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Interrelationships between reproductive cycle, age, body weight and steroid hormones in roe deer females (*Capreolus capreolus*)

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Abstract. The aim of present studies was to examine the interrelationships between reproductive events, age, body mass and steroid hormones in roe deer females (*Capreolus capreolus*). For this purpose we compared seasonal changes in body mass, blood levels of progesterone and estradiol (1) in young (1 year) and adult (2-4 years old) does and (2) in pregnant and non-pregnant animals. Monthly during 12 months all animals were weighed, blood plasma was collected, and concentration of progesterone and estradiol was analysed by RIA. Pregnant animals had significantly higher body weight, than non-pregnant ones, in November (before foetus implantation), and lower body weight in comparison with non-pregnant females in August (after parturition). In non-pregnant females high level of progesterone was observed from August (mating) up to December. Thereafter progesterone level declined up to minimum in summer months (April-July). Pregnant animals had increased progesterone level from February (foetus implantation) up to June (time after labour). In non-pregnant females, three peaks of estradiol concentration were observed in October, December and May. Pregnant animals, in contrast to non-pregnant females, had spring (January-March) gravidity-associated peak of estradiol level, but absence of summer (May) peak before parturition. Comparison of annual changes in body weight and plasma steroid hormone level in pregnant yearlings and old animals, as well as the number of offspring in these animals did not show principal age-dependent differences in these indexes, although yearlings had higher absolute progesterone (in December) and estradiol (in October and November) level than old animals. Our observations suggest significant seasonal changes in plasma progesterone and estradiol level and body weight in this species. Substantial differences in these changes in pregnant and non-pregnant animals demonstrate the involvement of steroid hormones in control of pregnancy in roe deer does. The absence of age-dependent differences in body weight and fecundity rate do not confirm previous hypothesis that age-dependent differences in metabolism and body mass can reduce fertility rate in yearlings. Moreover, our observations are the first demonstration of higher rate of steroidogenesis in young animals, than in adult females during early stages of gravidity and before embryo implantation. It is not to be excluded, that age-dependent reduction in ovarian steroid hormones level could be a cause of future infertility in old animals.

Key words: progesterone, estradiol, body mass, pregnancy, seasonal changes

Introduction

Roe deer (*Capreolus capreolus*) is numerous and popular game species around the Europe (Raesfeld et al. 1986, Prior 2000). Nevertheless, the maintenance and use of its populations is complicated by high age-dependent variability in female reproductive indexes. A large proportion of females does do not start reproduction in the first year of life (Kudlač & Elečko 1977, Gordon 1997, Andersen et al. 1998). The complications in roe deer reproduction could be due also to a short defined rutting season (from mid-July to mid-August), long latent period of embryogenesis (between mating in July-August and embryo implantation in November-December), relatively long post-implantation prenatal development (from November-December up to parturition in May-June), which represents potential risk for getting offspring in this species (Raesfeld et al. 1986, Komárek & Kočiš

1991, Andersen et al. 1998, Prior 2000, Klonisch et al. 2006).

The important factors affecting roe deer reproduction are age and age-dependent somatic indexes and energy metabolism. Start of reproduction occurs only after reaching advanced stage of somatic development and body weight. A large proportion of roe deer females does not start reproduction in the first year of life probably because of increased requirement in energy expenditure and body weight necessary for pregnancy (Kudlač & Elečko 1977, Mauget et al. 1997, 2003). According to Mauget et al. (1997), roe deer, in contrast to other cervid species, exposes no expressed seasonal rhythms in metabolic rate and parallel variations in body weight, but increases these indexes during reproduction (the last two month of gestation and during lactation in the first month post partum). These authors reported higher energy expenditure in primiparous females than in multiparous animals during reproductive period, therefore body mass is increasing during pregnancy more in yearlings than in old females (Mauget et al. 2003). Therefore, seasonal-, age- and reproduction-dependent changes in body weight are important for roe deer does reproductive success, but they were described only by one group of investigators, and they were not verified by independent studies.

The other physiological regulators of reproduction are reproductive hormones. Female reproductive efficiency – ovarian follicular development ovulation and embryo survival are controlled by steroid hormones progestagens (mainly progesterone) and estrogens (mainly estradiol) (Fortune et al. 2013, Sirotkin 2014). Therefore, alterations in these hormones could affect annual changes and timing of reproductive processes, as well as the age-dependent changes in reproductive efficiency in females roe deer. In male roe deer the increase in the accumulation of gonadal steroidogenic enzymes, estrogen receptors and blood level of LH and testosterone during mating suggests the involvement of steroid hormones in the control of reproductive cycles (Sempéré et al. 1992, Roelants et al. 2002, Schön & Blottner 2008). In cycling female roe deer LH and progesterone increased during a rut (July-August), whilst high progesterone level have been maintained until time of prospective implantation (December) (Sempéré et al. 1992). In cycling does Hoffmann et al. (1978) observed increased level of plasma progesterone from time of ovulation (July-August) up to *corpus luteum* maintenance (March), whilst pregnancy (December-February) was associated with the second peak of progesterone and increase in estradiol release. Schams et al. (1980) confirmed the increase in progesterone (together with gonadotropins) during ovulation, but failed to find the differences between pregnant and non-pregnant animals in hormones level. Lambert et al. (2001) studied plasma progesterone, estradiol and prolactin level in roe deer females during summer diapause and autumn initiation of embryogenesis and embryo implantation. The authors failed to find changes in progesterone release, but reported the increase in plasma estradiol and prolactin level after embryo implantation. Therefore, different studies of annual hormonal cycle in this species were focused on particular groups (either cycling or non-cycling animals), particular part of reproductive cycle (gravidity) or generated the contradictory data concerning association between steroid hormones level and pregnancy. To our knowledge, no comparison of whole annual cycle of plasma hormones in non-cycling and cycling/pregnant have been performed in one experiment previously.

The aim of present studies was to examine the involvement of steroid hormones and age-dependent changes in body weight in control of reproduction in female roe deer. For this purpose we studied the role of (1) season, (2) pregnancy and (3) age on body weight and plasma progesterone and estradiol level. In our experiments we compared seasonal changes in body mass and blood levels of progesterone and estradiol in pregnant and non-pregnant animals and in young (1 year) and old (2-4 years old) pregnant does.

Material and Methods

Experimental roe deer females were born in years (1998-2001) in deer farm in Research Institute of Animal Production in Nitra, and thereafter they were kept in pens with an area of 1000 m^2 in conditions described. The animals had permanent access to drinking water, in the daytime they received 500 g granulated feed mixture per animal (Garant-Tiernahrung Gesellschaft m.b.H, Pöchlarn, Austria) and alfalfa hay ad libitum. The content of nutrients in $g.kg^{-1}$ dry matter of granulated feed mixture was: crude protein 171.3, crude fibre 75.21, fat 26.85, ash 139.01, calcium 25.50, phosphorus 13.47 and the content of nutrients in $g.kg^{-1}$ dry mater of alfalfa hay was: crude protein 202.53, crude fibre 261.95, fat 18.04, ash 78.32, calcium 13.97, phosphorus 1.91. Only healthy females in good physical conditions were used in experiments. Before the experiment, the animals were divided into two groups – young (1 year old, total 5 animals) and old (2-4 years old, totally 12

animals) females. Experiment was performed between September 2002 and August 2003, i.e. during one whole year. Immediately before experiment (August 2002), during natural mating, 8 from 12 old animals and all 5 young animals were inseminated by male, which resulted pregnancy in 7 old and 5 young females respectively. In June all inseminated females (either young or adult animals) got twins. All manipulations were performed in accordance with Slovak and EU regulations concerning animal welfare under control of local ethical commission. All the animals used in experiments were grown from their birth in close contact with humans, therefore, as expected, they expressed no visible signs of stress during blood collection. During this collection the animals were lead to the cage corner and gently immobilised by using net. Blood from all (pregnant and non-pregnant, old and young) animals was collected by heparinized syringe without anaesthesia of animals one time per month during 12 months just prior to the morning feed. Ten ml of blood were obtained from each animal at the *vena jugularis*, using a 10 ml syringe with a 0.9 × 400 mm needle. Blood was collected into glass tubes containing 0.1 ml of heparin solution (5000 IU/ml, Zentiva a.s., Prague, Czech Republic). Plasma was separated by centrifugation for 10 min at 4oC and 300×g, and then frozen at –78 °C until analysis.

Concentrations of progesterone and estradiol in 25-50 µl of plasma were determined in duplicate without extraction by using commercial RIA kits from DSL Laboratories Inc. (Webster, TX, USA) according to the instruction of manufacturer. The characteristics of these assays are presented in Table 1.

Obtained results were subjected to statistical evaluation. The mixed model (Proc Mixed, SAS Institute Inc. 2009) was applied to study the influence of the sources of variation in the studied traits: body weight, estradiol and progesterone in roe deer does.

The model equation was the following:

$$y_{ijkl} = \mu + P_i + A_j + M_k + P_iM_k + A_jM_k + u_l + e_{ijkl}$$

where:

y_{ijkl} – individual observations of body weight (kg), estradiol (ng/ml plasma) and progesterone (ng/ml plasma)

μ – intercept

P_i – fixed effect of pregnancy (pregnant, non-pregnant does); $\sum_i P = 0$

A_j – fixed effect of age (primiparous, multiparous does); $\sum_j A = 0$

M_k – fixed effect of month (1, 2 to 12); $\sum_k M = 0$

P_iM_k – fixed effect of interaction between pregnancy and month; $\sum_{ik} PM = 0$

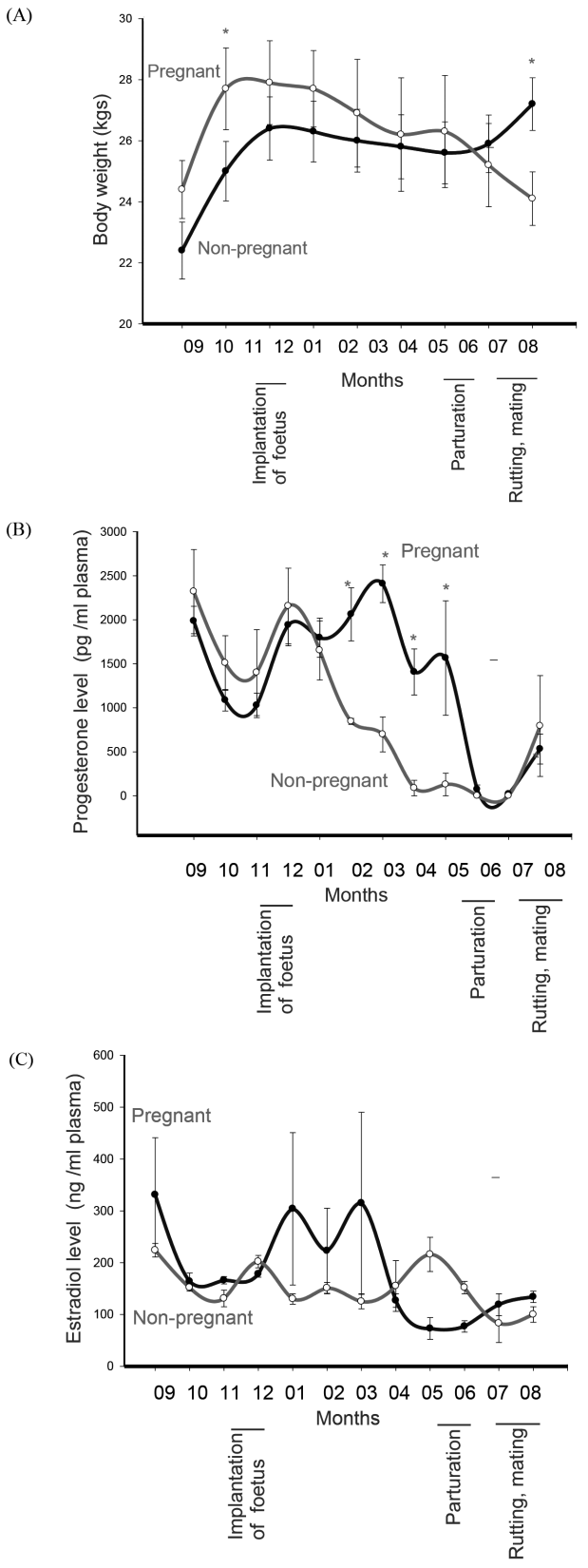

Fig. 1. Seasonal changes in body weight (A), plasma progesterone (B) and estradiol (C) level in pregnant and non-pregnant roe deer does irrespectively of their age. Values are LSM ± SE. Asterix denotes statistical significant ($P < 0.05$) differences between the groups in the corresponding month.

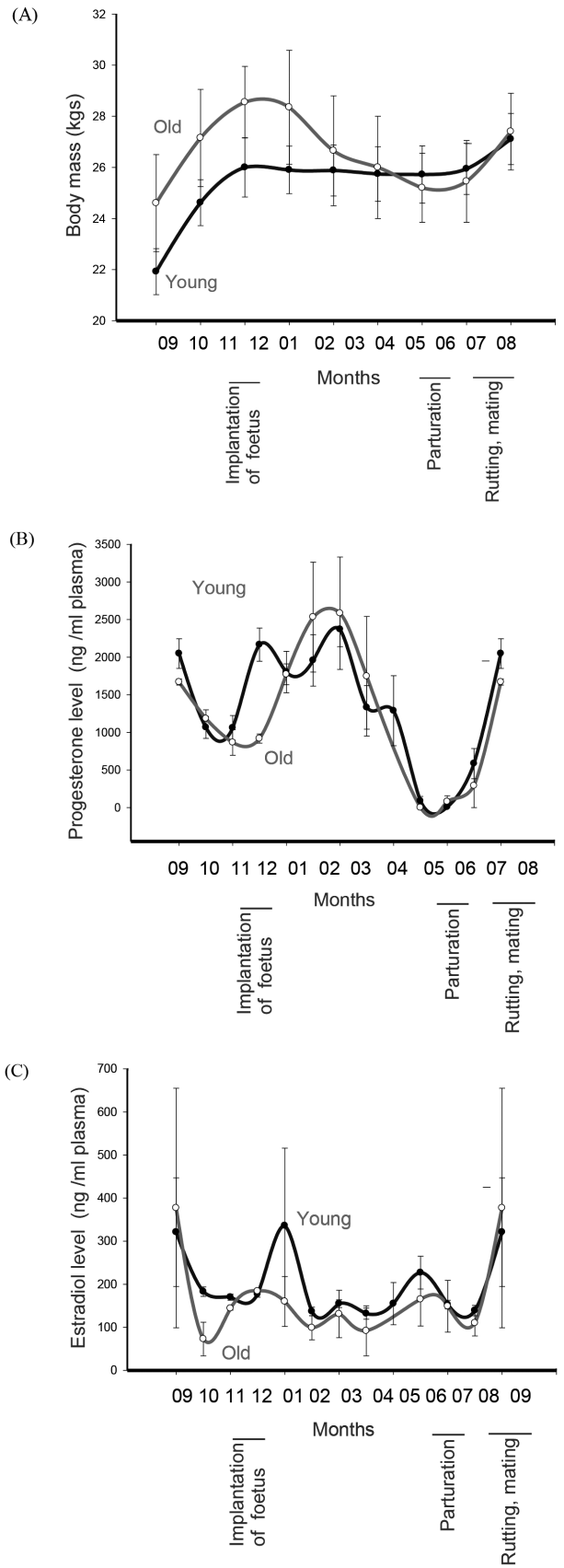

Fig. 2. Seasonal changes in body weight (A), plasma progesterone (B) and estradiol (C) level in young (1 year old) and adult (2-4 years old) pregnant roe deer does. See Fig. 1 for explanations.

A_jM_k – fixed effect of interaction between age and month; $\sum_{jk} AM = 0$

u_l – random effect of doe (1, 2 to 17); $u_l \sim N(0, I\sigma_l^2)$

e_{ijkl} – random error; $e_{ijkl} = N(0, I\sigma_e^2)$

Fixed effects included in the model were estimated using the LSM (Least Squares Means) method. Statistical significance was tested by Fischer's F-test and differences between the estimated levels of fixed effects were tested by Scheffe's multiple range tests. Differences between the groups with $P < 0.05$ were considered as significant. Doe and residual error variances were estimated using the REML (Restricted Maximum Likelihood) method. The estimated variances were used to estimate the repeatability of the studied traits within the individual doe:

$$r^2 = \frac{\sigma_l^2}{\sigma_l^2 + \sigma_g^2}$$

Results and Discussion

Analysis of variance and estimates of repeatability are given in Table 2. The higher values of coefficients of repeatability were found for body weight and progesterone, the lower value was found for estradiol (0.86, 0.32 *vs.* 0.03) indicating that the effect of individual doe was of higher importance in body weight and progesterone than in estradiol. When studying the influence of single effects of pregnancy, age of roe deer does and month of year, pregnancy showed no influence on the studied traits, whereas month of year was significant ($P < 0.01$) for body weight and progesterone (and tended to be significant for estradiol, $P = 0.18$). The effect of age of roe deer doe showed no influence estradiol and progesterone, however, it tended to be significant for body weight of roe deer doe ($P = 0.18$). When studying the influence of interaction effects of pregnancy × month and age × month, pregnancy × month of year was significant for body weight and progesterone; age × month of year showed no influence on the studied traits. Nevertheless, some individual levels of interaction effects tended to differ between one another, i.e. differences were found, although, these were insignificant.

Seasonal (mothly) and pregnancy-dependent changes in body weight in roe deer does are given in Fig. 1A. In all the animals minimal values of body weight were observed in September (after mating). In non-pregnant females high body mass was maintained from December to August with maximum in August (during new rutting season), whilst pregnant animals had high body weight from November up to August with maximum in November. Therefore, pregnant

Table 1. Characteristics of immunoassays.

Assay	Specificity (cross-reactivity of antiserum)	Sensitivity coefficient of variation (%)	
		Intra-assay	Inter-assay
Progesterone	< 0.001 % to cortisol, corticosterone, androstenediol, pregnenolone, estradiol, testosterone	0.12 ng/ml	< 8.0 < 13.1
Estradiol	< 0.01 % to DHEA, progesterone, cortisol, androsterone, testosterone, androstenedione, corticosterone, cortisone	6.5 pg/ml	< 9.4 < 19.2

Table 2. Analysis of variance (Fisher's test) for studied traits in roe deer does. DF: degrees of freedom, ++: $P < 0.01$.

Dependent variable/trait	Fixed effect	DF	F-value
Body weight	Pregnancy (P)	1	0.03
	Age (A)	1	2.55
	Month (M)	11	11.68++
	P×M	11	3.75++
	A×M	11	0.58
Estradiol	Pregnancy (P)	1	0.38
	Age (A)	1	0.25
	Month (M)	11	1.39
	P×M	11	0.35
	A×M	11	0.10
Progesterone	Pregnancy (P)	1	0.85
	Age (A)	1	0.27
	Month (M)	11	18.45++
	P×M	11	6.63++
	A×M	11	1.28

animals had significantly higher body weight than non-pregnant ones in November, and lower body weight in comparison with non-pregnant females in August. These observations confirm the reports of Mauget et al. (1997, 2003) about pregnancy-associated increase in body weight and related metabolism in this species. Good expressed seasonal changes in plasma progesterone level, which were dependent of animal reproductive state, were observed (Fig.1B). In non-pregnant females high level of progesterone was observed from August (mating) up to December. Thereafter progesterone level declined up to minimum in spring and summer months (April-July). In pregnant animals the level of progesterone in blood from August to January corresponded high level of this hormone in non-pregnant animals, but during pregnancy, after implantation of foetus (January), progesterone level not declined, but increased with maximum in March (mid-pregnancy). Reduction in progesterone level up to basal level was observed only in June, after labour (Fig. 1B). Our observations confirm the report of Hoffmann et al. (1978) on ovulation- and gravidity-associated increase in plasma progesterone level, but not the data of Schams et al. (1980) and Lambert et al. (2001), who did not observe such changes. Our observations, together with those of Hoffmann et al. (1978) suggest, that in roe deer does, like in other mammals (Fortune et al. 2013, Sirotkin 2014), progesterone plays an important role in ovulation and luteinisation of ovarian follicles, in maintenance the gravidity and embryo survival.

Seasonal changes in estradiol level in this species were observed. Differences in estradiol levels between non-pregnant and pregnant animals were also observed, however these were insignificant (Fig. 1C). In non-pregnant females, three peaks of estradiol concentration in the blood were observed in September, December and May. Minimal estradiol level was found in July-August (time of mating). In pregnant animals plasma estradiol level increased in September (during initial stages of gravidity) and January-March (advanced stages of gravidity after implantation of foetus), whilst decreased in April-June (before labour). Therefore, pregnant animals, in contrast to non-pregnant ones, had spring (gravidity-associated) peak of estradiol level, but absence of summer peak before parturition. This is the first observation of mating/ovulation-associated decrease in estradiol surge in roe deer females. Estradiol is considered as an important stimulator of ovarian follicullo- and oogenesis (Fortune et al. 2013, Sirotkin 2014), therefore the increase of this plasma hormone level during mating could be expected. It is not to be excluded, that estradiol controls these processes not (or not only) through increase in its output, but in its reception or postreceptor signalling pathways. In addition, seasonal decrease in estradiol release can be induced by negative feedback mechanisms described in other species (Sirotkin 2014). Nevertheless, our observations suggest the involvement of estradiol surge in control of roe deer doe gravidity and parturition, although the role and significance of estradiol in control of this process remain to be studied yet.

No non-pregnant young animals occurred in our experiment. Comparison of annual changes in body weight and plasma steroid hormone level of pregnant yearlings and old animals did not show principal

differences in these indexes (Fig. 2A-C), although young animals tended to have higher absolute progesterone (in December) (Fig. 2B) and estradiol (in October and November) (Fig. 2C) levels. Our observations do not confirm the report of Mauget et al. (1997, 2003) that pregnant young animals have higher metabolism, growth and body weight than the older females. Furthermore, our observations do not correspond to some previous reports (Komárek & Kočiš 1991, Prior 2000) concerning high age-related variability in somatic and reproductive indexes and high infertility rate in roe deer doe in the first year of life. Lack of such age-dependent differences observed in our experiments could be due to good care of experimental females studied in our experiments in comparison to wild animals.

Take together, our observation is the first demonstration of higher rate of steroidogenesis in young animals than in old females in autumn (during early stages of gravidity, before embryo implantation). The biological significance of such age-dependent differences remains unknown. They are probably not related to current gravidity because only pregnant animals were compared, and no substantial differences in fertility and number of offspring born by young and old animals was observed in our experiment.

Taken together, our observations suggest seasonal changes in plasma progesterone and estradiol level and body weight in this species. Differences in these changes in pregnant and non-pregnant animals demonstrate the involvement of steroid hormones in control of pregnancy in roe deer does. The absence of age-dependent differences in body weight and fecundity rate do not confirm previous hypothesis that age-dependent differences in metabolism and body mass can reduce fertility rate in yearlings. It is not to be excluded, that age-dependent reduction in ovarian steroid hormones level could be a cause of future infertility in old animals.

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