dimensions changed during pregnancy and after parturition producing a thicker secretion of a waxy substance/colostrum [6,8,15]. A similar set of findings was observed in Temminck's pangolin (*Smutsia temminckii*) bearing swollen teats after parturition to facilitate female nursing behavior [37]. However, while performing the morphometric examination of rescued females on arrival, no distinct changes in the mammary glands pertaining to lactation behavior in the focal individuals were found. Further, the deter-

mined bodyweight of rehabilitated females did not elicit any signs of pregnancy upon examination; thus confirmed only upon ultrasonic monitoring, i.e., exhibiting a fetus in the uterus.

Based on long-term field observations, Sun, Pei and Wu [27] concluded that Taiwanese pangolins exhibit postpartum estrus and mating between December and May to facilitate delayed implantation in females. No signs of parturition in the rescued females were observed in this study, but a seasonal rise in E2 and P4 concentrations were found during November–December, indicating a seasonal breeding pattern.

5. Conclusions

Despite several limitations, i.e., overall small sample size, missing monthly hormone data (Jan, Feb, and Oct), and frequent individual sampling, the study demonstrates basic endocrine correlates (E2, P4, and PRL) of the Taiwanese pangolin. The information presented still provides valuable information on the annual pattern of E2, P4, and PRL concentrations in Taiwanese pangolin. This valuable endocrinological information can advocate for scientists and support reproductive management initiatives to facilitate ex situ and in situ populations. These subjects warrant further investigation for future studies, i.e., vaginal cytology, in order to gain a comprehensive understanding of the reproductive biology in the Taiwanese pangolin.

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Institutional Review Board Statement: The study was ethically approved by the Laboratory Animal Center, National Pingtung University of Science and Technology (NPUST), following the Taiwan Forestry Bureau (permit numbers 0980129850, 0991616024, 1011701139, 1031700176, and 1050143346) as required by the Wildlife Conservation Act, 2013. The methodology used in the study complies with the ARRIVE guidelines and regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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