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Urinary 3α,17β-Androstanediol Glucuronide Is a Measure of Androgenic Status in Eld's Deer Stags (*Cervus eldi thamin*)[1]

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ABSTRACT

To determine the primary excretory by-products of testosterone (T), 85 μCi [^{3}H]T was administered i.v. to two adult Eld's deer stags. Blood (10 ml) was collected by jugular venipuncture at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 480 min after isotope infusion, and all urine and feces were collected for 96 h after injection. Seventy percent of labeled circulating steroid was conjugated by 30 min postinfusion. The majority (80.4 ± 3.2%) of T metabolites were excreted into urine, and 95.0 ± 0.9% of these were conjugated, 95.8 ± 0.2% being hydrolyzable with glucuronidase. Seven urinary androgen metabolites, including androstanediol (5α-androstan-3α-17β-diol and 5β-androstan-3α-17β-diol), were identified in glucuronidase-hydrolyzed, ether-extracted Eld's deer urine pools after gas chromatography/mass spectrometry. A double-antibody ^{125}I RIA for 5α-androstanediol-3α,17β-diol,17-glucuronide (3α-diol-G) was validated for unprocessed urine. Longitudinal assessments of urine samples collected from 13 stags for 3 yr revealed biological concordance between fluctuations in urinary 3α-diol-G and serum T, as well as seasonal changes in secondary sexual characteristics. Overall correlation between "same-day" matched serum T and urinary 3α-diol-G was 0.58, ($n = 6$; $p < 0.001$). Thus, monitoring urinary 3α-diol-G provides a noninvasive, alternative method for characterizing male endocrine interrelationships in an endangered ungulate species.

INTRODUCTION

Understanding reproductive-endocrine relationships in many nondomesticated ungulates often is impossible because of the difficulties associated with collecting repeated blood samples for hormonal evaluations. Instead, noninvasive urinary and fecal ovarian steroid metabolite assessments now are commonly used to evaluate female reproductive status in diverse mammalian species [1–15]. Not only is this information important for understanding the fundamental biology of these species, but hormonal data can help drive the development of successful assisted reproduction programs [5, 16, 17]. Interestingly, little effort has been focused upon developing noninvasive methods for monitoring androgenic status in males.

Most of our knowledge about reproduction in cervid species has been derived from studies of temperate species, whereas the majority of cervids exist in tropical or subtropical regions [18]. Unfortunately, financial constraints and poorly designed handling facilities in zoos make it impossible to conduct reproductive-endocrine studies in cervids that require repeated blood sampling. These studies are even less feasible in most range countries where topical cervid species originate. Noninvasive endocrine monitoring has the potential for being utilized to investigate the interaction among hormones, seminal traits, antler development, gross morphometry, and reproductive and social behaviors in both captive and free-living animals. Furthermore, this information is especially important for elucidating the effect of season on reproductive function and the extent of reproductive synchrony among males of the same species.

The Eld's deer (*Cervus eldi thamin*) is an endangered species studied extensively in our laboratory as a cervid model for validating noninvasive monitoring strategies in ungulates. The Eld's deer also is a striking example of a subtropical species that exhibits seasonal reproduction in its native habitat and after translocation to northern temperate latitudes [5, 18–20]. Unlike most temperate cervid species, maximal testicular growth, antler development, and behavioral aggression occur in Eld's deer as daylength increases during the winter and spring. Antlerogenesis corresponds to cyclic fluctuations in blood concentrations of LH, FSH, and testosterone (T) similar to that described for other cervids; however, hormonal rhythms are shifted 6 mo out of phase relative to most temperate deer [5, 18].

The objectives of the present study were to 1) determine the time course and dynamics of [^{3}H]T metabolism and the relative proportion of radiolabel excreted into urine and feces, 2) validate an RIA for 5α-androstan-3α-17β-diol,17-glucuronide (3α-diol-G) for stag urine, and 3) compare urinary 3α-diol-G excretion with same-day serum T concentrations. Once the approach was validated, we sought to demonstrate its utility for examining circannual androgen excretion patterns over a 3-yr period in 13 Eld's stags and to correlate endocrine data to the timing of rut.

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MATERIALS AND METHODS

Radiolabel Studies

To determine the primary excretory by-products of T, approximately 85 μCi [^{3}H]T (1,2,6,7-[^{3}H]testosterone; New England Nuclear, Boston, MA) was administered in June to two sedated (0.3 mg/kg xylazine hydrochloride i.m.; Rompun, Mobay Corp., Shawnee, KA), adult Eld's deer stags (2 and 3 yr of age, respectively) i.v. (10 ml 0.9% saline) into the recurrent tarsal vein. Immediately before administration, 50 μl of isotope was removed from each of the two injection syringes and mixed with 10 ml scintillation fluid (Ready Protein; Beckman Instruments Inc., Fullerton, CA) after which disintegrations per minute (dpm) were counted five times/sample on a Beckman LS5801 scintillation counter using a single-label quench compensation program. After isotope administration, syringes were rinsed successively with ethanol and scintillation fluid, and the residual radioactivity was counted and subtracted from the preinjection total. Stags 1 and 2 received 83.5 and 85.0 μCi, respectively.

Blood (10 ml) was collected by jugular venipuncture at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 480 min after isotope infusion. While animals were maintained within the metabolic holding stalls, all urine and feces were collected at 4-h intervals for 96 h after infusion and frozen (−20°C) until processed. The individual metabolism pens were 1 × 2 m, which was sufficient to allow each stag to stand freely, recline, and turn around. A center-positioned drain in the rubber-coated floor permitted urine to flow into a collection vessel mounted to the side of the stall. Feces were removed from the stall floor by a broom without removing animals from the pens. Aliquots of thawed samples (0.5 g feces, 0.5 ml urine) were initially mixed with 20 ml scintillation fluid and then further subdivided with additional scintillation fluid to achieve an "H" number (a measure of quench) for each vial below 120 (value at which little or no quench is observed for labeled steroid added to sample). Counts for all vials were summed to determine the total dpm excreted per unit measure of sample. The relative excretory rate of [^{3}H]T was determined by dividing the dpm detected in urine or feces by the total quantity of label detected in both compartments. The time course of steroid conjugation in blood circulation was determined by radioactive counting of aqueous and organic phases after ether extraction (1:10, aqueous:organic). Quantity of label present in each phase was compared to the total counts present in each sample before extraction and expressed as a percentage of the total.

The relative proportion of conjugated and unconjugated [^{3}H]T metabolites in urine (0.5 ml) was determined by radioactive counting of aqueous and organic phases after ether extraction (1:10, aqueous:organic). Residual aqueous extractants were enzymatically hydrolyzed with either β-glucuronidase/aryl sulfatase (2000 Fishman U glucuronidase activity, 16 000 Roy U sulfatase activity at pH 5; Boehringer-Mannheim Corp., Indianapolis, IN), glucuronidase (2000 Modified Fishman Units at pH 6.8, Type IX-A, G 7396; Sigma Chemical Co., St. Louis, MO), or sulfatase (30 Sigma Units at pH 5, Type VIII; Sigma), or they were subjected to acid solvolysis [21]. After hydrolysis, ether extraction (see above) was used to determine the proportion of hydrolyzable conjugates relative to the total counts contained in the aqueous phase before extraction.

Gas Chromatography/Mass Spectrometry (GC/MS)

Each of twenty 0.5-ml urine aliquots was extracted with 10 volumes of diethyl ether to separate free from conjugated steroids. Residual aqueous phase fractions containing conjugated metabolites then were enzymatically hydrolyzed with β-glucuronidase/aryl sulfatase (18 h at 37°C) and reextracted with diethyl ether as described previously. Remaining organic extractants containing enzyme-hydrolyzable metabolites were pooled, evaporated, and stored frozen (−70°C). Specific androgen metabolites were identified by means of GC/MS as described previously [22].

3α-diol-G RIA

A double-antibody ^{125}I RIA for 3α-diol-G (Diagnostic Systems Laboratory, Webster, TX) was validated for unprocessed urine. Serial dilutions yielded displacement curves parallel to those of standard preparations, and the mean recovery of added 3α-diol-G (range, 1.25–50 ng/ml) was 99.2 ± 9.1% ($y = 1.12x + 0.43$, $r^2 = 0.99$). Assay sensitivity was 0.5 ng/ml. Interassay coefficients of variation for two separate internal controls were 8.6% and 10.4%, respectively, and intraassay variation was < 10%. RIA of eluates after HPLC [4] revealed all immunoreactivity to be associated with a single peak that coeluted with standard 3α-diol-G. To compensate for variable fluid intake and urine concentration, creatinine (Cr) content in each urine sample was estimated by use of a modified Jaffe reaction [5, 23]. Hormone levels were divided by Cr concentrations, and values were expressed as mass units/mg Cr.

Circannual Evaluations

For assessing longitudinal androgen metabolite profiles, urine samples were collected one to three times weekly from 13 adult Eld's deer stags (1–10 yr of age; 70–120 kg BW) maintained at the Conservation and Research Center, Smithsonian Institution (Front Royal, VA; 38°N latitude). Males were housed individually in indoor stalls (3.4 × 4.6 m with skylights) connected to outdoor enclosures (3.6 × 36.6 m) and exposed to natural fluctuations in photoperiod. Individuals were maintained within visual proximity to other males and within auditory and olfactory proximity to conspecific adult males and females. Diet consisted of alfalfa hay and herbivore chow (12.5% crude protein, 3.0% crude fat, 16.0% crude fiber; Ralston Purina Co., St. Louis,

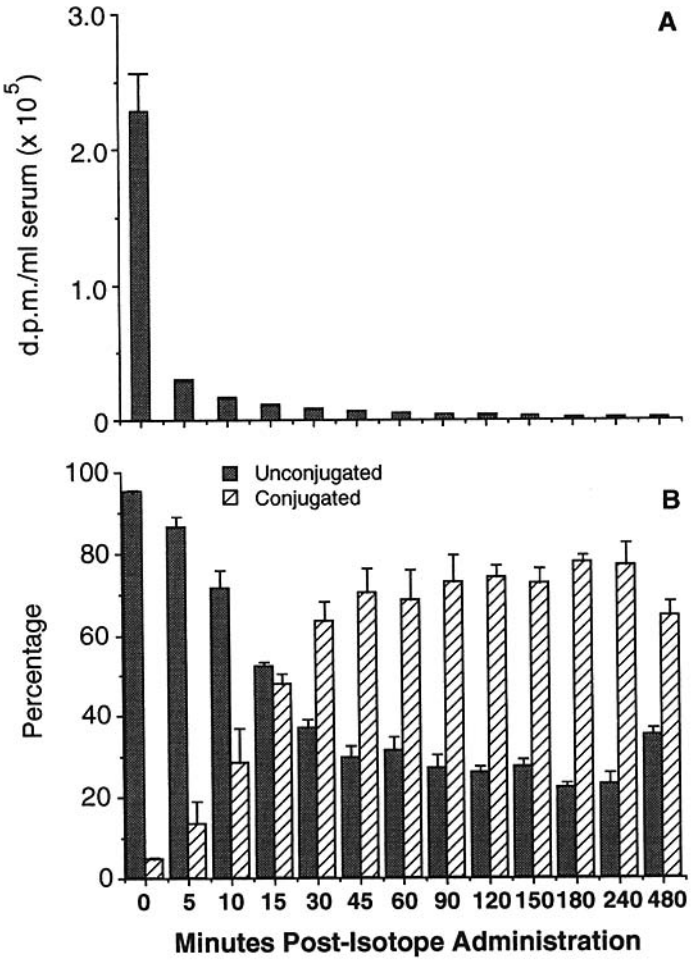

FIG. 1. A) Total radioactivity (dpm) in serum. B) Relative proportion of circulating radiolabel (expressed as a percentage of total) that either was unconjugated or conjugated during the first 8 h postinfusion. Data are mean ± SEM (n = 2).

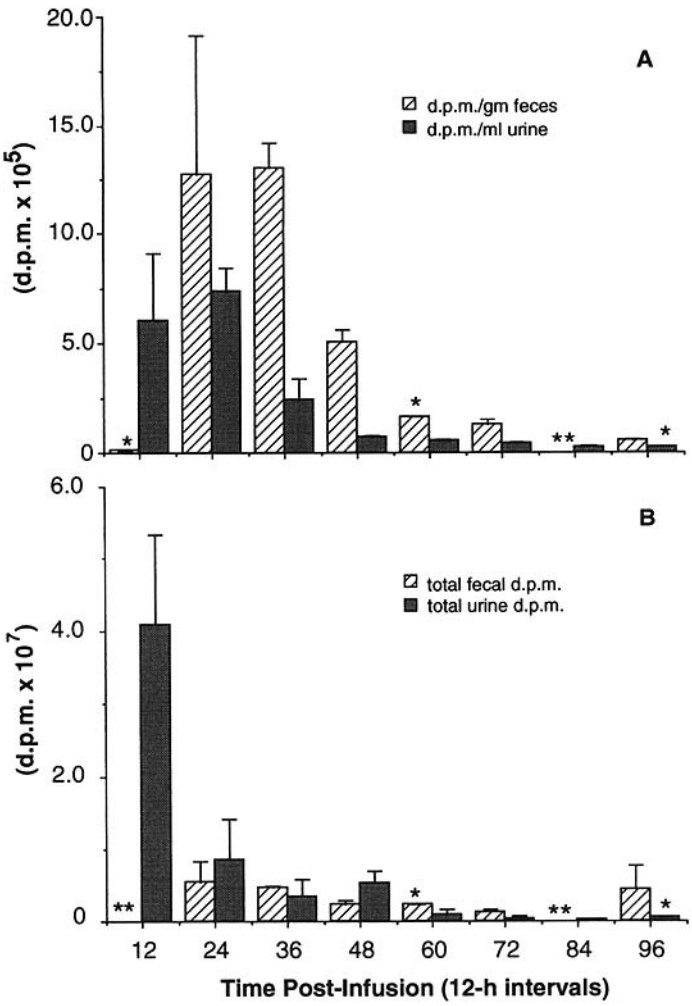

FIG. 2. Time course of radiolabel excretion from two stags during 96 h postinfusion. Data are expressed (A) as mean dpm per unit measure in urine or feces and (B) as total dpm excreted over time. *Time periods with data from only one animal. **Sampling periods when no samples were obtained.

MO) fed at the rate of 2–3% of body weight daily; animals had free access to a mineral block and water. All males were conditioned to enter an enclosed urine collection enclosure (1 × 2 m) constructed within their individual stall that was similar to the metabolism pen described previously. The rubberized floor of the urine collection platform was washed before males were introduced, and urine samples (3–5 ml) were collected from a center-positioned floor drain. Animals generally urinated within less than 1 h after entering the collection enclosure. Same-day blood samples, body weights, antler measurements, morphometric measurements, and behavioral data also were collected weekly from 6 of 13 stags during Year 1 and from 12 of 13 stags during Year 2 as part of another previously published study [18]. Only urine samples, body weights, and observations of antler status (velvet stripping and antler casting) were collected during Year 3.

Statistical Analyses

Hormone concentrations are represented as mean ± SEM. All statistical comparisons were performed with Statview 512+ (BrainPower, Inc., Calabasas, CA) on a Macintosh computer. Comparisons among weekly and seasonal parameters were determined by correlation analyses and repeated-measures analysis of variance. A posteriori multiple pair-wise contrasts were performed by Fisher's protected least significant difference test. Hormone concentrations that were below the limit of detection were assigned a value corresponding to the limit of detection for statistical calculations.

RESULTS

Radiolabel Studies

Radiolabeled T was cleared rapidly from blood circulation, and only 5.3 ± 1.6% of the quantity detected at time

0 in serum remained 15 min later (Fig. 1A). On the basis of the proportion of radiolabel detected in organic (unconjugated) or aqueous (conjugated) phases after diethyl ether extraction, free [^{3}H]T was conjugated rapidly, with the majority (63.3 ± 4.8%) of circulating label associated with the conjugate fraction by 30 min postinfusion (Fig. 1B). Mean total urine volume and feces weight were 2088 ± 596 ml and 1162 ± 669 g, respectively. Proportion of total radioactivity recovered in both urine and feces was 34.7% and 49.3%, respectively, for the two stags. Of these totals, 75.3 ± 2.1% of the radiolabel was excreted in urine and 23.7 ± 1.1% in feces. However, because more urine than feces (vol:wt ratio) was excreted, peak radiolabel concentrations (Fig. 2A) in feces exceeded those in urine. Peak radiolabel excretion (per unit volume or weight) was greatest during the first 36 h after radiolabel administration for both urine and feces. The majority of radioactivity (68%) in urine was excreted during the first 12 h (Fig. 2B), and 97% was excreted within 48 h. In contrast, no radioactivity was voided in feces during the first 12 h postinfusion, and although peak excretion (28%) occurred from 12 to 24 h, radioactivity was still detected in feces at 96 h postinfusion.

Because the majority of [^{3}H]T metabolites were excreted in urine, subsequent efforts focused solely upon identifying the predominant urinary androgen metabolites. Differential diethyl ether extraction of urine revealed that 95.0 ± 0.9% of [^{3}H]T metabolites were conjugated. Enzymatic hydrolysis with glucuronidase, glucuronidase/aryl sulfatase, or sulfatase liberated 95.8 ± 0.2, 95.4 ± 0.7, or 10.4 ± 0.4%, respectively, of label from the aqueous phase; acid solvolysis liberated 97.9 ± 0.3%. Urinary androgen metabolites identified in glucuronidase-hydrolyzed, ether-extracted Eld's deer urine pools after GC/MS included androsterone, etiocholanolone, androstanediol (5α-androstan-3α-17β-diol and 5β-androstan-3α-17β-diol), 11-oxo-etiocholanolone, 11-oxo-androsterone, and 11β-hydroxytestosterone.

Circannual Rhythms of 3α-diol-G Excretion

Overall correlation of urinary 3α-diol-G excretion to matched, same-day T concentrations was 0.58 (n = 6, $p < 0.001$, $df = 238$); two representative matched serum-urine profiles are depicted in Figure 3, A-D. Overall mean 3α-diol-G excretion profiles (n = 13 stags), aligned by the calendar year in which samples were collected, are presented in Figure 4, A and B. Between midsummer and mid-autumn, 3α-diol-G concentrations were low (July-October: mean range, 10–15 ng/mg Cr) and increased ($p < 0.05$) 2–4-fold above baseline as winter approached (mid-November and December: mean range, 20–60 ng/mg Cr). Peak 3α-diol-G concentrations were observed in winter from January to March (mean range, 120–340 ng/mg Cr). Thereafter, 3α-diol-G declined steadily to basal concentrations by early July. Figure 5, A-F, represents typical longitudinal urinary 3α-diol-G profiles from six stags from which urine was collected for 2–3 yr (1–3 urine samples/wk), demonstrating the variation in 3α-diol-G excretion among animals. Figure 6 depicts the same mean urinary 3α-diol-G data aligned by calendar date and stag age on the date sample collections were initiated. In general, the qualitative excretory profiles were similar for all stag ages. However, there was a steady increase in the

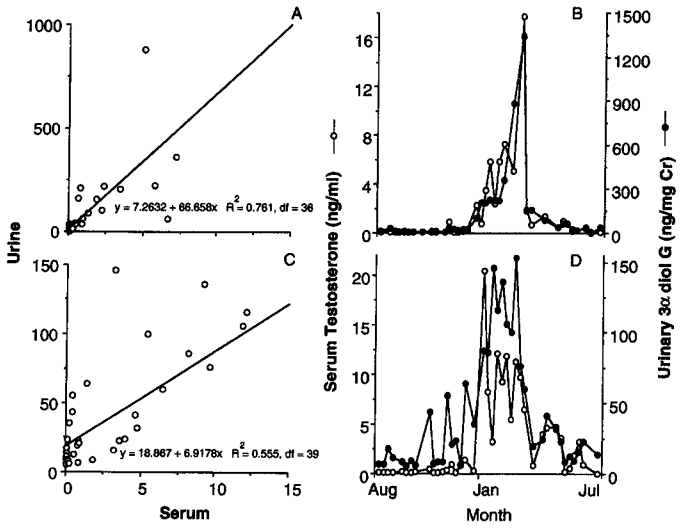

FIG. 3. A, C) Correspondence between serum T and urinary 3α-diol-G in two Eld's deer stags sampled weekly for 1 yr. B, D) Serum T and urinary 3α-diol-G profiles in the same two males.

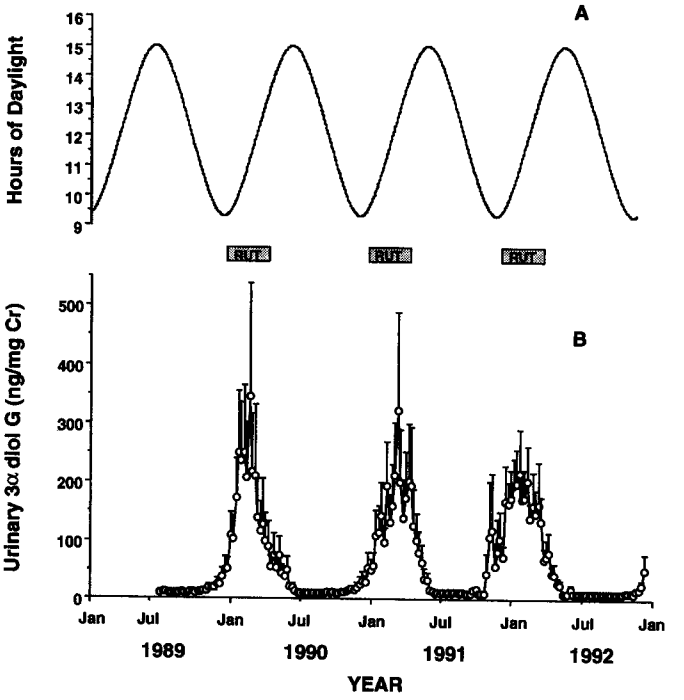

FIG. 4. A) Hours of daylight are plotted and periods of increased aggressive/reproductive behavior (RUT) are denoted by shaded boxes; data from [16]. B) Mean (± SEM) weekly urinary 3α-diol-G excretion aligned by calendar year in which samples were collected from 13 Eld's deer stags.

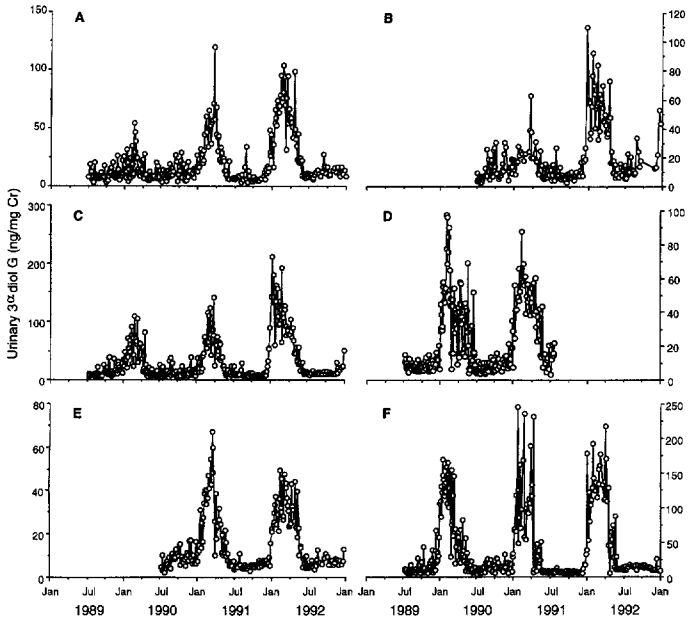

FIG. 5. Representative urinary 3α-diol-G excretion profiles in six (A-F) Eld's deer stags sampled 1–3 times/wk for 2–3 yr. Note different scales.

peak amount of 3α-diol-G concentrations during the breeding season (January-March) as stag age increased. From 1 to 5 yr of age, peak 3α-diol-G concentrations ranged from 20 to 190 ng/mg Cr, whereas from 6 to 10 yr, peak 3α-diol-G ranged from 450 to 1200 ng/mg Cr. Mean peak 3α-diol-G concentrations were greatest in 8–10-yr-old stags.

DISCUSSION

By determining the excretory fate of [^{3}H]T, we demonstrated that T in Eld's deer was rapidly metabolized and was excreted predominantly as glucuronidated urinary metabolites. Furthermore, one excretory byproduct of T metabolism was 3α-diol-G, which was quantifiable by an RIA for unprocessed urine. This method provided a valid, noninvasive tool for characterizing male endocrine interrelationships, and our data represented the first example of how this approach can be useful to evaluate circannual reproductive rhythms in an endangered male ungulate species.

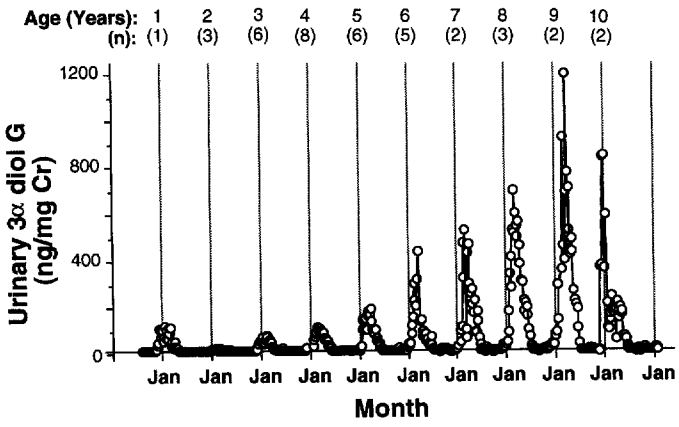

FIG. 6. Mean urinary 3α-diol-G excretion in 13 Eld's deer stags aligned by animal age and calendar month at time of sample collection.

In humans, secreted T is converted to dihydrotestosterone (DHT) by irreversible 5α reduction, which acts at the nuclear level to exert androgen action in sexual tissues [24, 25]. DHT serves as a paracrine hormone in target tissues, and most DHT is converted locally (by reversible 3α reduction/oxidation) to 3α-diol and 3α-diol-G, which then are released into the circulation [24, 25]. Accordingly, circulating 3α-diol, particularly 3α-diol-G, appears to provide an excellent index of whole-body DHT formation and, indirectly, 5α-reductase activity in humans [25]. Urinary 3α-diol-G also has been reported to be a major androgenic steroid in human urine [26], and plasma and/or urinary 3α-diol-G concentrations are elevated in patients with hirsutism or acne [25]. Furthermore, 3α-diol-G in men begins to increase during puberty, and young men have increased 3α-diol-G compared to elderly men [27, 28]. There seems to be consensus that urinary 3α-diol-G production in humans is a useful indicator of androgenic activity in peripheral tissues. However, it has been suggested that plasma 3α-diol may be a superior index of androgenicity [27, 29]. Urinary 3α-diol-G is presumed to reflect both extrahepatic metabolism and hepatic conversion/conjugation of steroid precursors, whereas plasma 3α-diol is derived almost exclusively from extrahepatic metabolism (i.e., within sexual tissues). On this basis, it has been argued that plasma 3α-diol may provide a more specific measure of androgenicity than urinary 3α-diol-G [27, 29].

To our knowledge, no study has investigated urinary 3α-diol-G excretion in any ungulate species. Like all cervid species, Eld's deer stags experience an annual testicular cycle whereby hypothalamic-pituitary signaling modulates T biosynthesis, which in turn influences spermatogenesis, antler development, and reproductive behavior [17, 18]. Antler growth occurs in the relative absence of T, and, after antler development is complete, T prevents antler casting [30, 31]. Once antlers become completely mineralized and the attachment with the antler pedicle is complete, reduced T production results in antler casting [32]. Although a clear relationship has been established between annual fluctuations in T secretion and antlerogenesis, it is unknown whether T modulates antler development directly or acts as a prohormone that is further metabolized in target tissues [32]. Although it has been shown that exogenously administered DHT or 5β-androstan-3α-17β-diol induces partial antler mineralization [32], no study has investigated the impact of exogenously administered 3α-diol on antlerogenesis.

Our results clearly demonstrate the biological concordance between fluctuations in urinary 3α-diol-G and serum T, as well as seasonal changes in secondary sexual charac-

teristics. First, we demonstrated that urinary 3α-diol-G excretion corresponded with matched, same-day serum T concentrations. In a previous study, Monfort et al. [18] demonstrated that longitudinal assessments of serum T revealed distinct seasonal oscillations that correspond with seasonal changes in antler growth, body eight, testicular size, ejaculate characteristics, and aggressive behavior. On the basis of analysis of matched, same-day urine and serum samples, we now have retrospectively demonstrated that urinary 3α-diol-G fluctuated in parallel with serum T, indicating that 3α-diol-G excretion provided an accurate, indirect measure of testicular steroidogenesis. Circannual urinary 3α-diol-G excretion profiles generated over a 3-yr period confirmed that, unlike most temperate cervid species, the testicular cycle in Eld's deer stags began during autumn and winter, with maximal androgen production, rutting behavior, and sperm production occurring during winter and spring as day lengths increased [5, 18]. The difference is that our earlier studies required more animal manipulation including, in some cases, restraint, sedation, or even anesthesia, all procedures that could potentially confound data interpretation due to stress. Although hormonal rhythms in Eld's deer stags were shifted 6 mo out of phase relative to most temperate deer, antler mineralization, peak aggressive behavior, and semen quality were observed when urinary 3α-diol-G concentrations were maximal [18]. Seasonal oscillations in serum T have been reported for numerous other cervid species including red deer (*Cervus elaphus* [33]), fallow deer (*Dama dama* [34]), white-tailed deer (*Odocoileus virginianus* [35]), black-tailed deer (*Odocoileus hemionus columbianus* [36]), roe deer (*Capreolus capreolus* [37]), chital (*Axis axis* [38]), reindeer (*Rangifer tarandrus* [39]), caribou (*Rangifer arcticus* [40]), rusa deer (*Cervus rusas timorensis* [41]), and wapiti (*Cervus elaphus nelsoni* [42]).

Although urinary 3α-diol-G may result from both hepatic conversion of androgen precursors and extrahepatic metabolism in peripheral tissues [25], this distinction appeared to be unimportant in tracking androgenic status in Eld's deer. However, it is tempting to draw parallels to literature on humans demonstrating that 3α-diol-G is elevated in highly masculinized individuals, presumably because of peripheral conversion of DHT in sexual tissues [25]. Because male deer undergo remarkable seasonal alterations in secondary sexual characteristics, it is likely that DHT produced in peripheral tissues plays an important role in modulating these changes. In fact, seasonal fluctuations in serum DHT parallel T in fallow deer bucks [43]. Although it is presently speculative, 3α-diol-G production in Eld's deer probably reflects, at least in part, DHT metabolism and androgen activity within sexual tissues.

Our observation that 3α-diol-G production increased with stag age was similar to the gradual age-related increase in peak T secretion reported in male wapiti [42], fallow deer [43], and white-tailed deer [44]. In general, peak T concentrations during the breeding season for these species increased by 5 yr of age, suggesting that males achieved "prime" reproductive status by this time. A similar pattern of T secretion was observed for Eld's deer stags, with peak T production observed by 4 yr of age with no apparent increases in T secretion thereafter ([16] and unpublished data). The temporal increase in urinary 3α-diol-G excretion for Eld's deer stags was striking because excretion of this androgen metabolite continued to increase up to 9 yr of age, with a pronounced increase in urinary 3α-diol-G excretion observed beginning at 5 yr of age. Subsequent 3α-diol-G excretion increased steadily, achieving peak concentrations from 8 to 10 yr of age. The qualitative differences in the trends of serum T and urinary 3α-diol-G further suggested that 3α-diol-G production in Eld's deer stags reflected DHT metabolism and androgen activity within sexual tissues rather than testicular androgen production alone. Although no males older than 10 yr were sampled in the present study, diminished antler development in stags > 10 yr of age has been observed in our study population, suggesting that androgen production probably declines in aged Eld's deer stags in a manner similar to that reported for white-tailed bucks [44].

From a conservation perspective, Eld's deer typify the challenges associated with managing captive, wild hoofstock populations. Their excitable temperament increases the risk of stress and injury during routine handling, and they frequently exhibit self-destructive behaviors in captivity. Such behavioral characteristics have historically imposed severe limitations upon the types of reproductive studies that can be conducted. Research initiatives have been further hampered because many nondomesticated ungulates are maintained in zoological institutions with husbandry and management schemes poorly suited for conducting detailed reproductive-endocrine studies. Thus, our efforts focused upon developing a noninvasive method for effectively and safely monitoring androgenic status. Although a substantial reproductive database now exists for Eld's deer, a wealth of valuable new information still can be accumulated by studying long-term circannual reproductive rhythms in this and other ungulate species. Perhaps most importantly, application of these noninvasive methods will permit studies designed to determine the impact of uniform photoperiod, seasonal rainfall patterns, and food availability on reproductive seasonality of Eld's deer living in native subtropical habitats. Noninvasive endocrine monitoring techniques already have been adapted for studying free-living animals [9, 45, 46], and this strategy holds enormous potential for improving our understanding of the reproductive biology of both captive and wild animals living in range countries. This is especially crucial because four of five mammalian species exist in the tropics, and, amazingly, the reproductive biology of virtually all of these species remains unstudied.

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