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STEROID HORMONES PROFILE DURING AN OVARIAN SYNCHRONIZATION PROCEDURE IN DIFFERENT AGE CATEGORIES OF RED DEER HINDS (*Cervus elaphus L.*)

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The objective of the present study was to compare estradiol/progesterone ratios of different age categories of red deer hinds and use it as a predictor of estrus synchronization success and consequently conception rate. To accomplish this we used 38 red deer hinds to establish serum progesterone and estradiol levels in young (21 animals), mature (10 animals) and old (7 animals) hinds during the estrus synchronization procedure (transvaginal/cervical AI). The following estrus synchronization was used: at the start of the experiment each hind received a controlled intravaginal drug-releasing device (CIDR, Pharmacia&Upjohn, New Zealand) containing 0.3 g of progesterone. The device was removed on day 11, simultaneously with an application of 250 IU of Pregnant Mare Serum Gonadotropin (PMSG, Folligon® Intervet International, Boxmeer, Holland). Transvaginal/cervical AI (artificial insemination) was performed 48 hours after CIDR withdrawal (day 13). Blood samples were obtained from the jugular vein using a Venoject® vacutainer without an anticoagulant for hormonal tests on the same experimental day (0, 11th and 13th day). A statistically ($p<0.01$) higher progesterone level was found in young hinds on the 11th day after controlled intravaginal drug-releasing device insertion. A significantly higher ($p<0.01$) estrogen level was observed in the young in regard to mature and old hinds on the expected day of estrus (13th day). Estradiol/progesterone ratios showed a statistically significant difference ($p<0.01$) on insemination day (13th day) between old and young hinds (98.67 : 46.59) and between old and mature hinds (98.67 : 51.79). Out of a total of 38 hinds only 9 had their offspring, 6 of the young and 3 of the mature hinds.

Key words: estrogen, estrus synchronization, progesterone, red deer

INTRODUCTION

The reproductive performance of hinds, especially the reproductive performance of yearlings and adult females is a major determinant of the productivity and economic viability of commercial deer farms (Audige *et al*., 1999). Success varies between yearling (primiparous) and adult hinds. There is a growing interest for propagation of genetically valuable wild animals using assisted reproduction techniques (McCorkell *et al*., 2006). One of these is artificial insemination (AI) which has been widely used in farmed animals. Both laparoscopic intrauterine AI and transvaginal/cervical AI techniques are being used in red deer (Fennessy *et al*., 1990, Asher *et al*., 2000). Also, different transvaginal AI procedures (the Gourley Scope method vs. standard speculum-guided AI gun) were investigated by Willard *et al*., (2002). Preparation for these procedures involves estrus synchronization procedures (Gruber *et al*., 2007). Synchronization of estrus and ovulation in a group of females can be induced artificially by altering the endogenous endocrine environment of the non-pregnant female through the exogenous administration of progesterone and gonadotropin (Asher *et al*., 1993). The most common method for this is the application of a controlled intravaginal drug-releasing device (CIDR) containing progesterone and subsequent administration of gonadotropin (Bowers *et al*., 2004). Both of these exogenous hormones are used to regulate the reproductive cycle. Estradiol and progesterone have been reported to be useful tests for pregnancy (Willard *et al*., 1998). Progesterone is an important hormone of pregnancy in red deer (Kelly *et al*, 1982). Plasma progesterone (P) concentration reflects the luteal function in red deer and it has been a useful parameter for determining the reproductive status (Plotka *et al*., 1980). Progesterone and estradiol (E_2) are required for successful conception, both to prepare the endometrium for blastocyst implantation and pregnancy. Estradiol initiates hypertrophy and hyperplasia of endometrial epithelia by increasing the blood supply. Progesterone is responsible for glandular development and endometrial glandular secretion. Progesterone and estradiol are also important feedback factors concerning the pituitary gland which has an indispensable role in the regulation of the reproductive cycle (Milošević *et al*., 2005). All this shows that estradiol and progesterone levels and estradiol and progesterone ratios (E/P) are important factors in the regulation of the normal reproductive cycle. However, there are little or insufficient data regarding E_2/P ratios of synchronized red deer hinds (Bowers *et al*., 2004), especially in different age categories. Therefore, our goal was to compare E_2/P ratios of different red deer age categories by determining serum progesterone and estradiol levels in young, mature and old hinds.

MATERIAL AND METHODS

Animals

The treated group consisted of thirty eight farmed red deer hinds between 1 and 11 years of age. They were raised on 7.5 ha at an altitude of 87 m, in a confined area, in Eastern Croatia and were fed with hay and feed mixture with

6.4MJ NEL/kg (Table 1). Frozen semen imported from New Zealand was used for AI. Semen characteristics after analysis according to the Croatian Center for Livestock Reproduction were as follows: density 112.5 spermatozoa /mL x10^6, motility 60%, live 59.21%, dead 40.79% (morphological assessment: normal 85%, pathological 15%, main defects 7.7%); general mark: good.

Table 1. Ingredients of the feed mixture for red deer hinds (%)

Feed ingredients	% in mixture
Corn grain	26
Barley grain	20
Wheat grain	1.5
Soybean meal, 44%	13.4
Dry beet sugar pulp	12
Wheat bran	10
Alfaalfa meal	4
Molasses	4
Limestone	3
Likra W6 (premix)	6

LIKRA W6: Crude protein 22.2%, Crude fat 4%, Crude fiber 4%, Ash 47.5%, Calcium 8%, Phosphorus 5.7%, Sodium 2.4%, Magnesium 2.1%, Vitamin A 669500 I.U., Vitamin D 95950 I.U., Vitamin E 900 mg, Biotin 1600 mg, Manganese 1500 mg, Zinc 3120 mg, Iron 1500 mg, Copper 1030 mg, Iodine 75 mg, Selenium 35 mg, Cobalt 12 mg

The average body condition of hinds was around 3. At the start of the experiment (31 August 2007, day 0) each hind received a controlled intravaginal drug-releasing device (CIDR, Pharmacia&Upjohn, New Zealand) containing 0.3 g of progesterone. The device was removed on day 11, simultaneously with an application of 250 IU of Pregnant Mare Serum Gonadotrophin (PMSG, Folligon® Intervet international, Boxmeer, Holland). Transvaginal/cervical AI (artificial insemination) was performed 48 hours after CIDR withdrawal (day 13). Blood samples were obtained from the *jugular vein* using a Venoject® vacutainer without an anticoagulant for hormonal tests on the same experimental day (0, 11th and 13th day). Pregnancy control was done on day 49. After blood clotting, the serum was separated by centrifuge at 1500g/10 min and frozen at -20^{o}C.

Analysis of hormones

Subsequently, the concentration of progesterone and estradiol in the serum was measured using electrochemiluminescence immunoassay "ECLIA" (Roche Elecsys 2010, Roche Diagnostics, Mannheim, Germany). Reproducibility was determined using Elecsys reagents 6 times daily for 10 days (n=60), and within-run precision on Modular Analytics E170 analyzer, n=21. The intra- and inter-assay variation coefficients were <2.7% and <5.5%, respectively for the lower

detection limit for progesterone and <5.7% and 6.2%, respectively for the lower detection limit for estrogen.

Results are presented according to age categories: 21 young hinds (1 and 2 years), 10 mature hinds (4, 5 and 6 years), and 7 old hinds (above 10 years).

Statistical analysis

The data were analyzed using the general linear model procedure of StatSoft, Inc. Statistica (2008). The differences between age groups were determined by the Tukey *post-hoc* test.

RESULTS AND DISCUSSION

The values of progesterone, estrogen and E_2/P ratio in different age categories are shown in Table 2.

Table 2. Serum progesterone (nmol/L), estradiol (pmol/L) and E_2/P ratios in different age categories of red deer hinds during an ovarian synchronization procedure

	Young $\bar{x} \pm$ Sd	Mature $\bar{x} \pm$ Sd	Old $\bar{x} \pm$ Sd
Progesterone, day 0	$5.31^{A} \pm 1.64$	$3.00^{B} \pm 0.99$	$3.82^{A,B} \pm 1.82$
Progesterone, day 11	$8.67^{A} \pm 3.65$	$3.44^{A} \pm 0.77$	$4.25^{B} \pm 1.09$
Progesterone, day 13	$3.04^{A} \pm 1.48$	$2.03^{A,B} \pm 0.68$	$0.93^{B} \pm 0.22$
Progesterone, day 49	$3.21^{A} \pm 1.74$	$5.42^{A,B} \pm 2.31$	$2.04^{B} \pm 0.75$
Estradiol, day 0	$125.85^{A} \pm 16.93$	$92.6^{B} \pm 10.39$	$93.35^{B,C} \pm 10.62$
Estradiol, day 11	$132.53^{a,A} \pm 22.5$	$101.41^{B} \pm 13.76$	$105.75^{b} \pm 17.88$
Estradiol, day 13	$117.42^{A} \pm 17.52$	$93.11^{B,C} \pm 13.44$	$86.05^{C} \pm 10.31$
Estradiol, day 49	$96.41^{A} \pm 14.86$	$69.03^{B,C} \pm 12.34$	$76.99^{C} \pm 5.34$
E_2/P ratio, day 0	26.72 ± 12.22	35.77 ± 19.94	35.53 ± 11.61
E_2/P ratio, day 11	$18.29^{A} \pm 9.4$	$31.57^{B} \pm 11.81$	$27.05^{A,B} \pm 10.57$
E_2/P ratio, day 13	$46.59^{A} \pm 20.83$	$51.79^{A} \pm 20.37$	$98.67^{B} \pm 34.4$
E_2/P ratio, day 49	$46.42^{a} \pm 16.18$	$18.33^{b} \pm 6.13$	$42.77^{a,b} \pm 19.08$

Different letters in the same row differ significantly: $^{A}p<0.01$; $^{a}p<0.05$; E_2 – estradiol

The CIDR device was retained by all hinds until removal on day 11. All the hinds on insemination day (13th day) showed intensive clinical signs of heat. Out of a total of 38 hinds only 9 had their offspring, 6 of the young and 3 of the mature hinds. The mean progesterone level on CIDR receiving day in young hinds was 5.31 ± 1.64 nmol/L, 3.00 ± 0.99 nmol/L in mature and 3.82 ± 1.82 nmol/L in old hinds (significantly higher in relation to mature hinds, $p<0.01$). The estradiol level was 125.85 ± 16.93 pmol/L in young, 92.6 ± 10.39 pmol/L in mature and 93.35 ± 10.62 pmol/L in old hinds. After CIDR removal (11th day), the level of progesterone in mature hinds (3.44 ± 0.77 nmol/L) was significantly ($p<0.01$)

lower than in the young (8.67±3.65 nmol/L). On insemination day (13th day), the hinds from all groups showed clinical heat signs, the progesterone level was significantly ($p<0.01$) lower in old (0.93 nmol.L^{-1}) compared to young hinds (3.04±1.48 nmol/L), while the estradiol level was significantly ($p<0.01$) higher in younger (117.42±17.52 pmol/L) than in mature (93.11±13.44 pmol/L) and old hinds (86.05±10.31 pmol/L) on the same day. E_2/P ratios showed a statistically significant difference ($p<0.01$) on insemination day between old (98.67±34.4) and young (46.59±20.83) hinds.

Initial progesterone level (Figure 1) indicated that all hinds had some ovarian cycle activity. McCorkell *et al.* (2007) reported a much higher progesterone level after CIDR application which, in our case, occurred only in young hinds. Garcia *et al.* (2003) signified that only an enhanced progesterone level of 3 nmol.L^{-1} after CIDR placement can be taken as a sign of synchronizations procedure effects.

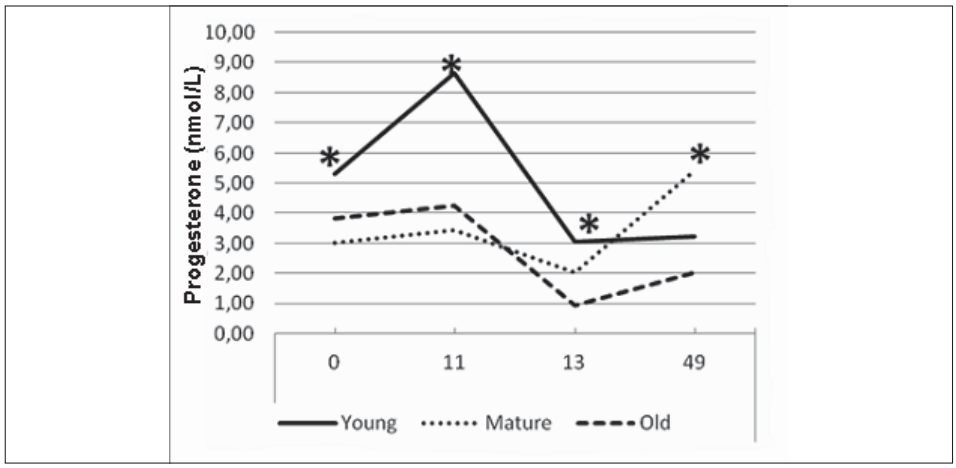

Figure 1. Profile of mean progesterone values in different age categories of red deer hinds during an ovarian synchronization procedure

As shown in Figure 1. the baseline level of progesterone concentration had been detected on the day of ovulation (day 13th) which is in correlation with Garcia *et al.* (2003). On the 36th day after AI, the progesterone level rose in all groups, while E level declined in all groups, which is in accordance with the results of Plotka *et al.* (1980) and Takahashi *et al.* (2001). Although other authors (Plotka *et al.,* 1980) state that estradiol concentration rises for three days and reaches a peak a few hours before estrus, our data (Figure 2) show even lower values on insemination day (13th day) compared to three days earlier.

Regardless of that all the hinds on insemination day (13th day) showed intensive clinical signs of heat.

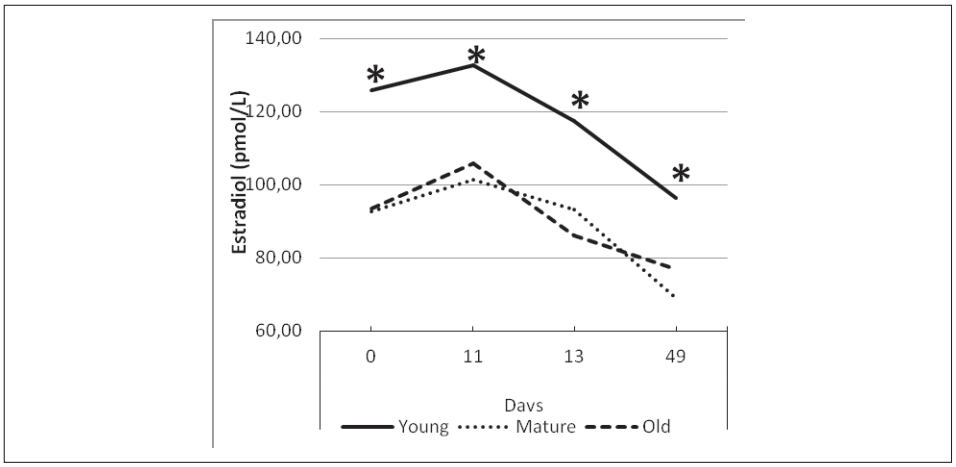

Figure 2. Profile of mean estradiol values in different age categories of red deer hinds during an ovarian synchronization procedure

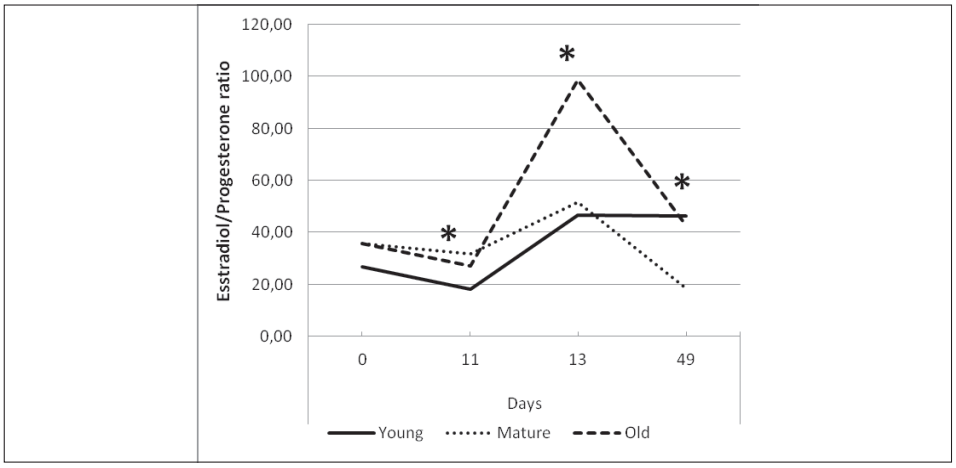

Figure 3. Estradiol to progesterone ratio (pmol/nmol) in different age categories of red deer hinds during an ovarian synchronization procedure

Figure 3. shows an E_2/P ratio peaking on insemination day, which is in accordance with Plotka *et al.* (1980). Relatively little is known about E_2/P ratio proportion in serum. There is a hypothesis in human medicine that moderately increased P values in the early luteal phase are associated with higher E_2/P ratios and better pregnancy outcomes, whereas a high increase in P values in combination with a decrease in E_2 values (reflected by a low E_2/P ratio) tend to indicate poor reproductive outcome (Gruber *et al.,* 2007). In contrast, Safro *et al.* (1990), in their experiment on mice concluded that a large elevation of the E_2/P

ratio inhibited implantation and created a uterine milieu that suppressed embryonic metabolism. They even suggest that an unfavorable E_2/P ratio could be corrected and embryo implantation re-established by injecting exogenous P. Therefore, further studies are needed to examine whether the E_2/P ratio could be used as a prognostic test provided with a full female hormones profile. In our trial the E_2/P ratio was growing towards the expected estrus day. On that day a statistically significant difference ($p<0.01$) was evident between old hinds in relation to young and mature hinds. However, out of a total of 38 hinds only 9 had their offspring, 6 of the young and 3 of the mature hinds. Gidley-Baird *et al.* (1987) found that the deer who failed to become pregnant had significantly higher E_2 levels and a lower ratio of E_2/P than those who became pregnant. They presume that the E_2/P ratio is a better predictor of implantation failure than are the absolute levels of either estradiol or progesterone hormones. It must be stated that those measurements were conducted a few days after conception, which might be the reason why they are not in correlation with ours. Many factors can influence AI success: duration of CIDR placement, stag exposure for modulating the timing of the preovulatory LH surge after synchronization, body condition, stress and insufficient grazing (McCorkell *et al*., 2007). Even handling stress during immobilization for estrus synchronization could be the reason that some hinds showed usual progesterone levels in the preovulatory phase, but did not ovulate (Budde, 1983). A lower conception rate in our investigation could be the outcome of a poor body condition score which was around 3 (Đidara *et al.,* 2008). There is also a wide range of individual hind characteristics such as weight and body condition score in yearlings and adult hinds (Audige *et al*., 1999b).

CONCLUSION

On insemination day the old hinds showed a significantly ($p<0.01$) higher E_2/P ratio compared to the young hinds. The old hinds did not have any offspring and the young had 6. Mature hinds which also showed a lower ($p<0.01$) E_2/P ratio compared to old hinds had 3 offspring. Taking this into account we can presume that a high E_2/P ratio has a negative effect on offspring count.

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PROFIL STEROIDNIH HORMONA TOKOM SINHRONIZACIJE ESTRUSA KOD RAZLIČITIH KATEGORIJA KOŠUTA (*Cervus elaphus L.*)

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SADRŽAJ

Farmski držani jeleni podvrgavaju se različitim tehnikama asistirane reprodukcije, kako bi se dobile genetski vredne životinje. Između ostalog ove tehnike uključuju i sinhronizovanje estrusa i veštačko osemenjavanje. Malo se zna o odnosu između estradiola i progesterona kod košuta. Cilj ovoga rada je bio da se uporede odnosi estradiola i progesterona različitih starosnih kategorija košuta crvenog jelena i da se koristite za predviđanje uspeha sinhronizovanja estrusa i koncepcije. Da bi smo ovo postigli proveli smo postupak sinhronizacije estrusa (0. dan aplikacija intravaginalnih uložaka progesterona (CIDR, Pharmacia&Upjohn, New Zealand), 11. dan uklanjanje intravaginalnih uložaka i aplikacija 250 IU seruma ždrebnih kobila (PMSG, Folligon® Intervet International, Boxmeer, Holand). Košute su umjetno osjemenjene 48 sati nakon aplikacije (13. dan) duboko smrznutim sjemenom uvezenim iz Novog Zelanda. Uzorci krvi za analizu hormona uzeti su iz jugularne vene pomoću Venoject® vacutainera bez antikoagulansa u iste dane tretmana (0., 11. and 13. dan) i pri tome smo određivali nivo progesterona i estradiola u serumu mladih, zrelih i starih košuta. Koncentracija hormona određena je imunoenzimskom kemiluminiscencijom "ECLIA" (Roche Elecsys 2010, Roche Diagnostics, Mannheim, Germany). Statistički ($p<0.01$) veći nivo progesterona primećen je kod mladih košuta 11 dana posle aplikacije progesteronskog intravaginalnog implantata. Statistički vrlo značajno viši ($p<0.01$) nivo estrogena primećen je kod mladih košuta u odnosu na druge. Trinaestog dana eksperimenta, odnosno na dan estrusa, odnos estrogen/progesteron se vrlo značajno razlikovao ($p<0.01$) između starih (98,67) i mladih (46,59) košuta, kao i između starih i zrelih košuta (98,67:51,79). Od ukupno 38 obrađenih košuta, 9 je imalo potomstvo, od čega je 6 iz kategorije mladih košuta i 3 iz kategorije zrelih.