Non-invasive Monitoring of Faecal Testosterone Metabolite Concentrations in a Northern Fur Seal (*Callorhinus ursinus*)

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ABSTRACT

We examined the relationship between serum testosterone and faecal testosterone metabolite levels in a captive northern fur seal (*Callorhinus ursinus*) using time-resolved fluoroimmunoassay to develop non-invasive testosterone monitoring technique. We found a significant relationship between faecal testosterone metabolite and serum testosterone levels when the faeces were collected approximately one day after blood withdrawal. In addition, faecal testosterone metabolite levels showed seasonal fluctuations that were similar to those found in serum testosterone levels, with higher testosterone levels occurring during the breeding season. These results indicate that serum testosterone levels can be estimated using faecal testosterone metabolite levels. These methods will be a useful tool to non-invasively monitor reproductive cycles of male northern fur seals.

Key words: faecal testosterone metabolite levels, seasonal changes, time-resolved fluoroimmunoassay

Non-invasive monitoring of steroid hormone metabolites has been developed in various taxonomic groups, including mammals [e.g. 1], birds [e.g. 2], reptiles and amphibians [e.g. 3, 4] to study environmental stress and reproductive cycles. Matrices such as faeces, saliva, hair, and urine are used to detect steroid hormones [e.g. 1, 3]. One of the main advantages of these matrices is that they can be non-invasively obtained. Faeces are often the easiest to collect, as it is unnecessary to train animals [1], and are useful for monitoring the stress levels [5] and reproductive status [6] of captive animals. The time lag between serum and faecal steroid hormone metabolite levels, however, is species-specific [6, 7]. It is thus important to determine the time lag of faecal metabolite levels with serum steroid levels when analyzing a new species [7].

Northern fur seals breed between June and August [8]. Reproductively intact male northern fur seals show seasonality in their testosterone levels [9, 10]. The size of seminiferous epithelia also increases during the breeding season [10]. Monitoring faecal testosterone metabolite levels in northern fur seals in captivity, as an alternative to measuring serum steroid levels, will eliminate the stress experienced by animals during capture and blood withdrawal. In addition, captive monitoring will increase our understanding of testosterone levels in wild northern fur seals. Faecal testosterone metabolites can provide an effective tool to monitor the physiology of northern fur seals; here, we provide the first study examining faecal testosterone metabolite levels in this species. We developed faecal testosterone metabolite monitoring in a captive male northern fur seal by determining the time lag between faecal testosterone metabolite and serum testosterone levels and by examining the seasonal relationship between faecal
testosterone metabolite and serum testosterone levels to test whether faecal monitoring provides a reliable replacement for information collected by sampling blood.

Samples were obtained from a reproductively intact 8-year-old male northern fur seal weighing an average of 86 kg at the Izu Mito Sea Paradise (Numazu, Japan). About 5 ml of blood was drawn from the hind flipper vein of the animal in the morning, twice a month, from April 2017 to January 2018, except that blood was taken only once in November 2017. The blood samples were centrifuged for 15 min at 1,550 ×g (H-26F, Kokusan, Japan), and the serum was frozen at –40°C. The animal was enclosed alone from 1530 hr to 0830 hr consecutively for three days after the blood withdrawal. Faeces were collected from a pool using a dip net at 0830 hr during the three mornings following the blood sampling. We were not able to find faeces once in April and September, twice in October, three times in November, and five times in January. All the faeces were also stored frozen at –40°C in the aquarium until the faeces and serum were shipped to our laboratory in Hakodate. Faecal samples were dried at 60°C (DKM600, Yamato Scientific Co., Ltd., Japan) until the faeces remain unchanged. The dried faeces were pulverized with a mortar and pestle and were kept frozen at –20°C until analysis.

Extraction methods of steroid hormones from serum and faeces were conducted as follows. The serum was centrifuged again at 21,600×g for 10 min (himac, CF15R, Hitachi, Japan). 1 ml of diethyl ether was added to 100 µl of serum, and it was vortexed for 30 sec. The serum was frozen in a cold methanol bath for 3 min, and the supernatant was decanted. This procedure was repeated three times. The supernatant was evaporated using a centrifugal concentrator (CC-181, Tomy Seiko Co., Ltd., Japan) until the weight of the faeces remain unchanged. The dried faeces were pulverized with a mortar and pestle and were kept frozen at –20°C until analysis.

Extraction methods of steroid hormones from serum and faeces were conducted as follows. The serum was centrifuged again at 21,600×g for 10 min (himac, CF15R, Hitachi, Japan). 1 ml of diethyl ether was added to 100 µl of serum, and it was vortexed for 30 sec. The serum was frozen in a cold methanol bath for 3 min, and the supernatant was decanted. This procedure was repeated three times. The supernatant was evaporated in a water bath at 45°C. Tris-HCl-based assay buffer with the same volume of the serum was added to reconstitute the sample for time-resolved fluoroimmunoassay. Steroids were extracted from 10 mg of each dried faecal samples using 1ml of 80 % methanol. The samples were shaken for 15 min, and centrifuged at 21,600 ×g for 5 min. The supernatant was evaporated using a centrifugal concentrator (CC-181, Tomy Seiko Co., Ltd., Japan). The extracts were reconstituted in the same volume of assay buffer.

Time-resolved fluoroimmunoassay (DEL-FIA®, PerkinElmer, Waltham, MA) was used to assay the samples [11]. The antigen, testosterone 3-(E-carboxymethyl)oxime-BSA IgG was obtained from Cosmo-Bio (FKA-102-E, Tokyo, Japan). The fluorescence of europium, Eu-N1 Goat anti-Rabbit IgG (AD0105, PerkinElmer Japan Co., Ltd., Kanagawa, Japan), was measured in the Wallac 1420 ARVOsx (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). The software WorkOut 2.5 (Dazdaq Ltd., UK) was used to calculate the concentrations. Manufacturer cross-reactivity data are as follows: testosterone (100%), 5α-dihydrotestosterone (7.0%), 4-androstenediione (2.0%), androstenolone (0.2%), 5α-androstenediol (0.15%), 5α–androstan-3α , 17B-diol (0.1%), 5ß-androstane–3α, 17β–dil (0.09%), cortisol (0.02%) and corticosterone (0.01%). Intra-assay and inter-assay variabilities of control samples were 11.9 % (n = 5) and 20.2 % (n = 4), respectively. Serial dilutions of serum (1:2, 1:4, 1:8, 1:16) and faecal samples (1:3, 1:6, 1:12, 1:24) were performed to obtain parallelism of the assay. Serum and faecal samples were spiked with known concentrations of standard solutions to determine the extraction efficiency. Known concentrations of standard solutions were added to these samples and extracted as previously described.

Parallelism of serum and faecal samples with standard solutions were compared using an analysis of covariance (ANCOVA) test. When the interactions between binding and standard, and between binding and samples were insignificant, the parallelism of the sample and standard solutions was confirmed. Faecal testosterone metabolite levels were fitted with serum testosterone levels using a generalized linear model (GLM). The models were fitted using gamma error distributions with a log link function. Significance of the models were tested with a null model using an analysis of deviance [12]. The testosterone metabolite levels from faecal samples taken the following morning (Day 1), two days (Day 2), and three days (Day 3) after the blood withdrawal (Day 0) were compared with the serum testosterone. The statistically significant level was set to a = 0.05. The software used for analysis was R 3.5.1 [13].

The results of the parallelism between the serially diluted serum of the fur seal and testosterone standards were similar (F = 0.029, p = 0.872, Fig. 1 (a)). In addition, the results of the parallelism between the serially diluted faecal steroid extracts of the fur seal and the testosterone standards were similar (F = 0.297, p = 0.614, Fig. 1 (b)). Extraction efficiency of serum (n = 5) and faecal metabolites (n = 4) were 104.6 ± 7.4 % and 106.8 ± 8.5 %, respectively. These results can be used as a
validation of assays. Although steroid hormones are completely metabolized in gut [14], the skeletal structure of steroids is kept in faeces [7]. In primates, several testosterone metabolites such as androsterone, epiandrosterone, and 5β-androstane-3a-ol–17-one/5α-androstan–3,17-dione are found in faeces [15]. In hyenas, the presence of epiandrosterone was also found [16]. It is possible that some of these metabolites were in the faeces of the seal, and they might cross-react with the antibody against testosterone.

The serum testosterone levels of the seal were significant predictors of faecal testosterone metabolite levels of Day 1 (Estimate = 0.414, SE = 0.122, p = 0.0048). An analysis of deviance showed strong support for the correlation between serum testosterone and faecal testosterone metabolite levels (Deviance = 1.15, F_{1,13} = 10.02, p = 0.007, Fig. 2). The serum testosterone levels were insignificant predictors of faecal testosterone metabolite levels of Days 2 and 3 (Estimate = 0.173, SE = 0.188, p = 0.375 for Day 2, Estimate = 0.415, SE = 0.219, p = 0.084 for Day 3, Fig. 2).

Once the species-specific time lag between serum testosterone and faecal steroid metabolite levels was known [6], we could relate it to the gut passage time [7]. The gut passage time of female northern fur seals is around 6–22 h [17], which is the same as the time between serum testosterone levels and faecal testosterone metabolite levels obtained from the male in this study. Although the gut passage time was determined in only female fur seals [17], a comparable study of Steller sea lions (Eumetopias jubatus) injected with adrenocorticotropic hormones showed the peaks in serum and faecal corticosterone metabolites after 1 h and 32 h of its administration.
respectively [18]. Cheetah (Acinonyx jubatus) injected with testosterone reached peak in faeces after one day [19]. Moreover, oestradiol, progesterone, testosterone, and cortisol that were injected in mammalian species appeared in faeces about 12–48 h after their administration [6,20]. These studies agree with our finding that serum testosterone and faecal testosterone metabolite levels of the northern fur seal were most highly related at about a 1-day time lag. No relationship between serum testosterone and faecal testosterone metabolite levels on both Days 2 and 3 indicate that Days 2 and 3 are unlikely to represent the same conditions as when the blood was drawn. The serum testosterone levels of northern fur seals thus are correlated to levels found in faeces deposited approximately 6–22 h after blood withdrawal.

Seasonal changes in serum testosterone levels and faecal testosterone metabolite levels were also examined (Fig. 3). The serum testosterone levels in this seal during May–August ranged from 1.18–6.31 ng ml\(^{-1}\). In a previous study, the testosterone levels of a male northern fur seal during May–August ranged from 1–4.26 ng ml\(^{-1}\), with the peak occurring in June, while testosterone during September–February was below detectable levels [9]. Although the greatest testosterone level measured in our study (6.31 ng ml\(^{-1}\)) occurred in May, relatively high testosterone levels were observed throughout the breeding months of June–August, a trend similar to that found in the previous study.

Faecal testosterone metabolite levels in the fur seal in this study ranged from 0.02–1.63 µg g\(^{-1}\). Higher peaks in testosterone metabolite levels (0.21–1.63 µg g\(^{-1}\)) occurred around the breeding season of June–August. In other marine mammal species that are seasonal breeders, faecal testosterone metabolite levels tend to be highest in males during the breeding season. For instance, two captive Australian fur seals (Arctocephalus pusillus doriferus) showed an increase in testosterone levels around the breeding season [21]. Similarly, free-ranging dugongs (Dugong dugon) showed a seasonal increase in faecal testosterone metabolite levels [22]. These results coincide with the seasonal increase in testosterone metabolite levels that we found in the northern fur seal. Both serum testosterone and faecal testosterone metabolite levels showed an increase during the breeding season. Faeces, therefore, are an invaluable tool to monitor the testosterone levels of this species. Since this study was performed on a single male, further testing of this relationship in other individuals could provide more evidence supporting the use of this methodology as a replacement for blood sampling.

In conclusion, the present study has demonstrated the following results: firstly, a protocol for the measurement of faecal testosterone metabolite levels in the male northern fur seal was established. Secondly, testosterone metabolite levels in the faeces of a captive northern fur seal were found to reflect the serum testosterone levels if faeces were collected after a 1-day time lag from the time of blood sampling. Lastly, the faecal testosterone metabolite levels as well as serum testosterone levels, were greater during the breeding season. Faecal testosterone metabolites, therefore, will be useful as a non-invasive method of studying the reproductive cycles of male northern fur seals.
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REFERENCES

キタオットセイ（Callorhinus ursinus）における糞中テストステロン代謝物濃度の非侵襲的モニタリング

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要約
飼育下キタオットセイのオスにおける糞中テストステロン代謝物濃度と血清中テストステロン濃度との関係を検証した。採血の約1日後に採取した糞を試料とした糞中テストステロン代謝物濃度は、血清中濃度において有意な傾向があった。糞中テストステロン代謝物濃度は、繁殖期に増加した。以上より、キタオットセイの糞中テストステロン代謝物濃度から血清中濃度の推定が可能となった。

キーワード: 季節変動, 時間分解蛍光免疫測定法, 糞中テストステロン代謝物濃度

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