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Fecal testosterone concentrations may not be useful for monitoring reproductive status in male blue-tongued lizards (Tiliqua nigrolutea: Scincidae)

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Assessment of reproductive status in animals generally depends on monitoring hormone concentrations in plasma, but blood sampling often involves significant stress to the subject. Monitoring steroid profiles by assaying excreted steroids in urine and/or fecal samples is non-invasive, but does pose some problems. Unlike plasma assays, urinary and fecal steroid analyses are of relatively little value in monitoring rapid, short-term changes in hormone concentrations (Heistermann et al., 1993) because there is a significant delay between production and excretion of steroids. However, such assays do enable measurement of "pooled" hormone concentrations over time (Wildt et al,. 1995; Brown, 1997).

Hormones do not usually appear unchanged in the urine or feces, but are present as a range of metabolites produced by the liver, the major site of steroid metabolism (Kime, 1987). The proportions and forms of steroid excreted differ between the two excretory pathways, and may be highly variable between species (Aldercreutz et al,. 1980). In the maned wolf (Chrysocyon brachyurus) 97% of testosterone (T) is excreted as unconjugated metabolites in the feces (Vellosso et al., 1998), while in African wild dogs (Lycaon pictus) 60 % of steroids are excreted in the feces and 40 % in the urine (Monfort et al., 1997). A proportion of the excreted steroid is present as water-soluble conjugates - glucuronidates and/or sulfates - which can complicate hormone analysis. In most mammals, however, voided feces contain a higher percentage of free than conjugated steroids (Wasser et al., 1996; Vellosso et al., 1998; Adams et al., 1994), so excreted steroids are measurable by standard assay procedures. However, even if the same hormones are produced, different liver enzymes may produce different metabolites for excretion (Hodges, 1986); fecal steroid assays therefore need to be validated separately for each species.

Most studies of fecal steroids have been conducted in mammalian species (Schwarzenberger et al., 1996), with limited work on birds such as the kakapo (Strigops habroptilus) (Cockrem & Rounce, 1995). This critically endangered, cryptic species is not amenable to invasive monitoring strategies, but Cockrem and Rounce (1995) have successfully utilized the ratio of fecal T to estrogen to assign sex in kakapo. Fecal steroid monitoring has also been used to follow the reproductive cycle in Japanese crested ibis (Nipponia nippon), and rock ptarmigian (Lagopus mutus) (Kikuchi & Ishi, 1997), and in the northern spotted owl (Strix occidentalis caurina) (Wasser et al.,1997; Brown et al., 1995).

There are, however, only two published reports of the application of fecal steroid monitoring to reptiles. The reproductive cycles, characterized by behavioral observations and ultrasound imaging, of four species of tortoise (Geochelone elephantopus, G. gigantea; Testudo gracea, T. hermanni) have been positively correlated with profiles of fecal steroid metabolites (Casares, 1995; Dobeli et al., 1992). Ideally, fecal steroid profiles should be validated against plasma steroid profiles for the same species (Pryce et al., 1993) but this was not achieved for the tortoises. No squamate reptile species has yet been monitored using fecal steroid assays, and further study of the potential of such techniques for monitoring reptilian reproductive cycles is clearly required.

Working from the premise that new techniques are best developed using a relatively common species, we collected fecal samples opportunistically during a study of the annual reproductive cycle and plasma concentrations of steroid hormones in male blotched blue-tongued lizards (Tiliqua nigrolutea) (Edwards and Jones, 2001). Here we report on the development of a method for assay of T in feces of this species. We compare the profile of

fecal T with that for plasma T through the reproductive cycle of blotched blue-tongued lizards, and also examine the relative proportions of conjugated and free T in feces.

We collected fecal samples from captive male Tiliqua nigrolutea from March 1997 – February 1999. The wild-caught animals were held at the University of Tasmania in semi-natural conditions. Male and female lizards cycle in synchrony with the wild population (Edwards, 1999; Edwards and Jones, 2001); details of animal husbandry are given in Edwards and Jones (2001). We collected fecal samples (N = 29) passed voluntarily while animals were being observed for reproductive behaviors. These samples were classified according to reproductive stage, and mean fecal T levels (Fig.1) were calculated using results obtained by assaying these samples. Plasma T values were derived independently from the more comprehensive data set presented as monthly mean values in Edwards and Jones (in press); standard errors are not therefore presented (Fig. 1.).

Upon collection, each fecal pellet was frozen at –20 ^{O}C in an individual, snap-lock bag until analysis. Prior to extraction of steroids, we thawed the fecal samples and mixed each one well to control for possible heterogeneous steroid distribution within the pellet. Samples were then lyophilized for 24 h to control for varying water content (Wasser et al., 1994; Thompson et al., 1998). The lyophilized samples were ground, and the fecal material separated from dried plant material and snail shells to produce a powder ready for extraction. We used the fecal steroid extraction method of Wasser et al. (1994), but some critical modifications were required. Initially our samples contained unknown, hydrophobic, interfering substances that resulted in very high non-specific binding values (‘blanks’) for samples in the radioimmunoassay (RIA). We needed to include purification steps additional

to those described by Wasser et al. (1994) in order to reduce the assay blanks to acceptable levels (i.e. equivalent to the RIA non-specific binding).

For each sample, 0.1 g of fecal powder was boiled in 5 ml of 90 % ethanol (Brown et al., 1994); 50 μl of tracer ([^{3}H]T: 4400 cpm) was added to the sample prior to extraction to measure recovery. The sample was boiled for 20 min, then centrifuged at 1500 g for 15 min. The supernatant was collected into a clean tube, and the pellet washed with 3 ml of 90 % ethanol, vortexed well and centrifuged at 1500 g for 5 min. Washes were dried down, and then the sample was reconstituted by standing for 24 h in 1 ml methanol, with frequent vortexing, at 4 ^{0}C.

At the second stage of the extraction, 0.5 ml of reconstituted sample was moved to a clean tube, dried down and reconstituted in 1 ml of 2 % ethanol in hexane for 24 h. After centrifuging for 5 min, the supernatant was collected; the 'pellet' was rinsed with 1 ml of 2 % ethanol in hexane, then centrifuged and the supernatants combined, dried down, and reconstituted in 1 ml of 2 % ethanol in hexane for 24 h. For the final purification step, we used Sep-Pak C18 cartridges (Waters) pre-wetted with 5 ml 2 % ethanol in hexane. The sample was applied to a column and eluted with 1 ml of 2 % ethanol in hexane. The eluate was dried down, and reconstituted for 24 h in 1 ml 2 % ethanol in hexane.

Duplicate sub-samples (50 μl) of the final extract were assayed for T by standard RIA, using [^{3}H]T as label, and charcoal separation, as detailed in Swain and Jones (1994). The cross-reactivity of the antiserum with T, dihydrotestosterone, and androstenedione is 100 %, 28.6%, and 6.2 %, respectively, and < 2.5 % with all other steroids tested, including epitestosterone (Bradley, 1990). This assay has been validated for assay of T in plasma of T. nigrolutea (Edwards and Jones, 2001). We included duplicate assay blanks (ie tubes without

antiserum) for every sample to measure sample non-specific binding; these counts were subtracted from the sample counts before calculation of assay results. Serial dilutions of fecal extract ran parallel to the standard curve suggesting that T was measured accurately. The sensitivity of the assay is 15 pg T; the intra-assay coefficient of variation for the fecal steroid assay is 5 %, while the inter-assay coefficient of variation is 13 %.

To determine the proportions of conjugated and free steroids in the feces, we used the ether/water extraction method of Velloso et al. (1998). Four fecal samples containing high concentrations of T were lyophilized and extracted as above. After the column purification step, the extracted steroids were re-dissolved in 1 ml distilled water combined with 10 ml ether: this separated conjugated, water-soluble steroid from unconjugated (free) steroid. The tubes were placed in a –20 ^{O}C freezer for 15 min to freeze the lower aqueous layer, then the upper ether fraction was decanted into clean tubes. We dried both fractions completely and re-suspended them in 1 ml methanol before taking sub-samples for RIA of testosterone. Data are reported as means ± standard errors.

We were able to detect T in all fecal samples analyzed. The overall mean recovery (extraction efficiency) was 25 % (N = 32). Fecal T concentrations ranged from ~ 500 ng g^{-1} to ~ 1500 ng g^{-1} of dry feces. The mean fecal T concentrations for each reproductive stage are shown in Figure 1. The mean fecal T concentration varied significantly between reproductive stages (ANOVA: $F_{3,17} = 5.58$, $P = 0.0075$); however, contrary to expectation, fecal T concentrations were lowest (554 ± 129 ng g^{-1} of dry feces) in animals during the mating period, rising significantly to 1398 ± 187 ng g^{-1} in reproductively quiescent animals. Differential ether/water extraction revealed that 12 ± 3.3 % of the fecal T measured by RIA

was free T associated with the organic solvent, with 88 ± 13 % of T associated with the aqueous phase, and presumably representing conjugated steroid.

Thus, our results show that it is possible to measure T in feces of male blue-tongued lizards. Testosterone was detected in all samples assayed, and fecal T concentrations ranged from ~ 500 ng g^{-1} to ~1500 ng g^{-1} dry feces. These concentrations are high compared with those reported for some other species, suggesting that the majority of T is excreted via this route; similarly, the gut is the major route of steroid excretion in Tiliqua rugosa (Bourne, 1972). By comparison, fecal T concentrations range from < 60 ng g^{-1} to a peak of 441 ng g^{-1} in the kakapo (Cockrem and Rounce, 1995) and from 400-800 ng g^{-1} in the male maned wolf (Velloso et al., 1998). In male siberian polecats (Mustela eversmanni) concentrations of T in the feces are similar to those in T. nigrolutea, ranging from 200 ng g^{-1} to about 1000 ng g^{-1} (Brown, 1997). Fecal T concentrations in four species of tortoise ranged from 25 ng g^{-1} to 694 ng g^{-1} (Dobeli et al., 1992; Casares, 1995) with high intra-individual variation. Plasma hormone cycles were not reported, but the patterns of variation in fecal T concentrations appeared to show some correlation with reproductive activity. In contrast, our results suggest that there is an inverse relationship between concentrations of T in feces and in plasma of male blue-tongued skinks, with fecal T concentrations being highest during the non-breeding season, when plasma T levels are low (Fig.1). The only published report of a similar occurrence is in the African elephant, in which fecal estrogens increase late in gestation (Fieβ et al., 1999) while plasma concentrations are decreasing (Hodges et al., 1983).

There are a number of factors that may contribute to this apparently anomalous result. Correlation of fecal and plasma hormone concentrations may be inherently more

problematic in ectotherms (particularly temperate species), than in birds and mammals. In reptiles, gut passage time varies through the year (McKeown, 1996) and therefore rates of excretion may also vary. For another study, we fed female blue-tongued lizards cat food colored with food dye. During late summer, they passed no more than two fecal pellets per week with some dye remaining in the pellets for at least a week, but during spring, pellets were passed daily (Atkins and Edwards, unpublished obs.). Male blue-tongued skinks reduce their feeding rates markedly during autumn, and cease feeding several weeks before hibernation. A slower gut passage time, resulting in accumulation of excreted steroid, could therefore explain the higher concentrations of T in feces during this quiescent period.

Also, temperature influences steroid metabolism profoundly through thermal effects on enzyme activity (Bourne et al., 1986; Huf et al., 1989; Kime & Hyder, 1983). Particularly in temperate reptiles, variation in mean body temperature through the annual cycle may be reflected in changing patterns of steroid excretion through thermal effects on steroid metabolism. For example, a change in the rate of catabolism or clearance of hormone from the plasma would also affect its rate of excretion. Finally, fecal steroid concentrations do not show a distinct annual pattern in all species. For example, in maned wolves there is no clear annual cycle of fecal T, although fecal T concentrations have been used to distinguish between the sexes (Velloso et al., 1998).

Over 80 % of fecal T was present as immunoreactive conjugates in Tiliqua nigrolutea, suggesting that limited deconjugation occurs in the gut in this species. In blue-tongued lizards, testicular tissue from quiescent males produces a larger proportion of conjugated steroids than tissue from reproductively active males (Edwards, 1999). Seasonal variation in rates of testicular steroid conjugation has been reported for some other reptilian

species (Huf et al., 1987). Further studies may reveal variations in the proportion of conjugated T in feces at different times of year.

Our results suggest, therefore, that assay of fecal T concentrations may not be useful for assessing reproductive status in blue-tongued lizards because of the lack of correlation between T concentrations in feces and plasma. As our experience has illustrated, extraction of fecal steroids is problematic in some species. Exploratory studies of fecal steroids in varanids at San Diego Zoo have proved unsuccessful (V. Lance, pers. comm.). For both Japanese crested ibis and rock ptarmigan, extraction of feces using standard organic extraction methods resulted in extracts containing a hydrophobic substance that interfered with RIA of fecal steroids (Kikuchi & Ishii, 1997); for each species, different chromatographic techniques were needed to remove the interfering substance. Our samples similarly contained unidentified interfering substances that were eliminated only by a complex series of purification steps that reduced the extraction efficiency. Further work is needed to investigate the usefulness of fecal steroid monitoring in reptiles, and to identify the physiological reasons for the pattern of fecal T concentrations observed in this study.

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Figure legend

Fig. 1. Comparison of mean fecal testosterone (T) concentrations (ng g^{-1} ± standard error) and plasma T concentrations (ng ml^{-1}) in male blue-tongued lizards (Tiliqua nigrolutea) at four stages of the annual reproductive cycle. (Late spermatogenesis: Sept, N=1; Mating period: Oct-Nov, N = 9; Late reproductive period: Dec, N= 3; Reproductive quiescence: Jan-Mar, N=8.). Mean plasma T concentrations are derived from data presented in full in Edwards and Jones (in press); for clarity, error bars are not shown.