Fecal testosterone in bighorn sheep (*Ovis canadensis*): behavioural and endocrine correlates

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Abstract: Noninvasive endocrine techniques allow repeated sampling of the same individual to study causes and consequences of variation in individual behaviour and physiology. In this study, radioimmunoassay was used to measure fecal testosterone and to assess the repeatability of the testosterone assay for bighorn rams (*Ovis canadensis*). Fecal samples were collected from marked males during the pre-rut and the rut over 2 years. Results were highly repeatable for samples of the same ram within a day ($r = 0.93$). Fecal testosterone peaked during the pre-rut (when social relationships are established) and then declined from the pre-rut to the rut. For both years of study, fecal testosterone was correlated with social rank (2001: $r = 0.73$, $P < 0.0001$; 2002: $r = 0.54$, $P = 0.007$) and age (2001: $r = 0.65$, $P = 0.002$; 2002: $r = 0.53$, $P = 0.008$) of individual rams. When age was accounted for, however, the relationship between social rank and testosterone was no longer significant. Aggressiveness (measured as hourly interaction rate) was weakly correlated with fecal testosterone ($r = 0.44$, $P = 0.039$). There was no association between aggressiveness and social rank ($r = 0.13$, $P = 0.591$). To our knowledge, this is the first report of an association between testosterone levels and individual social rank in wild ungulates.

Résumé : De nouvelles techniques non-invasives permettent de faire des dosages répétés des concentrations hormonales sur les mêmes individus afin d'étudier les causes et les conséquences de la variation individuelle du comportement et de la physiologie. Nous avons dosé par radio-immuno-essais la testostérone fécale et vérifié la répétabilité des analyses dans des échantillons prélevés sur de mâles marqués du mouflon d'Amérique (*Ovis canadensis*) pendant 2 ans durant le pré-rut et le rut. Nos résultats indiquent une très forte répétabilité des mesures de testostérone faites sur des échantillons prélevés sur un même individu au cours d'une même journée ($r = 0{,}93$). Les concentrations de testostérone atteignent un maximum au cours du pré-rut (période durant laquelle les mouflons établissent leur rang social) suivi d'une diminution graduelle jusqu'au rut. Au cours des 2 années de notre étude, les concentrations de testostérone fécale étaient en corrélation positive avec le rang social (2001 : $r = 0{,}73$, $P < 0{,}0001$; 2002 : $r = 0{,}54$, $P = 0{,}007$) et l'âge (2001 : $r = 0{,}65$, $P = 0{,}002$; 2002 : $r = 0{,}53$, $P = 0{,}008$) des mouflons. Cependant, lorsque l'effet de l'âge est pris en compte, la corrélation entre le rang social et la testostérone disparaît. Il y a aussi une faible corrélation entre l'agressivité (mesurée par le nombre d'intéractions à l'heure : $r = 0{,}44$, $P = 0{,}039$) et la testostérone. Cependant, il n'y a aucune corrélation entre le rang social et l'agressivité ($r = 0{,}13$, $P = 0{,}591$). À notre connaissance, notre étude est la première à démontrer l'existence d'une relation entre le rang social et la concentration de testostérone chez les ongulés sauvages.

Introduction

The ability to monitor hormonal levels in free-ranging animals may increase our understanding of the causes and consequences of variation in individual behaviour and physiology. Knowledge of hormonal levels can provide insights into the evolution of life histories and the factors shaping social organization and mating systems (Ketterson and Nolan 1992). Testosterone is a steroid hormone whose secretion by the testes (Binkley 1995) varies in response to diverse environmental stimuli such as day length, aggressive encounters, or female sexual behaviour (Wingfield et al. 1990; Ketterson and Nolan 1992). In polygynous mammals, testosterone stimulates muscle and skeletal growth, promotes secondary sexual characteristics, and increases aggressive behaviour and metabolic rate, all traits that may enhance intrasexual competitive ability (Sapolsky 1993). For example, testosterone levels are correlated with aggression (Creel et al. 1992; Sapolsky 1993; Creel et al. 1997), male social dominance (Wickings and Dixson 1992; Sapolsky 1993; Koren et al. 2002), and courtship (Adkins-Regan 1981). Because testosterone promotes aggressive behaviour, its secretion is expected to peak during periods of male–male aggressive contests (Wingfield et al. 1990; Sapolsky 1993). Mating also stimulates testosterone secretion (Sapolsky 1993); therefore, testosterone is also expected to peak during the mating season.

Recent studies of the androgens–behaviour relationship in mammals have mostly examined primates (Wickings and Dixson 1992; Sapolsky 1993; Brockman et al. 1998; Kraus

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et al. 1999; Strier et al. 1999; Cavigelli and Pereira 2000; Lynch et al. 2002) and rodents (Sachser and Pröve 1984; Wee et al. 1988; Boonstra et al. 2001; Trainor and Marler 2002). The lack of field data on the androgens–behaviour relationship in ungulates is particularly striking. The inherent difficulties in conducting long-term research on marked individuals (Gaillard et al. 1998) and the difficulties of assessing hormone levels of free-ranging animals (Monfort et al. 1997) are most likely responsible for this situation. Nevertheless, ungulates are a good model for studying how androgens may affect behaviour because they are long-lived (Gaillard et al. 1998) with a wide variety of mating systems (Jarman 1983; Clutton-Brock 1989) where access to estrous females is often determined by social rank (Clutton-Brock et al. 1982; Hogg 1984, 1987; McElligott et al. 2001). Therefore, if testosterone promotes fighting ability (Sapolsky 1993), one should expect a positive association between social rank and testosterone for male ungulates when intrasexual contests are most frequent, which for many species would be the pre-rut, when social rank is established (Geist 1971). The few studies conducted on ungulates revealed an autumn increase in testosterone secretion (Inga 1984; Lincoln 1989; Stevenson 1994), but none investigated the relationship between social dominance and testosterone.

Bighorn sheep (*Ovis canadensis*) are polygynous and highly dimorphic (Festa-Bianchet et al. 1996). They are sexually segregated except for the rut (Ruckstuhl 1998), and males switch from nursery groups to bachelor groups at 2 or 3 years of age (Ruckstuhl 1999). In late September and October, rams congregate and challenge each other with agonistic behaviours that may escalate in dominance fights (Geist 1971). This period is called the pre-rut and it is used by males to establish their social status (Geist 1971). Male social rank then becomes the main determinant of access to estrous ewes during the rut (Hogg 1984, 1987). In early November, rams begin to join female groups and start sexual displays.

The rut begins at the end of November and ewes show a high degree of synchrony of estrus (Hogg 1984). Males 2 years old and older usually participate in rutting activities (Shackleton 1991; Hogg and Forbes 1997), but the largest yearling males also show sexual behaviour during the rut. Male mating strategies depend on social rank. Dominant males defend or "tend" females, trying to prevent other males from mating with the defended female. Subordinate males use two alternative strategies, coursing and blocking. Coursing rams try to breach the tending ram's defence, chase the ewe, and attempt to copulate. Blocking rams coerce anestrous females away from other rams (Hogg 1984, 1987).

Hormonal levels historically have been measured from blood samples, requiring capture and restraint. For free-ranging wildlife, however, capture and restraint are stressful, time-consuming, and costly. Consequently, repeated blood samples of many individuals are impractical (Monfort et al. 1997). In addition, capture stress may affect hormone concentrations, rendering the measurements unreliable (Boonstra et al. 2001). Over the last decade, noninvasive techniques have been developed to assess hormone levels from saliva (von Engelhardt et al. 2000), urine (Lasley and Kirkpatrick 1991; Venturelli et al. 1995), feces (Creel et al. 1992; Monfort et al. 1997; Ziegler et al. 1997; Kapke et al. 1999), and hair (Koren et al. 2002). These promising methods allow the collection of daily samples for longitudinal endocrine studies of wild animals, and because their results reflect hormone secretion over composite periods of time (Creel et al. 1997), they may give a better daily "picture" of individual hormonal levels compared with blood samples.

This paper presents a method for fecal testosterone sample collection and analysis. To assess the repeatability of fecal testosterone analysis in bighorn sheep, several samples of the same animal were collected during the same day and a repeatability index was estimated. Then, to test whether testosterone was related to social rank and aggressive behaviour, fecal samples were collected during the pre-rut and the rut over 2 years. We expected testosterone concentrations to peak during the pre-rut, when dominance interactions are most frequent, and during the mating season, when males interact frequently with females. We also expected fecal testosterone levels to increase with male age and social rank. Finally, we expected a positive association between aggressiveness and testosterone.

Methods

Study area and sample collection

Bighorn sheep wintering in the Sheep River Provincial Park (Alberta, Canada) were studied in 2001 and 2002. This population has been marked and monitored since 1981 to study mating system and population dynamics (Festa-Bianchet 1986; Hogg and Forbes 1997; Hogg 2000). More than 95% of the sheep are marked with ear tags. The park was searched for ram groups every day during the pre-rut and the rut (mid-September to mid-December). Observations were made with binoculars and spotting scopes (15–45×) on 31 males in 2001 and 30 in 2002. In 2001 and 2002, feces from all males present in the park were collected on 15 September, 15 October, 15 November, 1 December, and 15 December. Because some rams, particularly midranking ones, leave the park to rut elsewhere (Hogg 2000), not all males sampled during the pre-rut could be sampled during the rut. Therefore, sample size for testosterone assays ranged from 17 to 31 in 2001 and from 7 to 30 in 2002 depending on the date of sampling. Social rank and testosterone levels during the pre-rut were documented for 19 adult males in 2001 and 24 in 2002.

To estimate the repeatability of the testosterone assay, in 2002, 10 rams were sampled up to five times during the same day. Samples were collected from marked individuals soon after defecation. Except for the repeat samples, feces were always collected in the afternoon to minimize potential effects of daily variation in steroid levels (Binkley 1995). For each sample, the date, time of day, age, and identity of the sheep were noted. On the day of collection, feces (2 g of solid feces) were stored in a solution of absolute ethanol (6 mL) and distilled water (6 mL) (Ziegler et al. 1997; Kapke et al. 1999) and kept frozen at –20 °C until the assay procedures. All samples were analyzed within 2–4 months after collection, a storage time recommended to limit any potential effects of storage on steroid concentration (Khan et al. 2002).

Laboratory analyses

On arrival at the laboratory, the tubes were vigorously shaken to resuspend the fecal material and then placed on a laboratory shaker at 200 rpm overnight. The contents were allowed to settle and then, a portion of the extract was poured into a 12 mm × 75 mm tube and centrifuged for 1 h at 4000 rpm to remove fine particles. The supernatant was decanted into a 3.6-mL cryotube and kept frozen until assay.

Samples were assayed using reagents (standards, antitestosterone-coated tubes, ^{125}I testosterone) from Diagnostic Systems Laboratory (Webster, Tex.). Testosterone calibration standards were diluted in phosphate-buffered saline, pH 7.0. The volume of samples and standards added to the antibody-coated tubes was 50 µL. To ensure equal incubation conditions, 50 µL of phosphate-buffered saline was added to the ethanol–water sample extracts and 50 µL of ethanol–water was added to standards. All tubes received 0.5 mL of ^{125}I testosterone solution. The tubes were gently mixed and incubated at 37 °C for 3 h. The supernatant was decanted and the bound radioactivity determined in a Packard Cobra AutoGamma counter. Data were analyzed using a log–logit plot. Concentrations were determined as nanograms per millilitre and then divided by the mass of feces extracted to give the results as nanograms per gram of feces. Initial binding (percentage of total bound by the zero standard) was 53 ± 1.4% and nonspecific binding was 2.6 ± 0.3%. The 50% displacement point for testosterone was 0.4 ng/mL.

Sensitivity (the lowest detectable dose) of the assay was 0.05 ng/mL. Specificity data provided by the manufacturer include cross-reactivity as follows: 5-α-dihydrotestosterone, 6.6%; 5-androstene-3β,17β-diol, 2.2%; 11-oxotestosterone, 1.8%; androstenedione, 0.9%. There is no detectable cross-reactivity to estrogens, progestins, or corticoids. It is possible that androgen metabolites other than the compounds tested were present in the feces and contributed to the assayed result.

All samples were assayed in duplicate. Intra-assay variability of the duplicates was 5.9 ± 3.9%. The assay was repeated for any sample in which the duplicates differed by 15% or greater. Quality controls containing low, medium, and high testosterone levels were run in each assay; inter-assay variability (n = 6 assays) was 12.9%, 6.4%, and 7.8%, respectively. Four samples with relatively high testosterone values were serially diluted 1:2, 1:4, and 1:8 to test for parallelism with the standard curve. Calculated results (expressed as percent observed/expected) gave a value of 101.7 ± 9.8%. Two of the samples with the highest testosterone values were further diluted. The observed/expected value was 96.3 ± 6.3% over the range of 1:2 through 1:128.

Behavioural observations

Two types of behavioural observations were conducted. First, focal animal observations were used to construct time budgets and estimate aggressiveness of individual rams. In 2002, for 23 rams aged 2 years and older, two to four focal samples (averaging 8 h in duration) were collected for a total of 877 sheep-hours of focal observations (range 29–49 h per ram). During focal samples, six types of agonistic interactions were recorded, front kick, rubbing, homosexual mount, clash, butt, and noncontact displacements (Geist 1971; Hogg 1984, 1987), and were used to calculate the hourly rate of interactions for each male.

Second, dominance rank was assessed using agonistic interactions recorded during focal watches and ad libitum observations during the pre-rut in 2001 and 2002. Repetitions of the same behaviour (e.g., a series of front kicks) during an encounter were recorded as a single interaction. A dyadic interaction matrix was constructed for each year using Matman 1.0 (Matrix Manipulation and Analysis, Noldus®) (de Vries et al. 1993). The method (described in detail by de Vries 1995, 1998) tests the statistical significance of the linearity h' of the dominance hierarchy (h' ranges from 0 to 1, with h' = 1 being a perfectly linear hierarchy) using 10 000 randomizations (de Vries 1995, 1998). The dominance hierarchy is then reorganized by a two-step iteration (1000 sequential trials) with rank assignments minimizing first the number and then the strength of inconsistencies. An inconsistency occurs between two individuals, *a* and *b*, if *b* dominates *a* but *b* is ranked below *a* in the hierarchy. The strength of an inconsistency is the absolute difference in rank between *a* and *b* (de Vries 1998). Only males that interacted with at least five other males were included in the matrix. Because the number of males in the matrix varied between years, social ranks were transformed as suggested by Côté (2000):

$$1 - (\text{rank}/n_x)$$

where n_x is the number of rams included in the matrix for year x. Social ranks vary between 0 (most subordinate) and 1 (most dominant). In 2001, 23 rams were included in the matrix, and interactions for 60% of the possible dyads (n = 737 interactions) were recorded. In 2002, 27 rams were included, and interactions for 57% of the dyads (n = 741 interactions) were seen.

Data analysis

All statistical analyses were conducted using SPSS, version 10 (SPSS Inc. 1999). Data were checked for normality and homoscedasticity. A repeatability index was calculated for individual rams following the algorithm proposed by Lessels and Boag (1987). Repeatability (r) is used in quantitative genetics to describe the proportion of variance in a character that occurs among rather than within individuals and it is useful in assessing the consistency of multiple measurements of the same individual (Lessells and Boag 1987). We used Student's t tests to compare testosterone levels between young males found in female groups or in male groups during the pre-rut. Because most sheep were seen every day, the group type of each individual young ram was known with confidence. Spearman correlations (r_S) were used to compare aggressiveness with fecal testosterone and social rank. Pearson correlations (r_P) were used to compare fecal testosterone with social rank and age. Because age and social rank are highly correlated in bighorn rams (Hass and Jenni 1991), a Pearson correlation was also calculated between the residual of social rank and testosterone levels to take into account the potential age effect on social rank. Testosterone concentrations were log transformed to approximate normality. All tests were two-tailed and the significance level was fixed at $P < 0.05$.

Fig. 1. Seasonal variation in fecal testosterone for bighorn rams (*Ovis canadensis*) in 2001 (open boxes) and 2002 (shaded boxes) during the pre-rut (15 September and 15 October) and the rut (15 November (just before the rut), 1 December (middle of the rut), and 15 December (just after the rut)), Sheep River, Alta. Means and standard deviations are presented and the sample size for each period is reported under the *x* axis.

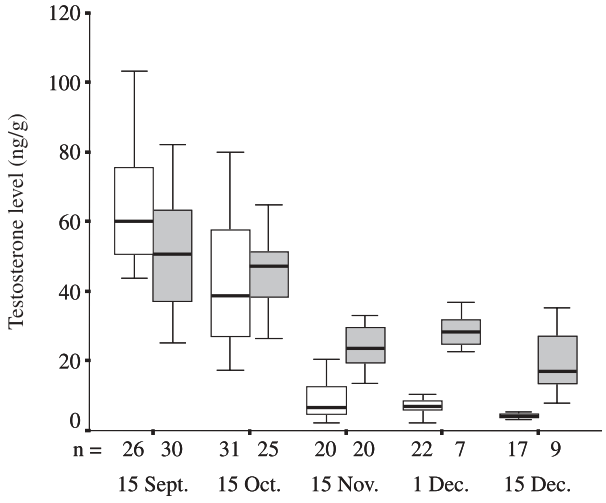


Table 1. Average fecal testosterone during the pre-rut for bighorn rams (*Ovis canadensis*) aged 1–3 years according of the type of group in which they spent most of this period in 2001 and 2002, Sheep River, Alta.

Type of group	Testosterone (ng/g)	SD	*n*
Nursery	49.29	2.18	11
Bachelor	41.13	3.12	8
Nursery (excluding yearling rams)	49.39	4.41	5

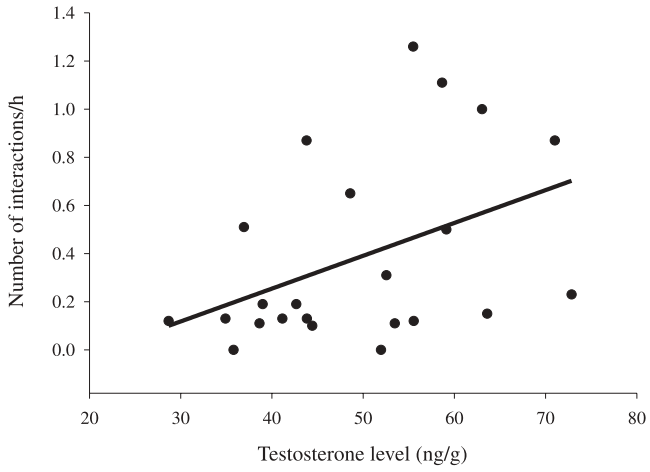
Note: Nursery groups included ewes, lambs, yearlings, and young rams. Bachelor groups included rams 2 years of age and older. *n* is the number of rams sampled in each group.

Because some rams were sampled in both years of the study, pseudo-replication may have occurred (Machlis et al. 1985); therefore, the 2 years were analyzed separately. To avoid pseudoreplication within a year, the mean testosterone level for the pre-rut for each animal was used in the analysis. When more than one time budget was available for the same ram, the mean of all observations for that ram was used.

Results

The minimum testosterone level (2.25 ng/g) was from a yearling ram 10 days before the start of the rut. The maximum (131.78 ng/g) was from an 11-year-old during the pre-rut. In 2002, only 3% of 124 samples had to be reanalyzed because duplicate measurements differed by more than 15%. Within-individual repeatability for sheep resampled within a day (two times: six cases; three times: three cases; five times: one case) was high ($F_{[9,16]}$ = 7.89, r = 0.73) and measurement error was 13% of total within-individual variance ($MS_w/MS_a \times 100$). One ram sampled twice in the same day, however, showed very high variation between its two measurements, with a standard deviation four times higher than for the nine other individuals (range of SD from 0.79 to 11.93). When repeatability was estimated excluding that male, it was very high ($F_{[8,15]}$ = 33.19, r = 0.93) and measurement error was only 3% of total within-individual variance.

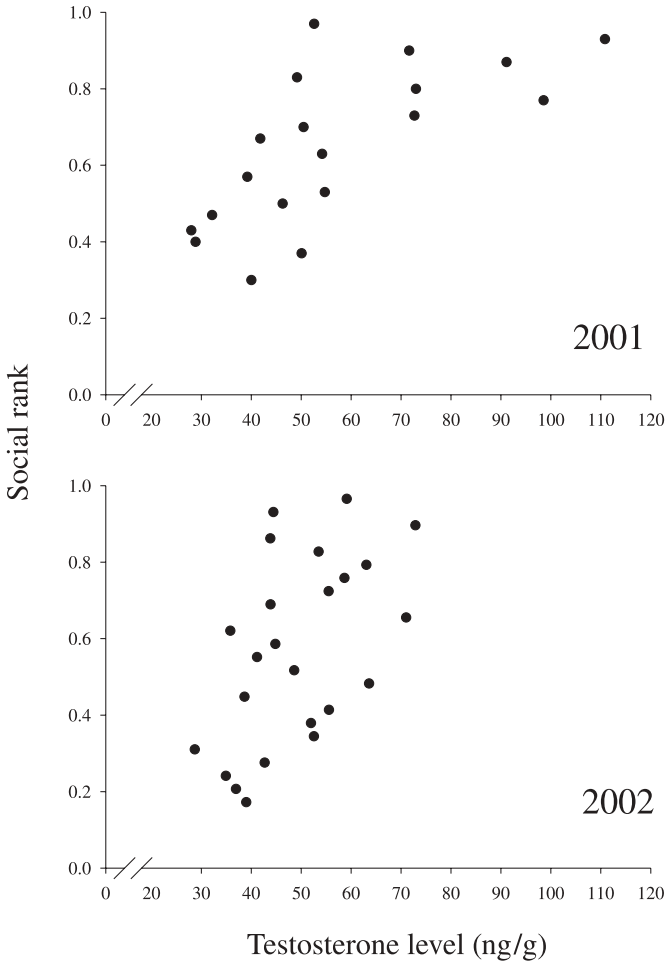
Fig. 2. Average individual hourly rate of agonistic interactions compared with average fecal testosterone during the pre-rut (mid-September to early November) in 2002 for bighorn rams 2 years old and older (r_S = 0.44, P = 0.039, n = 23), Sheep River, Alta.



Each year, we found a conspicuous seasonal cycle in fecal testosterone pattern associated with the mating season (Fig. 1). The maximum level was found during the pre-rut. For the subsequent analysis, we excluded 11 males aged 1–3 years that remained in ewe groups during the pre-rut. These rams were not involved in rank establishment during the pre-rut, and testosterone levels were higher for young rams in ewe groups than for young rams in bachelor groups (P = 0.04) (Table 1). When the analysis of group type effects excluded yearlings, the difference was no longer significant (P = 0.144) but the effect size did not change (Table 1).

The relationship between fecal testosterone and hourly rate of agonistic interactions for individual rams was weak but significant (Fig. 2). The dominance hierarchy was linear in both years (2001: h' = 0.48, P < 0.0001, n = 23; 2002: h' = 0.42, P < 0.0001, n = 27) and social ranks were stable over the pre-rut and the rut. Social rank was not correlated with aggressiveness (r_S = 0.13, P = 0.59, n = 18). In both years, social rank and fecal testosterone were correlated (2001: r_P = 0.73, P < 0.0001, n = 19; 2002: r_P = 0.54, P = 0.007, n = 24) (Fig. 3). Ram age was also correlated with testosterone levels in both years (2001: r_P = 0.65, P = 0.002, n = 21; 2002: r_P = 0.53, P = 0.008, n = 24). Because age and social rank are highly correlated in bighorn rams (males 2 years and older; 2001: r_P = 0.95, P < 0.0001, n = 19; 2002: r_P = 0.95, P < 0.0001, n = 24), the residuals of the quadratic relationship between age and social rank were used to assess the age-independent relationship between testosterone levels and dominance rank. Once corrected for age, the relationship between testosterone and rank was no longer significant (2001: r_P = 0.21, P = 0.392, n = 19; 2002: r_P = 0.01, P = 0.985, n = 24).

Fig. 3. Social rank and fecal testosterone level for bighorn rams 2 years old and older during the pre-rut in 2001 (r = 0.73, P < 0.0001, n = 19) and 2002 (r = 0.54, P = 0.007, n = 24), Sheep River, Alta. Nontransformed data are shown.



Discussion

The seasonal pattern of testosterone concentration observed for bighorns was similar to that reported for other sheep species (Lincoln 1989; Stevenson 1994). Fecal testosterone peaked during the pre-rut, when males frequently challenge each other to establish their dominance rank. Both aggressive interactions and social dominance behaviour correlate with testosterone levels in male mandrills (*Mandrillus sphinx*) (Wickings and Dixson 1992), sifakas (*Propithecus verreauxi*) (Brockman et al. 1998; Kraus et al. 1999), ring-tailed lemurs (*Lemur catta*) (Cavigelli and Pereira 2000), rock hyrax (*Procavia capensis*) (Koren et al. 2002), and wild dogs (*Lycaon pictus*) (Creel et al. 1997). Because the frequency or the intensity of intraspecific aggression can have an effect on testosterone level, and testosterone may promote aggressive behaviour (Wingfield et al. 1990; Sapolsky 1993), it is not surprising that testosterone should peak when social rank is being established through aggressive interactions. For many ungulates including bighorn sheep, rank is established during the pre-rut and similar seasonal patterns of testosterone variation have been reported for some cervids (Lincoln et al. 1972; Haigh et al. 1984; Inga 1984).

In contrast with the results for ungulates, several studies of primates reported that testosterone peaked during the breeding season (Sapolsky 1993; Brockman et al. 1998; Cavigelli and Pereira 2000; Lynch et al. 2002). The reason why testosterone levels slowly decrease from the pre-rut to the rut in bighorn sheep could be related to the timing of social dominance establishment. For bighorn rams, the social rank established during the pre-rut determines priority of access to estrous ewes during the rut (Hogg 1984, 1987; Hogg and Forbes 1997). Agonistic encounters during the rut are mostly used to displace the tending ram during coursing chases. Interactions during coursing chases are brief and independent of social rank: coursing rams are subordinate to the tending ram and do not challenge it but rather attempt to separate it from the estrous ewe, often as a group (Hogg 1988). Established social ranks are respected during the rut: a tending ram will give up defending an estrous ewe if a more dominant ram approaches. On the contrary, in species such as ring-tailed lemurs, testosterone peaks sharply during the mating period when social challenges appear to be more frequent than during the period preceding mating (Cavigelli and Pereira 2000). Consistent with this idea, testosterone levels failed to exhibit significant changes at the onset of sexual behaviour for male muriquis (*Brachyteles arachnoids*) that display a low level of aggression for access to mates (Strier et al. 1999).

In both years of study, testosterone levels were correlated with social rank; however, when age was accounted for, the relationship was no longer significant. As they age, bighorn rams develop larger body size and massive horns, which are important for attaining high dominance rank and defending ewes during the rut (Hogg and Forbes 1997; Coltman et al. 2001). These rams also had the highest fecal testosterone concentrations. Fecal testosterone was weakly correlated with aggressiveness, measured as ram interaction rate. Individual interaction rates were highly variable, possibly because bighorn used highly ritualized behaviours to establish their social rank. Interactions escalated only when opponents were very similar in rank. Moreover, aggressiveness was not correlated with social rank, which seems to be more closely associated with testosterone levels.

The method of testosterone analysis presented in this paper was highly reliable as indicated by the consistency of results for individual bighorns sampled repeatedly in the same day. Because hormone secretion in blood shows a strong circadian cycle (Binkley 1995), fecal testosterone may vary according to time of day. Therefore, it is preferable to concentrate sampling effort during a specific period of the day over the entire study. In this study, all ram groups were usually located by the afternoon; therefore, sampling effort was concentrated during that period. It was not possible to compare fecal testosterone with serum testosterone because this comparison would have required repeated recapturing of several rams over time. However, the seasonal changes in fecal testosterone levels are consistent with those documented in other species of sheep (Lincoln 1989; Stevenson 1994) in studies that used blood samples, suggesting that fecal testosterone is correlated with blood testosterone. The assay results described in this paper may be affected by cross-reactivity with others metabolites because not all of these have been identified, and therefore, other hormones may

have been measured. However, an assay that measures a group of hormones instead of just a specific one may be more effective at discriminating physiologic states (Lasley and Kirkpatrick 1991; Kapke et al. 1999).

This study is the first to report an association between testosterone levels and social dominance in individual ungulates. Because social dominance and age are correlated with reproductive success in bighorn rams (Hogg and Forbes 1997; Coltman et al. 2001), high levels of testosterone may have a positive effect on fitness. However, if high levels of androgen involve physiological costs (Zuk 1996), it is possible that only high-quality individuals can afford that cost.

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