Theriogenology 81 (2014) 403–406

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Evidence of higher levels of testosterone during the velvet period in muntjac than in other cervids

Chiu-Shuang Lin [a], Kurtis Jai-Chyi Pei [b,*], Paulus S. Wang [c,d,e,f], Bing-Tsan Liu [g]

[a] Graduate Institute of Bioresources, National Pingtung University of Science and Technology, Neipu, Pingtung, Taiwan
[b] Institute of Wildlife Conservation, National Pingtung University of Science and Technology, Neipu, Pingtung, Taiwan
[c] Department of Physiology, National Yang-Ming University, Taipei, Taiwan
[d] Graduate Institute of Basic Medical Science, Ph.D. Program for Aging, College of Medicine, China Medical University, Taichung, Taiwan
[e] Department of Biotechnology, College of Health Science, Asia University, Taichung, Taiwan
[f] Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan
[g] Department of Animal Science, National Pingtung University of Science and Technology, Neipu, Pingtung, Taiwan

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form 6 October 2013
Accepted 11 October 2013

Keywords:
Cervus nippon
Rusa unicolor
Deer reproduction physiology
Leydig cells
Muntiacus reevesi
In vitro testosterone production

ABSTRACT

Previous studies have shown that despite having a clear seasonal fluctuation in fecal testosterone concentration, the significantly lower testosterone levels found in velvet stags of the nonseasonal breeder muntjac (*Muntiacus* sp.) apparently did not stop their spermatogenesis as in other deer species. In the present study, *in vitro* cultivated Leydig cells isolated from adult stags of three native deer species of Taiwan were treated with androstenedione, with or without adding human chorionic gonadotropin. Results showed that, unlike the two seasonal breeders, sika deer (*Cervus nippon*) and sambar deer (*Rusa unicolor*), Leydig cells of velvet muntjac had no dramatic reduction in or even maintained the full capability of their testosterone productivity compared with the hard-antlered stage. The decrease in fecal testosterone level observed earlier in muntjac during the velvet period was probably due to a reduction of number of Leydig cells. These results support the hypothesis that testosterone production in muntjac during its velvet period might never be low enough to trigger the quiescent phase of the reproduction cycle.

1. Introduction

It is well known that in male cervids, serum testosterone concentration in the blood fluctuates annually following seasonal changes in testicular activity, which then dictates, simultaneously, the reproductive cycle, also known as annual repuberty phenomenon, and the antler replacement cycle. Stags are fertile and behaviorally active in mating, together with the regaining of the pair of hard antlers, only during periods of high serum testosterone concentrations in autumn [1–14].

Male muntjacs (genus *Muntiacus*), however, despite demonstrating a regular annual testosterone and antler replacement cycle like other deer species, do not exhibit the annual repuberty phenomenon and remain fertile throughout the year, even when they are in the velvet period, which occurs from the beginning of antler regrowth until it is mineralized and cleaned [15–21]. As a result, births can occur every month of the year [22–27].

It was proposed that, unlike other deer species, the serum testosterone concentration threshold for initiating the casting and regrowing of the antler in muntjac might be set higher than that required to stop spermatogenesis [16]; in other words, the testosterone production in muntjac during their velvet period, although significantly lower than in the hard-antlered stage, might never be low enough to trigger the quiescent phase of the reproduction cycle. In

* Corresponding author. Tel.: +886 8 7740285; fax: +886 8 7740388.
E-mail address: kcjpei@mail.npust.edu.tw (K.J.-C. Pei).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.10.011

this study, we used the *in vitro* approach [28] to test this hypothesis by comparing the testosterone productivity of the cultivated Leydig cells isolated from Reeves' muntjac (*Muntiacus reevesi*) with that of the other two seasonal breeders, namely sika deer (*Cervus nippon*) and sambar deer (*Rusa unicolor*).

2. Materials and methods

For comparison purposes, comparative data in the hard-antlered stage from our previous study [28] are also presented in this report. All procedures (i.e., concentrations, formulas, durations of cell incubation, and testosterone assay) followed the same study.

Testes of one adult male of each of the three species from deer farms in central Taiwan were castrated, 1 testicle in the velvet period and another in the hard-antlered stage, every year during 2006 to 2008. All individuals were older than 2 years and in normal condition. Direct observations in the deer farms showed that, besides sambar deer's velvet period occurring in January to May, sika deer and muntjac's were both in May to September (C-SL, personal observation). Velvet period samples were collected in the middle section of the velvet period (Sambar deer: February and March; sika deer and muntjac: July and August), and hard-antlered stage samples were collected during October to December.

After removal, the testicular Leydig cells were isolated and cultivated as described in Lin et al. [28]. Cell cultures of the Leydig cells purified from each individual were considered one sample, therefore, sample sizes were three for all treatments in the present study.

For evaluating the ability of the testosterone production by the cultured Leydig cells, steroidogenic precursor androstenedione (10^2 nM) was added to Leydig cell suspensions of 10^5 cells per mL, in 35-mm culture dishes (Costar, Cambridge, MA, USA) in medium 199 containing 1.4 g/L $NaHCO_3$ and 10% BSA, and incubated at 34 °C, 95% air and 5% CO_2 for 3 days. Androstenedione has been shown to be the most effective steroidogenic precursors in inducing the testosterone production by the cultivated Leydig cells [28]. Two kinds of treatments were conducted, one with and one without human chorionic gonadotropin (hCG; 0.5 IU/mL) added. The hCG was used in this study to replace LH as the original stimulatory agent for testosterone biosynthesis. The culture medium was then centrifuged at 4 °C and 200 × *g* for 10 minutes and the supernatant was stored at −20 °C until analyzed for testosterone using enzyme immunoassay (EIA) [28].

Testosterone concentrations were measured using an Immulate 2000 assay (Siemens Medical Solutions Diagnostics Inc., Los Angeles, CA, USA) using a commercial kit (EIA L2KTW2, Siemens Medical Solutions Diagnostics Inc.) (the quality of Siemens Medical Solutions Diagnostics Inc. is certified with ISO 13485: 2003). The antiserum had the following cross-reactivities (provided by the company): 100% testosterone, 2.0% 5α-dihydrotestosterone, 0.5% 5α-androstan-3β, 17β-diol, 0.6% androstenedione, and 0% 5α-androstan-3, 17-dione. Assay sensitivity was 0.2 ng/mL, and the inter- and intra-assay coefficients of variation were 5.1% and 7.2%, respectively. The Immulite 2000 assay is an automatic two-site sandwich immunoassay with chemiluminescent detection [28,29].

3. Results

All treatments, except plain velvet Leydig cells of the sika and sambar deer, produced traceable testosterone after the 3-day incubation. Without androstenedione, the control groups of plain Leydig cells or Leydig cells plus hCG only produced insignificant amounts of the testosterone (average 0.7–1.3 nM/10^5 cells per 3 days for the velvet period; average 0.7–2.0 nM/10^5 cells per 3 days for the hard-antlered stage).

As expected, the supplementation of the androstenedione in this study significantly increased the production of the testosterone for all three species in the hard-antlered stage (average 17.6–19.0 nM/10^5 cells per 3 days for plain Leydig cells; average 23.8–44.4 nM/10^5 for Leydig cells plus hCG); however, only the muntjac's velvet Leydig cells produced large amounts of testosterone as in the hard-antlered stage (average 13.0 nM/10^5 cells per 3 days for plain Leydig cells; average, 45.4 for Leydig cells plus hCG) (Figs. 1 and 2). Furthermore, the Leydig cells' testosterone productivity in muntjac showed no differences between the velvet period and hard-antlered stages when treated with the hCG and androstenedione (Mann-Whitney-Wilcoxon test, $Z^* = 0.218$; $P > 0.05$).

4. Discussion

The results of the present *in vitro* study suggest that, as predicted by the hypothesis, male muntjac sustained their ability to produce relatively high amounts of testosterone during their velvet period (Figs. 1 and 2), and, therefore, could maintain a year-round capability of generating fertile sperm. Apparently, the function of the muntjac testicle will not revert to a quiescent phase during the velvet period as

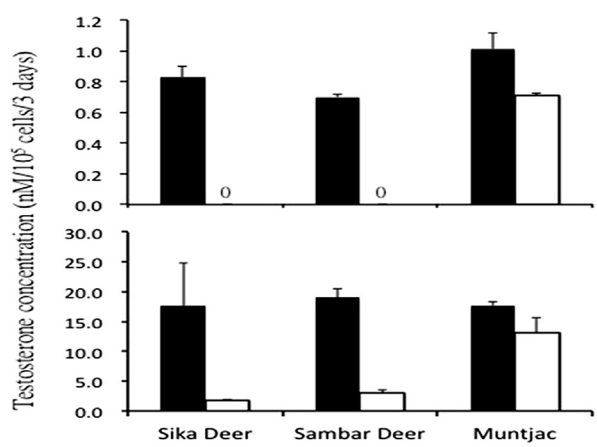

Fig. 1. Comparison of the average testosterone production (ng/mL per 3 days) using cultivated Leydig cells (LC; 10^5 cells per mL) purified from sika deer, sambar deer, and muntjac in the hard-antlered stage (black bar) or velvet period (white bar). The top figure shows the result of plain LC and bottom figure shows the result of LC treated with androstenedione (10^2 nM). Sample size was three animals for all treatments and data are mean with 1 SD.

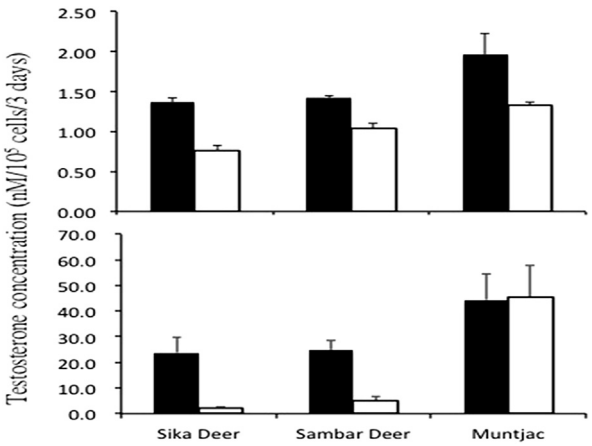

Fig. 2. Comparison of the average testosterone production (ng/mL per 3 days) using cultivated Leydig cells (LC; 10^5 cells per mL) purified from sambar deer, sika deer, and muntjac in the hard-antlered stage (black bar) or velvet period (white bar) with hCG (0.50 IU/mL) added. The top figure shows the result of plain LC and bottom figure shows the result of LC treated with androstenedione (10^2 nM). Sample size was three animals for all treatments and data are mean with 1 SD.

in other deer species, a phenomenon which calls for further investigation at the cellular or molecular levels of their Leydig cells. The study also demonstrated the important role of the LH (i.e., hCG) in controlling the Leydig cell's production of testosterone as in other deer species [14,30].

Nevertheless, the high amount of, or even no reduction in, testosterone production during the velvet period, which is featured in the study, was not expected because our previous study did show significant annual fluctuations in the fecal testosterone level in male muntjac [17]. The concentration and number of Leydig cells used in this *in vitro* study were the same for all treatments; therefore, a possible explanation is that although the function of producing testosterone by the muntjac Leydig cell does not regress during the velvet period, the number of Leydig cells might decrease significantly, which caused the decrease in concentrations of fecal testosterone of muntjac during the velvet period compared with in the hard-antlered stage [17]. It has been shown that the intratesticular testosterone level was significantly related to the number of Leydig cells and the concentration of Leydig cells per testis was 53% greater during the breeding season in stallions [30].

4.1. Conclusions

Pei et al. [17] and the present study both show that although testosterone might play a critical role in antlergenesis and spermatogenesis, the magnitude of its seasonal fluctuation in male muntjac seems to regulate only their antler cycle. Seasonal changes in testosterone production by male cervids are well known to be regulated by photoperiod through the hypothalamus-pituitary-gonad axis [10,12,13,30–35]. However, the surprisingly synchronous antler cycle in male muntjac over a broad geographical region, including zoos in Northern and Southern Hemispheres [36–42], suggests a different mechanism, other than photoperiod and testosterone-controlled, must be involved. It might be that the regular antler cycle in adult muntjac is endogenous, as proposed by Bedford and Marshall [43], Goss [44], and Lincoln [1] for the deer of tropical and subtropical origins. If this is the case, the antler and reproduction cycles in muntjac might not be dictated by photoperiod.

Interestingly, unlike other cervids, muntjacs do not stand on their hind feet and "box" when they are in the velvet stage [45,46]. The standing and "boxing" behavior of cervids not only serves as an antagonistic or threatening expression when they are antlerless, but also avoids damage to the sensitive tissue of the velvet. Sparring with the long pedicles covered with fresh, growing velvet in male muntjac usually results in abnormal hard antlers (KJ-CP, personal observation). Presumably, it has little value in fighting and displaying. This suggests that antler characteristics might not be as important to reproductive success in the muntjac as in other deer species.

Acknowledgments

The authors thank Mr. Hui-Wen Shaw, Mr. Chen-Shih Chen, Mr. Jung-In Chen, and Mr. Kuen-Feng Lin for their help in sample collection. Thanks also to the Reproductive Physiology Laboratory of the Department of Animal Science, National Pingtung University of Science and Technology, Taiwan, for kindly providing facilities and technical support for sample preparation. The Lezen Reference Laboratory (http://www.lezen.com.tw) conducted the EIA assays for testosterone measurements in the study. Funding was provided by the Forestry Bureau, Council of Agriculture, Taiwan (Projects: 96 Forest Management-02.1-Conservation-17).

References

[1] Lincoln GA. Seasonal breeding in deer. 165–80. In: Fennessy PF, Drew KR, editors. Biology of deer production. Wellington: R Soc NZ Bull; 1985.
[2] Bubenik AB. Das Geweih. Hamburg: Paul Parey Verlag; 1966.
[3] Bubenik GA, Brown GM, Bubenik AB, Grota LJ. Immunohistochemical localization of testosterone in the growing antlers of white-tailed deer (*Odocoileus virginianus*). Cal Tiss Res 1974;14:121–30.
[4] Bubenik GA, Bubenik AB, Brown GM, Trenkle A, Wilson DA. Growth hormone and cortisol levels in the annual cycle of white-tailed deer (*Odocoileus virginianus*). Can J Phys Pharm 1975;53:787–92.
[5] Bubenik GA, Bubenik AB, Brown GM, Wilson DA. The role of sex hormones in the growth of antler bone tissue. I. Endocrine and metabolic effects of antiandrogen therapy. J Exp Zool 1975;194:349–58.
[6] Bubenik AB, Tachezy R, Bubenik GA. The role of the pituitary-adrenal axis in the regulation of antler growth processes. Saugetierk Mitt 1976;24:1–5.
[7] Bubenik GA. The endocrine regulation of the antler cycle. In: Brown RD, editor. Antler development in *Cervidae*. Kingsville, TX: Caesar Kleberg Wildl Res Inst; 1982. p. 73–107.
[8] Bubenik GA, Reyes E, Dieter S, Lobos A, Bartos L. Seasonal level of LH, FSH, Testosterone and prolactin in adult male Pudu *(Pudu pudu)*. Comp Biochem Physiol 1996;115:417–20.
[9] Asher GW, Berg DK, Beaumont S, Morrow CJ, O'Nell KT, Fisher MW. Comparison of seasonal changes in reproductive parameters of adult male European fallow deer (*Dama dama dama*) and hybrid Mesopotamian × European fallow deer (*D. d. mesopotamica* × *D. d. dama*). Anim Reprod Sci 1996;45:201–15.
[10] Li C, Jiang Z, Jiang G, Fang J. Seasonal changes of reproductive behavior and fecal steroid concentrations in Père David's Deer. Horm Behav 2001;40:518–25.

[11] Loudon AS, Curlewis JD. Cycles of antler and testicular growth in an seasonal tropical deer (*Axis axis*). J Reprod Fertil 1988;83:729–38.
[12] Monfort SL, Brown JL, Bush M, Wood TC, Wemmer C, Vargas A, et al. Circannual inter-relationships among reproductive hormones, gross morphometry, behavior, ejaculate characteristics and testicular histology in Eld's deer stages (*Cervus eldi thamin*). J Reprod Fertil 1993;98:471–80.
[13] Rolf HJ, Fischer K. Serum testosterone, 5α-dihydrotestosterone and different sex characteristics in male fallow deer (*Cervus dama*): a long-term experiment with accelerated photoperiods. Comp Biochem Physiol 1996;115:207–21.
[14] Yamauchi K, Hamasaki S, Takeuchi Y, Mori Y. Assessment of reproductive status of sika deer by fecal steroid analysis. J Reprod Dev 1997;43:221–6.
[15] Pei K, Liu HW. Reproductive biology of male Formosan Reeves' muntjac (*Muntiacus reevesi micrurus*). J Zool Lond 1994;233:293–306.
[16] Pei K, Taber RD, O'Gara BW, Wang Y. Breeding cycle of the Formosan Reeves' muntjac (*Muntiacus reevesi micrurus*) in northern Taiwan, R. O.C. Mammalia 1995;59:223–8.
[17] Pei K, Foresman K, Liu BT, Hong LH, Yu YL. Testosterone levels in male Formosan Reeve's muntjac: uncoupling of the reproductive and antler cycles. Zool Stud 2009;48:120–4.
[18] Chaplin RE. The antler cycle of muntjac deer in Britain. Deer 1972;2:938–41.
[19] Chaplin RE. Antler cycle in muntjac. Deer 1973;3:51.
[20] Liu BT, Cheng CY, Chou MH, Liu SS. Annual changes of semen characteristics in the Formosan muntjac bucks. Proceedings of the 11th AAAP Animal Science Congress, Malaysia. 2004;2:467–469.
[21] Barrette C. Fighting behaviour of muntjac and the evolution of antlers. Evolution 1977;31:169–76.
[22] Chapman DI, Dansie O. Reproduction and foetal development in female muntjac deer (*Muntiacus reevesi*). Mammalia 1970;34:303–19.
[23] Chapman DI, Chapman NG. The antler cycle of adult Reeves' muntjac. Acta Theriol 1982;27:107–14.
[24] Chapman DI, Chapman NG, Dansie O. The periods of conception and parturition in feral Reeves' muntjac (*Muntiacus reevesi*) in southern England, based upon age of juvenile animals. J Zool (Lond) 1984;204:575–8.
[25] Chapman DI, Chapman NG, Colles DM. Tooth eruption in Reeve's muntjac (*Muntiacus reevesi*) and its use as a method of age estimation. J Zool (Lond) 1985;205:205–21.
[26] Chapman N, Claydon GK, Claydon M, Harris S. Distribution and habitat selection by muntjac and other species of deer in a coniferous forest. Acta Theriol 1985;30:287–303.
[27] Chapman NG, Harris S. Evidence that seasonal antler cycle of adult Reeves' Muntjac (*Muntiacus reevesi*) is not associated with reproduction quiescence. J Reprod Fertil 1991;92:361–9.
[28] Lin CS, Pei JC, Wang PS, Liu BT. An *in vitro* test on testosterone production of deer's Leydig cells. Tw J Biodivers 2011;13:323–31.
[29] Owen WE, Roberts WL. Performance characteristics of the IMMULITE 2000 erythropoietin assay. Clin Chim Acta 2004;340:213–7.
[30] Johnson L, Thompson DL. Effect of seasonal changes in Leydig cell number on the volume of smooth endoplasmic reticulum in Leydig cells and intratesticular testosterone content in stallions. J Reprod Fertil 1987;81:227–32.
[31] Bartoś L, Reyes E, Schams D, Bubenik G, Lobos A. Rank dependent seasonal level of IGF-1, cortisol and reproductive hormones in male pudu (*pudu pudu*). Comp Biochem Physiol 1998;120:373–8.
[32] Gómez JA, Garcia AJ, Landete-Castillejos T, Gallego L. Effect of advancing births on testosterone until 2.5 years of age and puberty in Iberian red deer (*Cervus elaphus hispanicus*). Anim Reprod Sci 2006;96:79–88.
[33] Goeritz F, Quest M, Wagener A, Fassbender M, Broich A, Hildebrandt TB, et al. Seasonal timing of sperm production in roe deer: interrelationship among changes in ejaculate parameters, morphology and function of testis accessory glands. Theriogenology 2003;59:1487–502.
[34] Blouch RA. Reproductive seasonality of the white-tailed deer on the Colombian Llanos. In: Wemmer CM, editor. Biology and management of the Cervidae. Washington, DC: Smithsonian Inst Press; 1987. p. 339–43.
[35] Branan WV, Marchinton RL. Reproductive ecology of white-tailed deer and red brocket deer in Suriname. In: Wemmer CM, editor. Biology and management of the Cervidae. Washington, DC: Smithsonian Inst Press; 1987. p. 344–51.
[36] Tzeng R M. Breeding records of mammals at Taipei Zoo in 1987-1988. Bull Taipei Zoo 1989;1:21–9.
[37] Bray DW. Taking muntjac seriously. Deer 1981;5:184–5.
[38] Mishra HR, Wemmer C. The comparative breeding Ecology of four cervids in Royal Chitwan National Park. In: Wemmer CM, editor. Biology and management of the Cervidae. Washington, DC: Smithsonian Inst Press; 1987. p. 259–71.
[39] Bray DW. Muntjac management. Deer 1986;6:252–4.
[40] Humphrey J. The control of muntjac. Deer 1981;5:181–3.
[41] Yahner RH. Time budgets in captive Reeves' muntjac (*Muntiacus reevesi*). Appl Anim Ethol 1980;6:277–84.
[42] Wang MN. The biological study on Formosan muntjac (*Muntiacus reevesi micrurus*)-distribution, sexual dimorphism and age determination. MS Thesis. Natl Taiwan Normal Univ, Taipei, Taiwan, ROC; 1989.
[43] Bedford D, Marshall FH. On the incidence of the breeding season in mammals after transference to a new latitude. Proc R Soc (Lond) (B) 1942;130:394–9.
[44] Goss RJ. Photoperiodic control of antler cycles in deer. III. Decreasing versus increasing day-lengths. J Exp Zool 1976;197:307–20.
[45] Barrette C. Social behaviour of muntjac. PhD Diss, Univ. Calgary, Alta; 1975.
[46] Walther FR. Communication and expression in hoofed mammals. Bloomington, IN: Indiana Univ Press; 1984.