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Non-invasive Assessment of the Annual Gonadal Cycle in Free-living Kakapo (*Strigops habroptilus*) Using Fecal Steroid Measurements

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The Kakapo (*Strigops habroptilus*) is a unique New Zealand parrot that is threatened with extinction. It is large, flightless, and nocturnal. The breeding biology of the Kakapo is characterized by a lek mating system with all parental care of the eggs and young performed by the female, and by the occurrence of egg laying and successful breeding at intervals of several years.

The Kakapo became extinct on the mainland in the early 1980s, at which time there was still a natural population of about 60 birds on Stewart Island. Today, the remaining population (ca. 33 males and 17 females) consists entirely of birds moved to other islands off the New Zealand coast. There are very few female Kakapo remaining, and it is clearly important for the conservation of the species to maximize the breeding output of every female. A management strategy to achieve this is dependent on knowledge of the activity of the reproductive system of female Kakapo during potential breeding seasons. However, though male Kakapo show very obvious sexual behavior (booming), the only direct measure of female reproductive activity is evidence of mating at booming bowls (i.e. feathers pressed into the ground; Powlesland et al. 1992), and the discovery of nests with eggs. Measures of this type provide no information about changes in ovarian size in birds that do not lay eggs. Plasma hormone levels can be used to assess ovarian activity in birds, but it is not practical to collect blood samples from free-living Kakapo.

The need for information on ovarian activity in Kakapo led us to consider an alternative, non-invasive approach. Steroid hormone levels can be measured in avian droppings (Bishop and Hall 1991), and Kakapo produce droppings that can be collected from the forest floor. These droppings are larger than those worked with by Bishop and Hall (1991), so we applied recently developed methods for measuring testosterone and estradiol levels in chicken droppings (Cockrem and Rounce 1994) to an analysis of Kakapo droppings. Droppings were collected for 16 months in order to determine the annual pattern of steroid levels and to relate this pattern to the events of the breeding cycle.

Kakapo droppings were collected from the forest floor on Little Barrier Island, New Zealand, by Department of Conservation staff. Droppings were collected from tracks during routine operations each day, returned to the field base, and frozen in liquid nitrogen or at −20°C. They were later transported to the laboratory for analysis. Collections generally, but not always, were made daily; therefore, the length of time between a dropping being produced by a bird and collection of the dropping varied from less than a day to several days. The droppings were subjected to natural, field weather conditions before collection.

Droppings were available for hormone assay for the months of September 1989 through December 1990, except that there were no samples from December 1989 or January 1990. The sample size was usually four or five droppings per month (major exceptions being one dropping in September 1989 and 24 in March 1990). Three of the droppings were collected from birds of known sex during a handling procedure. The identities of the birds that produced the other dropping were unknown, and more than one dropping in each month's sample could have come from a given bird.

The procedure for preparation of the fecal samples followed Cockrem and Rounce (1994). It was based on Bishop and Hall (1991) and consisted of the mixing of dried fecal samples with a phosphate buffer solution. This mixture was soaked overnight and then centrifuged, with aliquots of the buffer extract subsequently taken for hormone assay.

Frozen droppings were thawed, thoroughly stirred, and then dried at 55°C until constant mass was reached 4–7 days). Most droppings weighed 10 to 30 g; about 80% of the wet mass of each dropping was lost in drying. Droppings that initially weighed more than 10 g were divided and a portion taken for drying. Phosphate-buffered saline with gelatin (pH 7.4) was added to each dropping at a mass:volume ratio of 1:8. The fecal material was broken up with a glass rod, stirred, and left to soak overnight at 4°C. On the next day the solution was centrifuged (6,000 rpm, 10 min), the supernatant decanted, and aliquots removed and frozen for subsequent assay.

Estradiol and testosterone levels in fecal samples were measured by radioimmunoassay as described by Cockrem and Rounce (1994) for chicken droppings. Cross-reactions of the estradiol antiserum with other steroids included estrone (1.3%), estriol (0.24%), and testosterone, progesterone, and androstenedione (all <0.004%). The testosterone antiserum cross-reacted with: dihydrotestosterone (34%); 5β-androstan-3α, 17β-diol (3.8%); 11-hydroxytestosterone (3.3%); three other androgens (2–3%); and other steroids (<1%). Serial dilutions of buffer extracted Kakapo samples were parallel to the standard curves for both hormones.

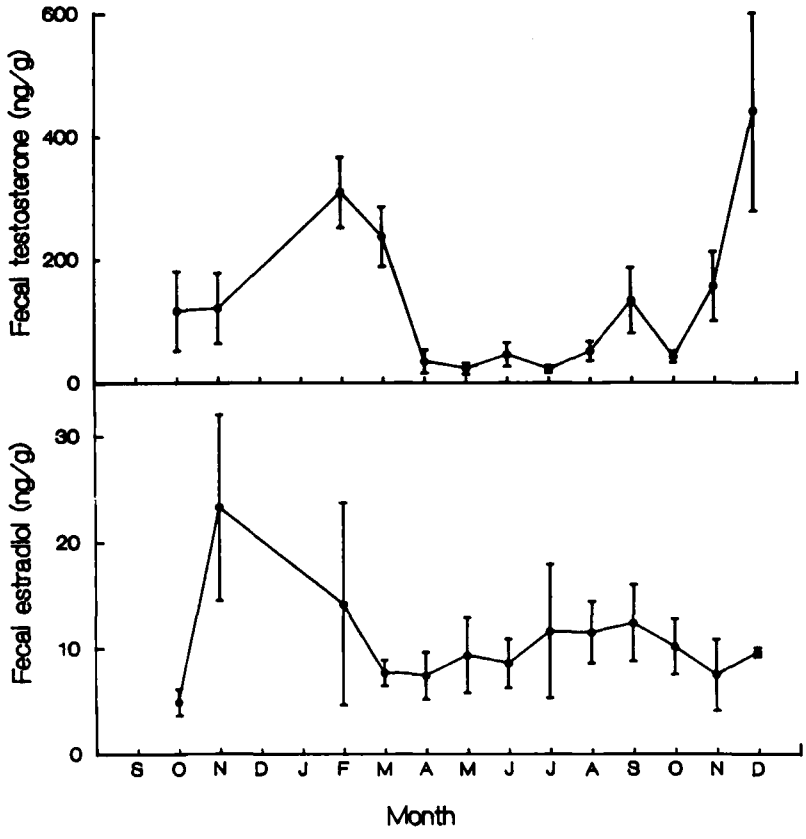

Fig. 1. Fecal testosterone and estradiol levels in droppings collected in 1989–1990 from Kakapo on Little Barrier Island. Data plotted as $\bar{x} \pm$ SE. Sample sizes were three to six droppings per month, except in October 1989 (9) and March 1990 (24).

Different amounts of hormone were added to buffer extracts of fecal samples and to plasma samples. The percent recoveries ($n = 5$ in each case) were 91.4 ± SD of 2.5% for estradiol and 90.8 ± 4.4% for testosterone. All samples were measured in one assay for each hormone. The sensitivities of each assay expressed as concentrations of hormone in the fecal buffer samples were 70 pg/ml for estradiol and 0.595 ng/ml for testosterone (calculated for each hormone from the concentration at the $\bar{x} - 2$SD from the zero hormone point on the standard curve).

Fecal hormone concentrations were measured in diluted buffer extracts. Buffer had been added to the droppings in proportion to the dry mass of the droppings, so the hormone concentrations in buffer were directly proportional to the amount of steroid per unit dry mass of the original dropping. The raw assay results, therefore, were converted to give final values as ng steroid/g dry mass dropping. The conversion included a correction factor for the recovery of labelled steroid from mixtures of fecal sample and buffer during the sample preparation procedure. The recoveries were measured using spikes of tritiated hormone and were 14.1 ± 1.8% for estradiol and 13.3 ± 1.9% for testosterone.

Comparisons between means were made after Bartlett's test for homogeneity of variances between groups had been applied. Analysis of variance (ANOVA) and Kruskal-Wallis nonparametric ANOVA were then used as appropriate. Regression analyses used the multivariate general linear model. All analyses were performed using SYSTAT and graphs plotted using SYGRAPH on a personal computer (Wilkinson, 1988a, b). Summary data are presented as $\bar{x} \pm$ SE.

There was an annual cycle of fecal testosterone levels (Fig. 1), and mean testosterone levels varied significantly between months ($P < 0.001$). Mean levels rose from around 120 ng/g during October–November 1989 to a peak of 309.5 ± 57.5 ng/g in February 1990. Levels declined in March and then dropped sharply to low levels in April. Mean testosterone levels remained low (<60 ng/g) from April–October, apart from a rise in September. Testosterone levels rose again in November 1990 to reach 441.1 ± 163.4 ng/g in December.

Elevated testosterone levels corresponded to the

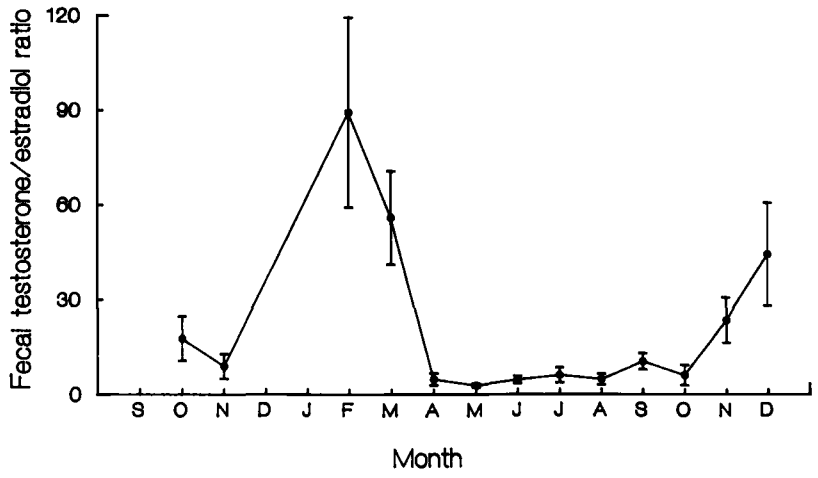

Fig. 2. Ratios of fecal testosterone to estradiol levels in droppings collected in 1989–1990 from Kakapo on Little Barrier Island. Data plotted as $\bar{x} \pm$ SE. Sample sizes were three to six droppings per month, except in October 1989 (9) and March 1990 (24).

period of male sexual activity in late spring and summer each year. The samples were from birds of unknown sex, but maximum levels of testosterone in each month are most likely to be in samples from male Kakapo. Monthly maximum testosterone levels in individual droppings were moderate in October and November 1989 and February 1990, with a peak in March. This was followed by a sharp decline to low levels from April–October and then a rise in November to a high level in December 1990.

There was no clear annual pattern of fecal estradiol levels, and no significant change in mean estradiol levels during the study (Fig. 1). Mean estradiol levels were high in November 1989 (23.3 ± 8.7 ng/g) and February 1990 (14.2 ± 9.5 ng/g), and were low thereafter. The coefficient of variation in estradiol levels in December 1990 was very small, and it is likely that all of these samples were from male Kakapo.

Maximum levels of estradiol in each month are most likely to occur in samples from female Kakapo. Monthly maximum estradiol levels in individual droppings rose from a low level in October 1989 to a moderate level in November. A peak occurred in February 1990 followed by a sharp decline to low maximum levels from March to December 1990. These data provide some limited evidence for an annual cycle of estradiol levels in female Kakapo.

The ratio of testosterone to estradiol levels in fecal samples has been used in previous studies of fecal steroids as a measure of gonadal activity that is independent of actual steroid concentrations. It is expected that in male birds this ratio will be at a maximum when they have large testes and plasma testosterone is high. In female birds this ratio is likely to be at a minimum during the period of greatest ovarian growth when plasma estradiol is high. For the Kakapo, there were significant differences between months in this ratio ($P < 0.01$), with a clear annual cycle (Fig. 2) that was more pronounced than the cycle of testosterone levels. Ratios were low in October and November 1989 (<20), followed by a peak in February (89.4 ± 32.0). Ratios dropped in March to be very low in April. They remained low (<11) from April–October and then rose in November to reach 44.7 ± 16.4 in December 1990. This cycle of testosterone/estradiol ratios presumably reflects changes in plasma steroid levels and gonadal activity in male rather than female Kakapo.

Our study has demonstrated an annual cycle in fecal testosterone levels in free-living Kakapo. This is only the second report of an endocrine cycle in a free-living bird species where samples for hormone analysis have been collected without birds having to be captured and handled (Kofuji et al. [1993] measured fecal steroids in the Brown Dipper, *Cinclus pallasii*). The Kakapo were living on a rugged island, were nocturnal, and could not be observed other than sporadically at a few fixed locations. The fact that from our data we can make inferences about the extent and timing of testicular activity in male Kakapo in this situation shows the power and value of fecal steroid analyses.

There were thought to be at least 12 male and 6 female Kakapo on Little Barrier Island during the study period. The available data on the movements of individual Kakapo during this time were insufficient to assign droppings to particular birds based on knowledge of home ranges and the use of feeding stations. The excess of males in the population means that most droppings collected at any time of year were probably from males rather than females. Booming (the production of loud, resonant calls by male Kakapo) occurred from late December 1989 until mid-March 1990, and commenced again in mid-October

1990. Females visit booming sites only for copulation, which is thought to have occurred in mid- to late January 1990 (and at a similar time in 1991). Many droppings were collected around the track and bowl systems used by male Kakapo for booming displays, so most droppings collected during the breeding season (October–March) were likely to have been from males.

The annual cycle of testosterone levels in Kakapo feces corresponds well with the pattern of male sexual behavior. Fecal testosterone levels were moderate in October and November 1989, when male Kakapo were probably starting to visit the booming sites. It likely that booming sac growth was occurring at this time. Testosterone levels increased in December and January, with a peak in February 1990 at the time of greatest booming activity. Levels declined in March and then fell sharply to basal levels in April. This corresponds exactly with the time at which booming ceased, and there was evidence of postnuptial molt. Studies of other avian species in which testis size and plasma testosterone levels were measured concurrently have shown that annual cycles of plasma testosterone levels reflect similar cycles in testis size (Follett 1984), although testosterone levels can fluctuate according to stage of the breeding cycle, while testes remain large (Wingfield et al. 1987). If fecal testosterone levels in the Kakapo do reflect plasma testosterone levels, it is reasonable to presume that the present results reflect an annual cycle of testicular size in the Kakapo.

Strong support for the validity of the fecal-testosterone results as measures of plasma testosterone comes from a comparison of relative fecal levels of testosterone and estradiol, and of the Kakapo results with those from other species. Maximum testosterone levels in Kakapo droppings were nine times the maximum estradiol levels. This difference is of the same order of magnitude as the differences in plasma hormone levels reported in other species. Peak mean levels of fecal testosterone in Kakapo reached 440 ng/g, and Cockrem and Rounce (1994) found mean fecal levels in male chickens of 457 ng/g. Furthermore, the highest fecal estradiol level in a Kakapo dropping was 90 ng/g, which is the same as the mean level of 89 ng/g found by Cockrem and Rounce (1994) in laying chickens. These results also are consistent with those of Bishop and Hall (1991) for the Japanese Quail (*Coturnix coturnix japonica*). Mean fecal testosterone levels in male quail reached 480 ng/g, and mean fecal estradiol-3-glucuronide levels in female quail reached 180 ng/g. Lower levels of estradiol than of estradiol-3-glucuronide are to be expected given that free estrogens are present in droppings in smaller amounts than conjugated estrogens such as estradiol-3-glucuronide (Bishop and Hall 1991). It was not possible to collect blood samples from Kakapo. However, the fecal steroid results for both testosterone and estradiol are consistent across the three species of birds (chicken, Japanese Quail, and Kakapo). It is reasonable to assume that fecal measurements of both hormones in Kakapo reflect plasma levels of the hormones and, hence, changes in gonadal activity.

Most of the droppings probably were produced by male Kakapo, but the estradiol data do provide limited evidence for an annual cycle of estradiol levels in female Kakapo. Monthly maximum estradiol levels in individual droppings rose from October to November 1989 and were highest in February 1990. They then fell sharply in March to low levels in April. At least two female Kakapo laid eggs in early February 1990, so the pattern of maximum fecal estradiol levels reflected the likely pattern of ovarian growth and regression during this breeding season. There was no rise in estradiol in November and December 1990, but these samples were probably largely (November) or entirely (December) from male birds.

The annual cycle of testosterone/estradiol ratios was more distinctive than the cycle of testosterone levels. The ratio was used in earlier studies of fecal steroids when results were not expressed as amount of steroid per unit mass of dropping (e.g. Bercovitz et al. 1982). The ratio is still of value, but is influenced by changes in levels of both hormones; the primary information comes from the actual steroid levels in droppings for each hormone.

Cockrem (1989) suggested that the Kakapo is capable of breeding annually and that day length is the main proximate factor stimulating gonadal growth in spring. That Kakapo can breed annually has been confirmed by the occurrence of egg laying in two successive seasons on Little Barrier Island. The fecal steroid data are consistent with the hypothesis that male Kakapo have an annual cycle of gonadal growth and regression with a corresponding cycle of plasma levels of testosterone. It is suggested that testicular growth occurs in spring (September–November), and that gonadal regression occurs at the end of the breeding season, in March or April. The estradiol data are limited, but it is very likely that female Kakapo also have an annual cycle of gonadal growth and regression. Ovarian growth probably occurs over several months during late spring and early summer (October–December), with rapid yolk deposition and ovulation in January or February in those years when eggs are laid.

Egg laying does not occur every year, so clearly the appropriate stimuli are not naturally present every summer. An adequate food supply is essential for Kakapo breeding, as shown by egg laying on Little Barrier Island only after the provision of supplementary food from September 1989 onwards. However, supplementary food supply is permissive rather than stimulatory to breeding in Kakapo, as evidenced by the lack of egg laying by female Kakapo provided with supplementary food on Little Barrier Island in 1992 and on Maud Island in 1992 and 1993.

In this study we have successfully applied fecal

steroid measurements to the assessment of gonadal activity in free-living Kakapo. The survival of this endangered species depends on egg laying by female Kakapo. Thus, conservation efforts are focussed on the females. The shortage of female samples in our study means that further sample collection programs are needed to realize the potential of this new method to provide information on the reproductive condition of female Kakapo. In a wider context, fecal steroid measurements can now be applied to other free-living birds for which handling and blood sampling is difficult or inappropriate.

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Polygyny in the Asian Openbill (*Anastomus oscitans*)

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Although monogamy is the predominant mating system in birds, there are few strictly monogamous bird species (see review by Ford 1983). Other than the widespread occurrence of polygyny in various species, occasional polygyny also has been reported in normally monogamous species (Armstrong 1955, Verner and Willson 1969, Logan and Rulli 1981, Marks et al. 1989). Although Brown (1987) did not include any members of the Ciconiiformes in his list of 222 species of communally breeding birds, occasional polygyny also has been found in this group (Lancaster 1970, Cramp 1977, Fujioka 1986, McKilligan and McConnell 1989). However, storks always have been found to breed monogamously (Ali and Ripley 1968, Cramp 1977, Coulter et al. 1989).

We describe several cases of polygyny in a breeding population of a stork, the Asian Openbill (*Anastomus oscitans*). We also report information concerning the formation of such mating groups and the success experienced by them in comparison to monogamous pairs.

Methods.—Our study was conducted at the Raiganj Wildlife Sanctuary (25°36'N, 38°10'E), West Bengal, India, where Asian Openbills are found breeding from July through December, as are five other waterbird species.

We studied this population of openbills from 1987 to 1991. Nests with more than two adult birds were considered as a possible case of polygyny, and we kept close watch on those nests. Data from those nests