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Effect of Antiandrogen Cyproterone Acetate on the Development of the Antler Cycle in Southern Pudu (*Pudu puda*)

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ABSTRACT The antler cycle of pudu is similar to other cervids, but unlike most boreal deer, male Southern pudu (*Pudu puda*) exhibits two seasonal peaks of LH and testosterone. In that respect, pudu is similar to roe deer. Whereas the antler cycle in some deer species, such as roe deer or white-tailed deer, is very sensitive to variation of testosterone, in other cervids, such as fallow deer or reindeer, a blockade of androgens with cyproterone acetate (CA) has little or no effect on the timing of the antler casting. In order to test the sensitivity of pudu antlers to variations of androgens, CA (administered 2× weekly at 50 mg/buck) was injected intramuscularly for 3 weeks in 5 adult male pudu, starting February 19 (late summer). Four other males of similar age served as controls. The experiment was performed at the University of Concepcion, Chile, latitude 36.6°S. Blood samples were taken once a week between January 19 and April 3. In CA-treated bucks, the antlers were cast approximately 3 weeks after the initiation of CA treatment and a new antler growth began almost immediately. The antlers reached about 5 cm in length, before ceasing to grow at the end of April, when they became mineralized and were subsequently polished. CA had no effect on the already declining levels of LH. Plasma levels of testosterone in controls increased from February 15, whereas in CA-treated bucks remained depresses until March 21. It is concluded that similarly to white-tailed deer, the antler cycle of Southern pudu is very sensitive to manipulation of androgen levels. *J. Exp. Zool. 292:393–401, 2002.*

The antler cycle of male deer is closely associated with the seasonal variation of blood concentrations of reproductive hormones, particularly androgens (Bubenik, '90). The milestones of the antler cycle (such as the antler mineralization, velvet shedding, antler casting, and renewal of antler growth) are convenient external signs indicating internal changes of reproductive status of each individual deer. In most deer species, a rapid antler growth occurs during the spring when androgen levels are low. Late in the summer, an increase in concentrations of androgens, observed before the rut, causes rapid antler mineralization. A sudden decrease of androgen levels after the rut results in antler casting (Bubenik, '90; Suttie et al., '95), whereas the initiation of new antler growth indicates low concentrations of peripheral androgens. In large mammals, including most cervid species, the main male sex hormone testosterone (T) exhibits only one seasonal peak occurring around the rut (Bubenik, '86, '90). Conversely, in the smallest deer of the world, the Southern pudu (*Pudu puda*), LH and T exhibit two peaks of almost equal magnitude, spaced usually half a year apart. The first peak (spring)

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is associated with the mineralization of antlers; the other T peak (fall) is associated with the rut (Bubenik et al., '96, 2000; Reyes et al., '97). Such a bimodal rhythm of LH and T was first observed in the European cervid, the roe deer (Barth et al., '76; Schams and Barth, '82; Sempere, '90). It has been hypothesized that the bimodal variation of seasonal concentrations of reproductive hormones (found so far only in these two small cervids) is the result of evolution. Cervids may have developed in the peri-equatorial regions and then migrated into temperate and boreal latitudes (Acharjyo and Bubenik, '83). Although most tropical cervids are aseasonal breeders and are fertile year-round (Chapman and Chapman, '82; Loudon and Curlewis, '88), boreal deer shift their rut to the fall (Bubenik, '86) in order to secure the most optimal conditions for the survival of their offspring. Conversely, small cervids opted for the strategy of a delayed implantation of the embryo. Such adaptation is confirmed in European roe deer (Aitken, '74; Lambert et al., '99) and is suspected in pudu (Bubenik et al., 2000).

In view of the discrepancies between reproductive cycles of various cervids and the relationship to their antler cycle, we decided to investigate the response of male pudu to the experimental manipulation of circulatory androgen levels. Generally androgens play a crucial role in the development and maintenance of antler cycles of all cervids, but individual species respond quite differently to the manipulation of androgen concentrations.

In order to investigate the role of androgens in the regulation of the antler cycle of various cervid species, androgen receptor blocker, cyproterone acetate (CA) was utilized in several cervid species, including white-tailed deer, roe deer, red deer, fallow deer, and reindeer. In white-tailed deer (*Odocoileus virginianus*), a small amount of CA induced an antler casting within 2–3 weeks after the initiation of the treatment (Bubenik et al., '75, '87). In roe deer (*Capreolus capreolus*), moderate doses of CA prevented antler mineralization (Schams et al., '86) and caused casting within 3 weeks after the first CA injection (Schams et al., '92). In red deer (*Cervus elaphus*), a moderate dose of CA induced antler casting about 3 weeks after the initiation of CA treatment (Suttie et al., '95). In fallow deer (*Dama dama*), casting was achieved only after 9 weeks of treatment with large doses of CA (Kierdorf et al., '93). Finally in reindeer, even large doses of CA did not result in antler casting (Bubenik, unpublished). In order to determine whether the antler cycle of pudu is androgen-dependent, two studies (one pilot one and one final) were performed. We tested the hypotheses that small doses of CA applied in adult male pudu shortly after the natural mineralization of antlers will induce premature antler casting and the effect will be dose and time course dependent. In both experiments the progress of the antler cycle was monitored by photography and the determination of LH and testosterone levels in plasma.

MATERIALS AND METHODS

The experiments were performed at the University of Concepcion breeding colony of pudu, (Concepcion, Chile, latitude 36.6°S). The bucks (live weight 9–10 kg) were kept in small outdoor enclosures, exposed to natural photoperiod, and fed the native shrubs, leaves, and plants, supplemented with corn and oats (Bubenik et al., '96, 2000). All experimental procedures on animals conformed to NIH guidelines and were approved by the Animal Care Committee of the University of Concepcion.

Experiment 1

In this pilot study, three adult male pudu (ages 2, 5, and 6 years) that were carrying hard antlers were treated with androgen receptor blocker cyproterone acetate (CA) (Schering AG, Berlin, Germany). Twice a week, after a manual restraint, each buck was injected subcutaneously with 30 mg of CA. The antiandrogen was dissolved in a mixture of olive oil, benzyl benzoate, and propylene glycol (5:1:3) and injected at concentrations of 100 mg/mL (Bubenik et al., '75). The CA administration began on 20 February and ceased on March 13. After an interruption of several weeks, the antiandrogen was applied again from April 5 until May 22. As in the first period, a dose of 30 mg of CA per deer was used, but this time it was injected every 10 days. The progress of the antler cycle (casting and a new antler growth) was recorded, and the length of new antlers was measured by a caliper every 10 days and documented by photography. The deer were restrained manually on February 20, March 20, May 5, and July 20 so that 3 mL of blood could be drawn from the jugular vein of non-anesthetized bucks into preheparinized tubes. The blood was immediately placed on ice and centrifuged within 1 hr after the sampling. After the separation, plasma was stored at –20°C for a later determination of testosterone and LH.

Experiment 2

Nine adult male pudu (ages 1.3–7 years), all carrying hard antlers polished in November, were divided into two groups, similar in age and rank. Group 1 (control) included 4 bucks (average age 4.5 years), and Group 2 (experimental, CA-treated) included 5 bucks (average age 4.4 years). The animals were caught and manually restrained once a week (starting on 19 January) so that blood samples (3 mL) could be taken from the jugular vein. Blood was treated identically to the first experiment. Beginning on February 8, each animal in the experimental group received 50 mg of CA, injected intramuscularly. The CA treatment continued twice a week until March 3, when the last CA injection was given. Blood sampling continued once a week until April 3, when the last blood collection was performed. The progress of the antler cycle and plasma levels of hormones was monitored as described in Experiment 1.

Radioimmunoassays

Concentrations of luteinizing hormone (LH) were determined by a homologous bovine assay with no cross reactivity (<0.1%) against other anterior pituitary hormones such as prolactin, ACTH, follicle-stimulating hormone, or thyroid-stimulating hormone. This assay was also adopted for roe deer plasma (Schams et al., '80). The reference preparation was bovine pituitary preparation (LH-DSA; biological activity 1.0 IU of NIH-bLH-S1). The sensitivity was 0.2 ng/mL; intra-assay coefficient of variation (CV) averaged 7.4%, and intra-assay CV was 9.2–13.8%. The assay was validated for pudu by recovery studies of four different concentrations added to plasma and was on average 98.4% ± 8.2%. Dilution curves of pudu plasma with a higher LH content run linearly to bovine standard as determined by linear regression analysis (regression coefficient 1.0 and $r^2 = 0.96$).

Testosterone was measured by a highly sensitive enzyme immunoassay after extraction with 30/70 *tert*-butyl methyl ether/petroleum ether (v/v) (Karg et al., '76). The sensitivity was 50 pg/mL. The inter-assay CV was 7.8–13.8%.

Statistics

The data were subjected to the General Linear Models Procedure (GLM) for unbalanced ANOVA (the SAS System for Windows 8.01). The effects were classes and one continuous variable. Classes were "Date" (11 dates between January 19 and April 3) and "Group" (CA vs. "Controls"). The continuous variable was "Age" (1.3–7 years). Interaction between "Group" and "Date" was also added into the model. Least-square means (LSMEAN) were computed for each class and differences were tested by *t*-test. We used a Tukey–Kramer adjustment for multiple comparisons. To calculate the relationship between testosterone and LH levels, residual correlation coefficients were computed.

RESULTS

Experiment 1

In this group of CA-treated bucks most antlers were cast between 22 and 32 days after the initiation of treatment. The left and right antlers, were respectively cast on the following days: 2-year old buck, March 16 and 24; 5-year old buck, March 14 and 16; and 6-year old buck, March 12 and 14. In all animals new antler growth began almost immediately; at the peak of growth activity the antlers grew at a speed of approximately 3 mm/day. The growth ceased around May 22, and the antlers mineralized in September, about 1 month ahead of intact bucks living with the same group. These antlers of CA-treated deer were then carried until the following winter (June), when they were cast at the normal time.

Experiment 2

In the second experiment, the antlers in the CA-treated group were cast 17–26 days after the initiation of CA treatment. The antlers were cast on the following days: February 25 (3 bucks) and March 3 and 6. A new antler growth began to grow almost immediately; in some cases a wall of new growth around the rim of the pedicle was already visible at the time of casting (Fig. 1). In one buck, an incompletely mineralized antler was separated almost 2 cm above the pedicle (Fig. 2). Within the first month after casting (March) the antlers grew on average to the length of 4 cm; the growth rate then slowed down, and the antler grew another 0.5 cm in April (Fig. 3). The final length (between 4.6 and 5.0 cm) was achieved in May (Fig. 4), when the velvet of antlers was polished. The antlers were cast at the normal time in June. In the control group the antlers finished the growth in October when they reached maximal length of around 9 cm (Fig. 5). After they were polished in November, they remained hard until they were cast in June of the next year. Since antlers were cut off for safety reason right after their mineralization, we could not compare the progress of the

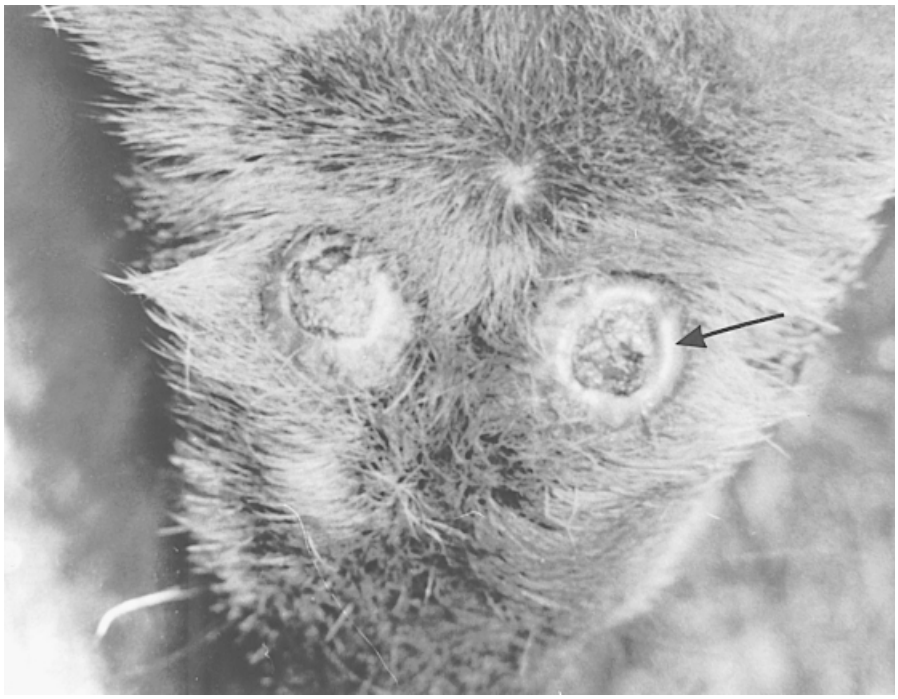

Fig. 1. Photo of pudu head taken immediately after CA-induced casting of antlers. Note the growing rim of tissues around the pedicle (arrow).

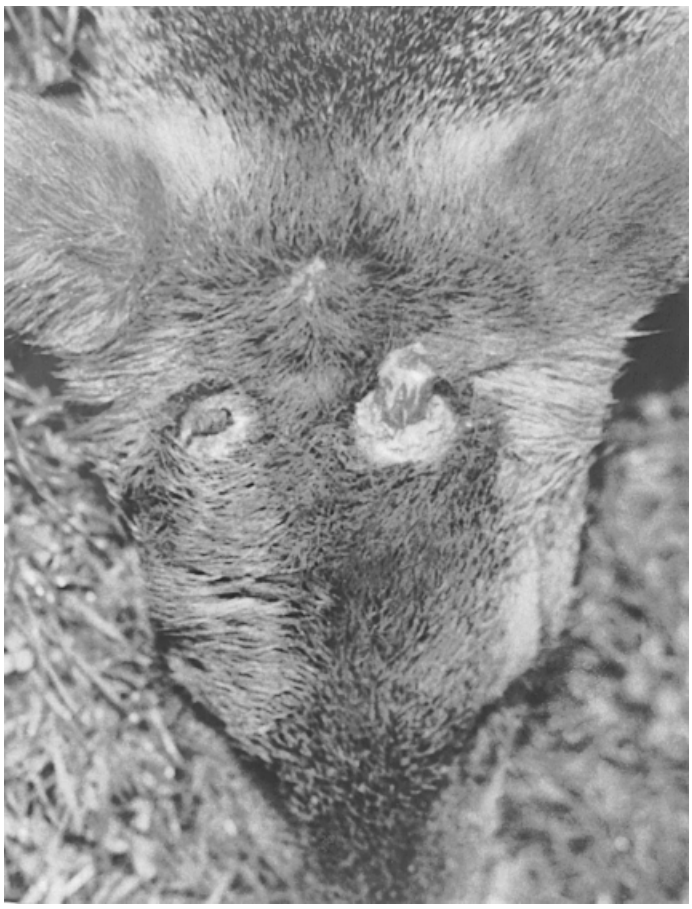

Fig. 2. Irregular casting of a left antler resulting from CA treatment. Surviving antler bone was later overgrown with a new antler tissue.

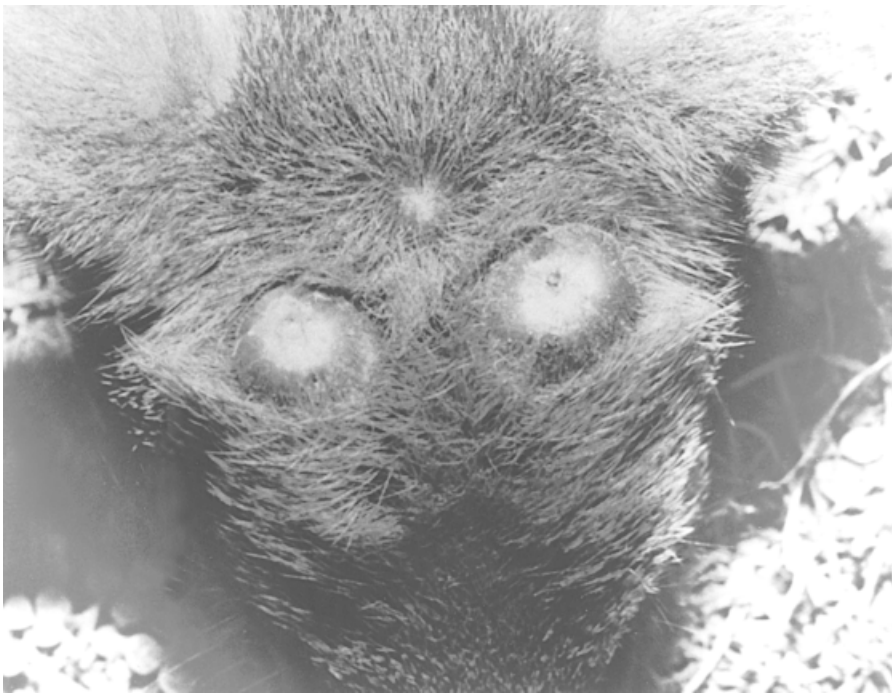

Fig. 3. Regular antler growth in the CA-treated buck.

Fig. 4. Almost complete antler regeneration after CA treatment.

Fig. 5. Finished antler growth (average size) in a control buck of prime age.

antler development between control and CA-treated bucks.

To compare the influence of a CA-treatment regime on the date of antler casting, GLM was constructed. We applied the effects of a continuous variable "Age" and a class "Experiment" (Experiments 1 and 2). The factor "Age" appeared not significant, and hence the final GLM contained only a class "Experiment." CA-treated males of the Experiment 1 cast their antlers later than males of Experiment 2 (LSMEANS ± SE, Experiment 1, March 15 ± 2.6 days; Experiment 2, February 27 ± 2 days, $F_{(1,7)} = 26.45$, $P < 0.01$).

Testosterone levels in the control group began to increase after February 15 (Fig. 6, top), and in three mature bucks it reached peak rutting concentrations of 13–15 ng/mL. Only in immature buck # 5 were peak levels <6 ng/mL. In the CA-treated group, average T levels remained low throughout the entire experimental period and indicated the return to higher levels only in the last sample. That final increase was most conspicuous in the mature buck # 8, who exhibited almost 4 ng/mL on April 3. GLM appeared highly significant ($F_{(25,98)} = 10.91$, $P < 0.01$) with all factors influential (Age $F_{(4,98)} = 6.66$, $P < 0.001$, Date $F_{(10,98)} = 6.93$, $P < 0.001$, Group $F_{(1,98)} = 82.33$, $P < 0.001$, Group × Date $F_{(10,98)} = 7.77$, $P < 0.001$). The time course of T values in the experimental group differed significantly from the values in the control group from March 3 on.

LH levels did not differ substantially between CA-treated and control groups. As LH concentrations are naturally peaking in January and February (Bubenik et al., '96), in the control group seasonal LH levels declined coincidentally with the initiation of CA treatment. In the experimental group levels of LH declined to control values shortly after the initiation of CA treatment (Fig. 6, bottom). In general, GLM appeared significant ($F_{(25,98)} = 5.52$, $P < 0.001$) also for LH levels. All three individual factors were influential (Age $F_{(4,98)} = 3.36$, $P < 0.05$, Date $F_{(10,98)} = 8.68$, $P < 0.001$, Group $F_{(1,98)} = 1.53$, NS). Throughout the entire period of blood sampling, the control group exhibited higher LH levels than CA-treated bucks (LSMEANS ± SE, controls 1.42 ± 0.06, CA 1.22 ± 0.04). The relationship between the two hormones was calculated before the initiation of CA treatment, during the

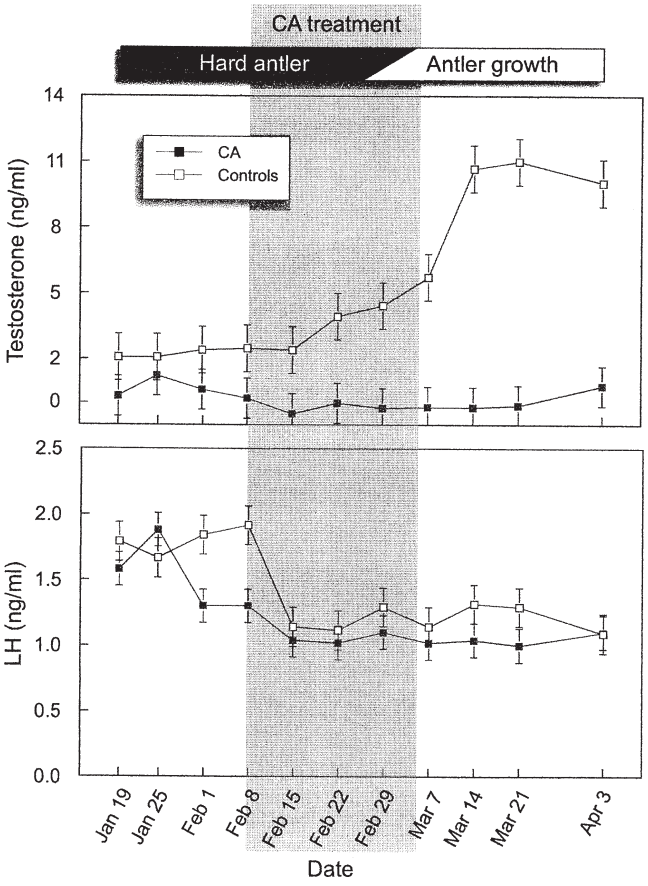

Fig. 6. Variations of testosterone (**top**) and LH concentrations (**bottom**) in plasma of four controls and five experimental male pudu before, during, and after treatment with cyproterone acetate (CA). Vertical shade indicates the period of CA treatment. Phases of the antler cycle presented on the top of the graph refer to the variation observed in CA-treated bucks. Control animals carried hard antlers throughout the entire experimental period.

treatment, and after the treatment was ceased. Whereas before the CA treatment there was an obvious relationship between testosterone and LH levels ($r_8 = 0.65$, $n = 27$, $P < 0.001$), during the CA treatment there was a trend toward the opposite relationship ($r_8 = 0.28$, $n = 45$, $P = 0.057$). After the CA treatment no relationship occurred between LH and T ($r_8 = 0.14$, NS).

DISCUSSION

Southern pudu that lives mostly in Chile is one of the less investigated cervid species. A basic determination of endocrine patterns, performed in the last 10 years, revealed that the seasonal variation of reproductive hormones in male pudu is most closely resembling that of a roe deer (Sempere, '90; Bubenik et al., '96; Reyes et al., '97; Bubenik et al., 2000). Whereas in most boreal cervids reproductive hormones, such as LH and testosterone, have only one peak per year (Bubenik et al., '82; Suttie et al., '84; Bubenik et al., '86), roe deer exhibits two circannual peaks of reproductive hormones. The first peak, usually a smaller one, occurring in the spring, is associated with the mineralization of antlers. The second peak, found in the summer, is related to the rut (Barth et al., '76; Schams and Barth, '82; Sempere, '90). Pudu also exhibit two seasonal peaks of reproductive hormones, but unlike in the roe deer, where the two peaks of LH and two peaks of testosterone are only few months apart (Sempere, '90), in pudu, these two peaks are almost of equal height and occur some 6 months from each other (Bubenik et al., '96).

Why do pudu and roe deer exhibit a biannual variation of reproductive hormones without having two ruts and two antler cycles? It can be hypothesized that such a bimodal rhythm is a vestigial remnant of the original year-round breeding pattern observed in tropical or temperate cervids. Similarly, a small short-term spring elevation of T and LH was reported in males of several species of boreal deer (Bubenik et al., '82). This elevation was coincidental with a much larger elevation of estradiol-17β detected in male white-tailed deer (Bubenik et al., '79). It was speculated that a conversion of T to estradiol prevented a spring rut, an occurrence rarely observed in boreal cervids (Bubenik et al., '85). Although no two antler cycles in one year were reported in pudu, a regular biannual antler growth was reported in penned Pere's David deer by Erna Mohr ('62) and in a wild red deer by Kierdorf and Kierdorf ('98).

Two seasonal peaks of reproductive hormones in pudu might also be related to the extended period of testicular activation and fertility (Hershkovitz, '82; Reyes et al., '97). A similar extended period of fertility was also reported in tropical, temperate, and some boreal deer (for a review see Reyes et al., '97); despite this fact, most deer species, however, exhibit only one distinct rutting period.

It has been mentioned that the seasonal pattern of reproductive hormones in pudu is similar to roe deer. Roe deer antlers are very sensitive to variation of T concentration in blood.

Castrations of roe bucks result in a quick antler casting and a subsequent, massive proliferation of the velvet, which is often fatal (Bubenik, '90). The outcome of our present study with CA

indicates that a similar outcome might be expected in pudu.

The results of both our experiments revealed that pudu's antler cycle is also very sensitive to the manipulation of blood levels of androgens. The advanced hypothesis was confirmed in both points. Suppression of peripheral concentrations of testosterone by small doses of CA induced a quick antler casting in both treatment regimes. The CA effect was dose-dependent. The lower CA dose (30 mg/buck), given in experiment one, initiated casting within 20–32 days. The higher CA dose (50 mg/buck), used in experiment two, caused casting in only 17–26 days. In Experiment 1, the second period of suppression of blood levels of reproductive hormones (given from April 5 to May 22) lasted long enough to prevent a subsequent mineralization of antlers. As a result of the natural decline of testosterone at that time, these antlers were not mineralized until September. Consequently, they were then carried as hard antlers for nine more months and cast only at the normal time in June. Conversely, antler mineralization and a subsequent casting occurred much earlier in Experiment 2, where CA treatment began 1 month earlier than in Experiment 1 and had already ceased by March 13. The antler mineralization in May, caused by a rebound of testosterone levels (observed already in Fig. 6) was followed by casting in June. As a result, the hard antlers were carried for 10 months in Experiment 1, instead of the normal 8–9 months (Bubenik et al., '96, 2000), whereas in Experiment 2, the abbreviated, CA-induced antler cycle lasted only 4 months. As a result, two antler cycles were observed in one year in the CA-treated group.

LH levels declined sharply in both the control and the CA-treated groups at the beginning of CA treatment. This result was in agreement with our earlier study of seasonal concentration of reproductive hormones in pudu. We reported seasonal peak concentrations of LH in plasma in February, whereas peak concentrations of T were detected in March (Bubenik et al., '96). In the present study, T concentrations in controls began to increase in February and reached peak values in March. Testosterone concentrations in CA-treated bucks were suppressed for most of the duration of this study (Fig. 6, top). Conversely, LH levels in both groups were kept low from February 15 until the end of the study in April.

As expected, in both experiments antlers of pudu were cast in approximately 2½ to 3 weeks after the first administration of CA. In that respect, pudu reacted similarly to white-tailed deer, where CA-induced antler casting also occurred only 2–3 weeks after the initiation of CA treatment (Bubenik et al., '75, '87). In experiment one, despite the decline in photoperiodicity in March, new antler growth began almost immediately and continued for the duration of the summer and fall. Despite the extended period of low androgen levels, antler growth ceased when the species-specific antler length, expected for each age category, was achieved. In that respect, the growth of pudu antlers was similar to roe deer, where CA treatment also did not induce growth of antlers beyond their species-specific size (Schams et al., '92).

Statistically, T concentrations between control and experimental group did not differ before the initiation of CA treatment. After that date T concentrations in the experimental group stabilized and did not increase to levels detected in controls, which increased sharply just 2 weeks after the beginning of CA treatment (Fig. 6, top). Peak control levels were detected as usual during the rutting period in March (Bubenik et al., '96; Reyes et al., '97).

It can be concluded that pudu is a cervid species whose antler cycle is very sensitive to variation of blood levels of T but is also flexible in regard to the adjustment in androgen fluctuation.

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