——Original Article——

Season- and Age-related Reproductive Changes Based on Fecal Androgen Concentrations in Male Koalas, *Phascolarctos cinereus*

Satoshi KUSUDA¹, Hisashi HASHIKAWA², Masato TAKEDA³, Hideki ITO², Atsushi GOTO⁴, Jun OGUCHI⁴ and Osamu DOI ¹

¹Laboratory of Animal Reproduction, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan
²Nagoya Higashiyama Zoo, Aichi 464-0804, Japan
³Osaka Municipal Tennoji Zoological Gardens, Osaka 543-0063, Japan
⁴Awaji Farm Park England Hill Zoo, Hyogo 656-0443, Japan

Abstract. The purposes of the present study were to clarify age- and season- related androgen patterns, and to compare the reproductive physiology between Japanese captive koala populations and Australian populations. To measure fecal androgens, feces were collected from male koalas (4.2 to 13.8 years of age) kept in Japanese zoos. Fecal androgens were extracted with methanol from the lyophilized samples and determined by enzyme immunoassay using 4-androstene-3,17-dione antibody. Fecal androgen concentration in male koalas increased after sexual maturation and remained relatively high until old age. In the survey with the Japanese zoo studbook of koalas, copulation (conception) month showed a pyramid shape with a peak in March to June (60.7%) in koalas born and reared in Japanese zoos and from July to April with the highest concentration in September to January (69.7%) in Australian institutes. Japanese zoo koala populations have a characteristic physiological cycle adapted to Japan’s seasonal changes. The suitable month of year for copulation or conception in Japan is diametrically opposed to that in Australia. Mean fecal androgen concentrations by month in the males born and reared in Japan indicated annual changes with the highest concentration in May and the lowest value in November. Fecal androgen analysis may be a noninvasive alternative tool to monitor circulating testosterone and may be helpful in understanding reproductive activity and physiology in male koalas.

Key words: Androgen, Fecal hormone, Reproduction, Seasonality, Testosterone

Koalas (*Phascolarctos cinereus*), belonging to the family Phascolarctidae, order Diprotodontia, are unique Australian marsupials, which have a habitat range from northeastern to southeastern Australia. Currently, koalas are kept and exhibited at Australian sanctuaries and other zoos in Australia, Europe, America and Asia including Japan, Taiwan, China and Thailand. In Japan, koalas were initially imported from Toronga Zoo to Nagoya Higashiyama Zoo (Nagoya) and Tama Zoological Park (Tokyo) and from Lone Pine Koala Sanctuary to Hirakawa Zoological Park (Kagoshima) in 1984. As of December 31, 2011, 36 northern koalas and 12 southern koalas were being kept at 8 zoos in Japan [1]. Our study describes Queensland and New South Wales subspecies as northern koalas and the Victorian subspecies as southern koalas. In Japan, all zoos housing koalas have implemented management units since 2006, based on a recommendation by the International Union for Conservation of Nature (IUCN)/Species Survival Commission (SSC) Australian Monotreme and Marsupial Taxon Advisory Group [2].

Free-ranging male koalas reach sexual maturity at approximately 2 years of age; however, competition from larger or older males may restrict opportunities for mating until 4 to 5 years of age [3]. Victorian males reach full body size (12 kg) at over 4 years of age [4]. The North American captive male population was reported to produce the first joey at a mean of 4.3 years of age (a minimum age of 1.5 years of age) [5]. Free-ranging male koalas tend to have a shorter lifespan than females (females commonly live to 13 to 18 years of age) due to aggression during the breeding season [4]. It has been reported that most females produce one joey each year up until 10 to 15 years of age [2]; however, the reproductive lifespan in male koalas is not well known.

Although male koalas can mate throughout the year, they have a reproductively active season, and thus this season is reflected in the birth season: summer (October to May) in Australia [6]. Koalas kept at San Diego Zoo have been born in all months of the year with a peak during spring and summer (March to August) [5]. Similarly, the births of koalas kept at Japanese zoos have occurred throughout the year, but with a predominant season. In Japan, after the first successful breeding at Nagoya Higashiyama Zoo in 1986, 231 joeys (230 births including twins in one case) were recorded in Japanese zoos by December 31, 2011 [1], and the birth season is primarily from spring to summer.

Some studies on the reproductive endocrinology of male koalas have been performed, mainly in Australia. In male koalas, annual changes in plasma androgen concentrations have been reported [4, 7, 8]. It has been also reported that the plasma androgen concentration in male koalas housed with females is significantly high compared with males housed without females [7]. These studies were performed...
Animals, husbandry and fecal collection

Fecal androgen analysis and Australian populations. To clarify age- and season-related androgen patterns, and 3) to compare endocrine monitoring technique using feces from male koalas, 2) to management. To understand the reproductive cycle of koalas for captive breeding previous reports on the Australian population. It is important to well understand the reproductive cycle of koalas for captive breeding management.

The purposes of the present study were 1) to validate a noninvasive endocrine monitoring technique using feces from male koalas, 2) to understand the relationship and reproductive physiology between Japanese captive koala populations and Australian populations.

Materials and Methods

Animals, husbandry and fecal collection

Five male koalas, 4 southern and 1 northern (Table 1), were used in this study. These koalas were kept at Awaji Farm Park England Hill Zoo (AFZ) and Nagoya Higashiyama Zoo (NHZ) in Japan. All male koalas were kept individually in an indoor viewing area with natural light at 17 to 28°C (average yearly temperature).

Sexual behaviors, including copulations, were observed by animal keepers and veterinary staff. A male was introduced to an estrous female by the animal keepers several days after estrus had begun and was kept for a few hours in the same area. Mating was evaluated by male mounting and thrusting behaviors.

From all koalas, 5 to 10 fresh-looking grain-shaped feces were collected from the ground in the viewing area once a week for over one year (a minimum of 13.5 months for male #137 and a maximum 41.5 months for male #216) and stored at –30°C until assay. The husbandry and management of the koalas were reviewed and observed the Code of Ethics of the Japanese Association of Zoos and Aquariums (JAZA) and the management guidelines for koalas at each institute.

Fecal androgen analysis

Fecal androgens were extracted using a methanol extraction method [9]. Briefly, 5 to 10 grain-shaped frozen feces were lyophilized for approximately 24 h, crushed using a hammer and pooled. A portion of the powder (0.1 g) was then extracted with 5 ml of 80% methanol by vortex-mixing for 30 min and placed overnight at 4°C. After centrifugation at 2500 rpm for 10 min, the supernatant (methanol fraction) was transferred to a clean tube and then diluted at a ratio of 1:4 with assay buffer (0.04 M disodium hydrogen phosphate dihydrate buffer containing 0.1% bovine serum albumin and 0.05 M sodium chloride). Fecal androgens were determined by enzyme immunoassay using 4-androstene-3,17-dione (androstenedione) standard (A9630, Sigma-Aldrich, St. Louis, MO, USA), 4-androstene-3,17-dione-3-CMO-HRP (FKA137; Cosmo Bio, Tokyo, Japan) and anti-4-androstene-3,17-dione-3-CMO-BSA antiserum (FKA138-E; Cosmo Bio). In general, androstenedione is a type of testosterone metabolite, and androstenedione antiserum cross-reacts with some androgen metabolites. The cross-reactivity of the antiserum was analyzed for some available androgen metabolites: 100% for androstenedione, 15.9% for epitestosterone, 6.34% for androsterone, 1.19% for testosterone and 0.26% for 5α-dihydrotestosterone (our analysis data). The main cross-reactivity from the product data sheet was 100% for androstenedione, 35.0% for 5α-androstenedione, 4.5% for dehydroepiandrosterone, 1.5% for androstenetione, 1.2% for progesterone, 6.0% for testosterone, 0.32% for cortisol, 0.20% for corticosterone and 0.01% for estradiol. The intra- and interassay coefficients of variation were 2.8 and 6.3%, respectively. All fecal data are expressed as per gram of dry feces.

Investigation of breeding season

To investigate the breeding season in koalas kept at Japanese zoos, the number of births was counted by month based on all birth records registered with the Japanese zoo studbook of koalas [1]. Breeding koalas has been successful at 8 zoos in Japan. At these 8 zoos, koalas are housed in indoor areas with natural light from windows, and the temperature is artificially controlled throughout the year (approximately 17 to 28°C).

The investigated number of koalas included 66 (except 3 for which the birth month was unknown) born at Australian institutes and imported to Japan and 231 registered animals (including twins in one case) born in Japanese zoos from 1986 (Japan's first successful breeding) to the end of 2011. With the exception of 2 animals for which the birth month was unknown, 228 births (one birth was twins) in Japanese zoos were separated into two patterns, 1) one in which the dam had been born in an Australia institute and imported into Japan and 2) one in which the dam had been born and reared in a Japanese zoo. Conception month was estimated by back calculation of the mean gestation period (34 days [9]) from the birth date.

Data analysis

Data on fecal androgen concentrations are presented as the mean ± SEM. Mean concentrations of fecal androgen were calculated by koala (age) and month. Mean fecal androgen concentrations by koala (age) were calculated from data of one year or several full years from the start of the study (that is, the last several months of data at the final age during this study period of each male were omitted from the calculations) because the mean fecal androgen concentrations by age may reflect the effect of a seasonal change in concentration if they are not calculated for a ‘full’ year. The mean concentrations by month were calculated from 3 koalas, #087, #121 and #137, born and reared in Japan. Analyses for differences in mean fecal androgen concentrations by age (5 koalas, #087, 121, 137, 216 and 224) and season (3 koalas, #087, #121 and #137) were performed with the Kruskal-Wallis and Friedman test, respectively, using Microsoft® Office Excel 2010 and SSRI Ekuseru-Toukei 2010.
Results

Age-related fecal androgen concentrations

The mean concentration of fecal androgens in the 5 male koalas are shown in Fig. 1. Mean (± SEM) fecal androgen concentrations were lowest for #224 (219.35 ± 8.72 ng/g), in which the investigation was performed at 4.2 to 6.1 years of age, and highest for #087 (629.43 ± 21.14 ng/g), in which the investigation was performed at 11.7 to 13.6 years of age. Mean concentration of fecal androgen had a tendency to increase with age (Fig. 1).

Fecal androgen concentrations and profiles

Profiles of fecal androgen concentrations in the 5 male koalas are shown in Fig. 2. Fecal androgen concentrations showed an annual change with a high from January to August compared with other months. This tendency was especially prominent in males #087, #121 and #137. In the profiles of males #216 and #224, fecal androgen concentrations were markedly high from October to December, 2003, and August to October, 2004, for #216. Fecal androgen concentrations were elevated in December (measurement start) through June for male #224; however, subsequently, there was a tendency toward slightly high values during January and August. Male #224 copulated on a total of 12 days during the present study period when it was introduced to an estrous female by the animal keepers several days after estrus had begun. Although the fecal androgen concentration showed a high tendency around copulation, a relationship between fecal androgen and copulation could not be identified.

Breeding season and season-related fecal androgen concentrations

The conception month in Australia and Japan and monthly mean concentrations of fecal androgens in 3 male koalas born and reared in Japanese zoos are shown in Fig. 3. The conception month in Australian institutes was from July to May with a peak in October and was concentrated in between September and January (69.7%) (Fig. 3a). In koalas born in Australia and imported into Japan, conception in Japan occurred throughout the year, with two marked peaks of March and November (Fig. 3b). In koalas born and reared in Japan, the majority of conceptions (60.7%) occurred from March through June (Fig. 3c). Mean fecal androgen concentrations by month in 3 males, #121, #216 and #224, born and reared in Japan, are also shown in Fig. 3c. Mean (± SEM) fecal androgen concentrations indicated an annual change with the lowest value (339.39 ± 14.06 ng/g) in November and the highest value (711.12 ± 103.92 ng/g) in May; however, significant differences among months were not observed (P>0.05).

Discussion

Male koalas are reported to be capable of producing viable sperm at 2 years of age [4]. Based on their ages, all the male koalas had probably reached sexual maturity at the beginning of our study. Mean fecal androgen concentrations in sexually mature male koalas increased with age from 4 to 12 years of age, the range of the investigated period. This result may indicate that the production of testosterone increased with age even after sexual maturation and that testosterone production or reproductive activity persists until old age (at least approximately 9 years of age).

Although male koalas can mate throughout the year, they have a reproductively active season [6], and thus the reproductively active season is reflected in the birth season. Males attempt to defend their territory containing females during the active season: September to January in New South Wales and November to February in Victoria [12]. In Australia, births occur during summer (e.g., October to May [4]; October to March [13]). It has been reported that 80% of births are concentrated in between October and January at Lone Pine

Table 1. Male koalas used in this study

| Studbook No. (name) | Koala type | Birth date | Age during the study (year) | Arrival date in Japanese zoo | Institute |
|---------------------|------------|------------|----------------------------|-----------------------------|-----------|
| #087 (Miku)         | Southern   | July 26, 1991 | 11.7–13.8                 | Born in Japan               | AFZ       |
| #121 (Riku)         | Southern   | April 20, 1994 | 9.2–10.3                  | Born in Japan               | AFZ       |
| #137 (Arun)         | Southern   | April 19, 1996 | 8.9–11.0                  | Born in Japan               | AFZ       |
| #216 (Justine)      | Southern   | February, 1997 | 6.1–9.6                   | March 21, 2003              | AFZ       |
| #224 (Forest)       | Northern   | October, 1999  | 4.2–6.9                   | October 29, 2003            | NHZ       |

AFZ: Awaji Farm Park England Hill Zoo. NHZ: Nagoya Higashiyama Zoo.

Fig. 1. Age-related changes in mean (± SEM) fecal androgen concentrations in 5 male koalas (4.2–13.6 years of ages). Different letters for each bar indicate a significant difference (P<0.01).
Koala Sanctuary [14] and that 67.5% of births are concentrated in November to January in Victoria [3]. In Australia, the estimated season for mating or conception is probably September to April based on counting backwards for the 34 to 36 days of the gestation period [6] from the birth season (October to May [4]). Our investigation for koalas in Australia (range, July to May; peak, September to January, 69.7%) was completely consistent with other results from a survey in Australia. Although estrus and/or ovarian luteal activity in female koala populations in Japanese zoos is observed throughout the year [9], strong estrous signs and increased behavioral activity has been observed empirically from spring to summer (H Hashikawa, personal communication). The peak months of successful conception in Japanese zoos were March to June in our study. This result indicates that the period for mating or conception is diametrically opposed to that in Australia (but the breeding season of koalas kept in both countries is in spring).

On French Island (Victoria, Australia), plasma testosterone concentrations show an annual change with a marked increase in

Fig. 2. Profiles of the fecal androgen concentrations in 5 male koalas kept at Japanese zoos. Plus signs indicate copulation. Arrows indicate the day when a koala was introduced to Japan from Australia.
adaptation to Japan’s climate changes, which are the opposite of those in Australia, but not completely. Copulations in Japanese zoos were seen throughout the year in koalas born in Australia, and the month distribution indicates a mixed type (in Australia and Japan) with peaks in March and November. This distribution may reflect the degree of adaptation to Japan’s season.

Our study also showed that the fecal androgen concentration starts to increase beginning in December before the peak breeding season (April). It has been reported that the serum testosterone concentration in male blue sheep (*Pseudois nayaur*), a seasonal breeder, started to increase several months before the onset of the female breeding season (start of progesterone cycle) and reached a peak around the onset of the female breeding season [15]. Similarly, in male Sika deer (*Cervus nippon*), the fecal testosterone concentration showed a peak preceding the onset of ovarian cyclicity in the female [16]. It has been considered that increased testosterone might be responsible for triggering aggressive behavior to obtain as many females as possible in the territory of the male Sika deer [16]. Free-ranging male koalas attempt to defend their territory containing females during the active season [12], and the increased period of testosterone production also coincides with the increased frequency of aggressive male-male interactions before the start of reproductive activity [6]. A large amount of androgen secretion before the active breeding season is probably associated with male-male competition and the acquisition of many females, rather than spermatogenesis and breeding behavior.

In conclusion, fecal androgen analysis is a noninvasive alternative tool to monitor circulating testosterone and is helpful in understanding reproductive activity and physiology in male koalas. The production of androgen in male koalas increased to some extent with age after sexual maturity and remained high at approximately 9 years of age. Koala populations housed in Japanese zoos have a characteristic physiological cycle adapted to Japanese seasonal changes, which are the opposite of those in Australia.

**Acknowledgments**

We gratefully acknowledge F Tamamura, H Naito, N Suzuki, Y Tojima, Y Nomura, M Kurobe and Y Satō (Nagoya Higashi-yama Zoo); R Tanaka, H Nakano and H Takeda (Awaji Farm Park England Hill Zoo); and H Noguchi, H Aono, A Hayakawa, T Matsushita and T Yoshida (Osaka Municipal Tennoji Zoological Gardens) for fecal collection, behavioral observation and their kind assistance. This study was supported by a Grant-in-Aid (No.18-1116) for JSPS Research Fellows from the Japan Society for the Promotion of Science.

**References**

1. Kurobe M. Internal Studbook of Koalas, *Phascolarctos cinereus* (2011). Tokyo: Japanese Association of Zoos and Aquariums; 2011.

2. Jackson S. Koala. In: Bell CE (ed.), Encyclopedia of the World’s Zoos, Vol. 2. Chicago and London: Fitzroy Dearborn Publishers; 2001: 687–690.

3. Martin RW, Handasyde KA. Population dynamics of the koala (*Phascolarctos cinereus*) in southeastern Australia. In: Lee AK, Handasyde KA, Sanson GD (eds.), Biology of the Koala. Sydney: Surrey Beatty & Sons; 1990: 75–84.

4. Martin R, Handasyde K. The Koala: Natural History, Conservation and Management. Malabar: Krieger Publishing Company; 1999.

5. Bercovitch FB, Tobey Jr. JR, Andrews CL, Doyle L. Mating patterns and reproductive success in captive koalas (*Phascolarctos cinereus*). *J Zool* 2006; 270: 512–516. [CrossRef]
313

6. Blanshard W, Bodley K. Koalas. In: Vogelnest L, Woods R (eds.), Medicine of Australian mammals. Collingwood: CSIRO Publishing; 2008: 227–327.

7. Cleva GM, Stone GM, Dickens RK. Variation in reproductive parameters in the captive male koala (Phascolarctos cinereus). Reprod Fertil Dev 1994; 6: 713–719. [Medline] [CrossRef]

8. Handasyde KA, Medonald IH, Than KA, Michaelides J, Martin RW. Reproductive hormones and reproduction in the koala. In: Biology of the Koala. Sydney: Surrey Beatty and Sons; 1990: 203–210.

9. Kusuda S, Hashikawa H, Takeda M, Takeda K, Ito H, Ogata-Kobayashi Y, Hashimoto M, Ogata M, Morikaku K, Araki S, Makino T, Doi O. Non-invasive monitoring of reproductive activity based on fecal progestagen profiles and sexual behavior in koalas, Phascolarctos cinereus. Biol Reprod 2009; 81: 1033–1040. [Medline] [CrossRef]

10. Paris MCJ, White A, Reiss A, West M, Schwarzenberger F. Faecal progesterone metabolites and behavioural observations for the non-invasive assessment of oestrous cycles in the common wombat (Vombatus ursinus) and the southern hairy-nosed wombat (Lasiorhinus latifrons). Anim Reprod Sci 2002; 72: 245–257. [Medline] [CrossRef]

11. Hamilton RA, Stanton PG, O’Donnell L, Steele VR, Taggart DA, Temple-Smith PD. Determination of seasonality in southern hairy-nosed wombats (Lasiorhinus latifrons) by analysis of fecal androgens. Biol Reprod 2000; 63: 526–531. [Medline] [CrossRef]

12. Nowak RM. Order Diprotodontia. In: Walker’s Marsupials of the World. Baltimore: The Johns Hopkins University Press; 2005: 135–197.

13. Tyndale-Biscoe H, Renfree M. Breeding biology of marsupials by family. In: Reproductive Physiology of Marsupials. London: Cambridge University Press; 1987: 14–94.

14. Smith M. Notes on reproduction and growth in the koala, Phascolarctos cinereus (Goldfuss). Aust Wildl Res 1979; 6: 5–12. [CrossRef]

15. Kusuda S, Nagami H, Kusumoki H, Nishikaku T, Nakagawa D, Takida T, Kurita D, Uemichi K, Fukai M, Kabota H, Ueda K, Ooe T, Okuda K, Ueda K, Doi O. Annual changes in testicular size and serum and fecal testosterone concentrations in male bharals, Pseudois nayaur. J Vet Med Sci 2006; 68: 1093–1095. [Medline] [CrossRef]

16. Yamauchi K, Hamasaki S, Takeuchi Y, Mori Y. Assessment of reproductive status of sika deer by fecal steroid analysis. J Reprod Dev 1997; 43: 221–226. [CrossRef]