Conceptus-endometrial interactions and reproductive hormone profiles during embryonic diapause and reactivation of the blastocyst in the European roe deer (*Capreolus capreolus*)

Roger T. Lambert

Department of Zoology, University of Aberdeen, AB24 2TZ, Scotland, United Kingdom (rogerlam@roedeer.u-net.com).

Abstract: Roe deer blastocysts exhibit obligate embryonic diapause between early August and late December. The blastocyst then expands and elongates rapidly before implantation. The objective of this study was to ascertain the cues for reactivation of the diapausing blastocyst. Blood samples and reproductive tracts were collected from roe does during diapause, blastocyst expansion and subsequent implantation. Peripheral concentrations of oestradiol-17β, progesterone and prolactin were measured by radioimmunoassay. Luteal progesterone release was determined following *in vitro* incubation. Conceptuses and endometrial tissue were cultured with ^{3}H-leucine for 24 hours to measure *de novo* synthesis of secretory proteins. Endometrial secretory proteins were separated by two-dimensional electrophoresis. Results showed that peripheral progesterone concentrations declined by 55% just prior to expansion and did not rise until a 3-fold increase after implantation. Luteal progesterone release remained constant until expansion when it declined by 50% before increasing 2-fold at elongation and implantation. Concentrations of oestradiol-17β remained at a consistently low level during diapause and expansion until a 30-fold increase at elongation with concentrations remaining elevated after implantation. Plasma prolactin levels remained at basal concentrations during late diapause and then increased marginally at reactivation before decreasing again at elongation and implantation. Incorporation of radiolabel into both conceptus and endometrial secretory proteins was low during diapause, but incorporation in the conceptus increased 4-fold at expansion and by 24-fold at the expanded trophoblast stage. Incorporation into endometrial secretoty proteins remained constant until the expanded trophoblast stage and implantation when a 2-fold increase was recorded. Furthermore, the profile of endometrial secretory proteins was constant during diapause and expansion but changed qualitatively following implantation. These data indicate that both endometrial protein synthesis and sectetion did not change during late diapause and early expansion. The increase in conceptus protein synthesis not only precedes that of the endometrium but consistently low luteal progesterone release, peripheral progesterone and oestradiol-17β concentrations at early expansion suggests that reactivation is not in response to a maternal uterine trigger.

Key words: *corpora lutea*, delayed implantation, embryo, oestradiol, progesterone, prolactin.

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Introduction

The roe deer, *Capreolus capreolus* exhibits obligate embryonic diapause between early August and late December (Aitken *et al.*, 1973). The females have a single oestrous period and mate during late July and early August, (Short & Hay, 1966; Flint *et al.*,

1994). The blastocyst enters a period of diapause before reactivation in early January (Aitken, 1974). The zona pellucida is shed prior to the diapause period (Short & Hay, 1966) and the blastocyst undergoes a low level of mitosis during diapause (Lengwinat & Meyer, 1996). After elongation, the conceptus eventually forms a villous cotyledonary attachment to each of the uterine caruncular ridges (Aitken *et al.*, 1973), the placental link is formed and fetal growth follows, with parturition taking place in late May and early June (Aitken, 1974). During the 5 month period of diapause the *corpora lutea* remain active whether the doe is pregnant or not, the *corpora lutea* in the non-pregnant doe regressing after the period of diapause (Hoffman *et al.*, 1978). Broich *et al.* (1998) demonstrated in a pregnant doe, by using transrectal ultrasound, that the *corpora lutea* remain active until parturition. Peripheral progesterone remains at an elevated level throughout diapause, rising rapidly after implantation (Hoffman *et al.*, 1978). Aitken (1981), believed that the rise in progesterone was a result of implantation, not the cause and therefore suggested it was placental in origin. He was unable to identify the environmental cues for reactivation of the diapausing blastocyst. He did, however, detect in the uterine luminal flushings of roe deer large amounts of a uterine-specific protein, which in first dimension electrophoretic gels, appeared to migrate between transferrin and albumin during the period of elongation and implantation and to a lesser extent, during diapause (Aitken, 1974). Aitken (1981), also suggested that since a 5-month period of diapause was unlikely to be programmed into the embryonic genome, the timing of reactivation was not under genetic control because (1) endometrial secretory activity is observed at the end of diapause in the absence of direct contact with an elongating blastocyst and (2) there is anecdotal evidence of premature birth recorded in roe deer which may be explained by assuming that in some individual animals the delay phase has been attenuated or omitted. Another possibility was that prolactin was in some way involved in the induction of diapause (Aitken, 1981).

It is worth adding that genetic studies of the roe deer have found it to be polymorphic (Harrington, 1985). Roe deer also have a high level of heterozygosity (Hartl & Reimoser, 1988), which gives the animal a selective advantage. This suggests that the roe deer may vary genetically, between generations and populations (Mĺorsch & Leibenguth, 1994).

Photoperiod studies of the roe deer have shown that the doe has a fixed gestation period of 10 months and the five month period of diapause cannot be altered either by artificial changes in the natural photoperiod regime or by the use of melatonin implants. Increasing daylength in spring inhibits ovarian activity, with ovulation occurring in late July, indicating that the female roe deer can show refractoriness to long days and that the endogenous sexual cycle of the doe is about one year (Sempéré *et al.*, 1998).

The objective of the present study was to ascertain possible interactions between the conceptus and endometrium and reproductive hormone profiles during diapause, elongation of the blastocyst and subsequent implantation in roe deer. Such data could indicate the cues for re-activation of the diapausing blastocysts.

Material and methods

Animals

Twenty-two pregnant roe does were culled on Glen Tanar, Learney and Dunecht Estates and by Forest Enterprise at Durris, in north east Scotland (57.15°N, 2.52°W), between 9 November and 29 January over a period of three seasons (1995/96, 1996/97, 1997/98). The ages of the animals were estimated by the Forest Enterprise Head Ranger and the Head Gamekeepers on the three Estates as between eighteen months and four and a half years. Nine of the twenty-two animals, culled between 20 November and 29 January, were used to study endometrial-conceptus interactions *in vitro*. Blood samples were taken from all 22 animals for hormone radioimmunoassay. Three orphan female infant kids and two male infant kids were collected from the wild and hand reared.

Ethics of experimentation

The cull animals were obtained during the period authorised under the Deer (Close Seasons) (Scotland) Order 1984 and were part of management culling practice. No animal was culled for the sole purpose of the study. Live animals were obtained as day old kids with the authority of the Secretary of State for Scotland, in terms of Section 33(3B) of the Deer (Scotland) Act 1959. The three females and two males were hand reared under veterinary supervision. The live animals were used primarily for the observation of reproductive behaviour and in 1997/98 a manipulation of photoperiod trial,

which was exempt from the conditions of the Animals (Scientific Procedures) Act 1986.

Blood samples

As soon as the roe deer was shot, the jugular vein was cut and blood collected in sterile pots to harvest either plasma or serum. The pots for collecting blood samples for plasma contained heparin and the sample was placed immediately in ice. The samples were centrifuged within an hour and the plasma aspirated and stored at - 20 °C. Serum samples were collected in sterile pots and allowed to separate out at room temperature over a 24 hour period. The serum was then aspirated and stored at - 20 °C.

Processing of reproductive tract

As soon as possible after death, the whole reproductive tract was removed under aseptic conditions and placed in a sterile vacuum container warmed to roe body temperature of 38.9 °C and despatched to the laboratory, where under sterile conditions the conceptuses were recovered by irrigating the uterine lumen with 15 ml of sterile 0.9% NaCl. Individual conceptuses were measured under the microscope. The 15 ml of recovered uterine flush was centrifuged at 1000 rpm for 10 minutes at 4 °C and stored at -20 °C. The conceptuses and endometrial tissue were then transferred to a laminar flow hood. Each individual conceptus was placed in a small petri dish with 3 ml of leucine free MEM (GibcoBRL) culture medium (at 38.9 °C) supplemented with antibiotic-antimycotic solution (ABAM) and L-leucine made up in the ratio of 500:1 and 5:1 by volume respectively. Finally, 50 µl of ^{3}H-leucine (specific activity: 5.88 TBq/mmol. 159 Ci/mmol.) (Amersham Life Science) was added to radiolabel *de novo* protein synthesis during culture. Approximately 300 mg of endometrial tissue was dissected from the uterus and cut into 1mm sections, placed in a petri dish and accurately weighed under aseptic conditions. Fifteen ml of supplemented MEM at 38.9 °C was added followed by 50 µl of ^{3}H-leucine. Endometrial tissue and conceptuses were cultured for 24 hours at 38.9 °C in a controlled atmosphere chamber on a rocking platform and gassed with a mixture of 45% O_2/50% N_2/5% CO_2 for ten minutes at 8 hourly intervals. After 24 hours the culture medium was aspirated and stored at -20 °C. Each conceptus was placed in a sterile Eppendorf tube containing 1 ml of sterile phosphate-buffered saline (4 g NaCl/0.1 g KCl/0.72 g Na_2HPO_4/0.12 g KH_2PO_4 made up to 500 ml with distilled H_2O) and frozen at -70 °C prior to oestradiol-17β assay. The ovaries were placed in sterile 0.9% NaCl. The *corpora lutea* were dissected from the ovaries, weighed, and minced. Individual minced *corpora lutea* were cultured in leucine free Medium 199 (GibcoBRL) supplemented with ABAM in a ratio of 500:1 at 38 °C for two hours. After centrifugation, the media were aspirated and stored at -20 °C prior to assays for progesterone and prolactin. The minced luteal tissue was stored at -20 °C prior to Bradford microprotein estimation (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Dialysis of conceptus, endometrial and uterine flush proteins

Uterine flushings and endometrial and conceptus culture media samples were dialysed in individual molecular porous membrane tubes (Spectra/Por) with a 3500 molecular weight cut off, against three changes of 1.0 mol Tris/HCl at pH 8.2 at 4 °C. The uterine flushes were frozen at -2 °C prior to Bradford protein estimation. Culture media samples were then precipitated with trichloro-acetic acid (Mans & Novelli, 1961) to determine *de novo* synthesis and secretion.

Isoelectric focusing (IEF) and two-dimension electrophoresis

Samples of radiolabelled endometrial proteins containing 400 000 dpm were separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide and visualised by fluorography. Bradford microprotein estimation was carried out on the uterine flushes and the amount of sample required for IEF was determined and freeze dried. The technique for producing first dimension separation (IEF), two-dimensional PAGE and fluorography was as described by Roberts *et al.* (1984). The molecular weights of the separated proteins were within the range 22 000 to 97 400.

Radioimmunoassay of blood samples, uterine flushings & luteal secretions

Progesterone was determined without prior extraction using a ^{125}I - labelled progesterone double antibody radioimmunoassay (McNeilly & Fraser, 1987). The assay was modified for use with rabbit anti-progesterone, donkey anti-rabbit IgG and normal rabbit sera, obtained as gifts from the Scottish Antibody Production Unit, Carluke, Scotland. Progesterone stripped bovine plasma was added to

the standard curves for plasma samples and progesterone stripped roe deer serum was added to the standard curves for serum samples. Culture medium (Medium 199) was added to the standard curves and used to dilute luteal culture medium samples. The sensitivity of the assay was 0.6 ng ml^{-1}. For the plasma and serum sample assays, the intra- and inter-assay coefficients of variation QC low, medium and high were 20.5, 11.0, and 8.9% and 21.5, 10.8, and 9.0% respectively. For the culture media sample assays, the intra- and inter-assay coefficients of variation for QC low, medium and high were 16.5, 6.4 and 5.8% and 21.7, 6.3 and 5.8% respectively.

Each individual minced CL tissue sample was used to determine luteal protein content using Bradford microprotein estimation. From the radioimmunoassay values of the matching samples of progesterone secreted *in vitro,* it was then possible to express luteal progesterone release as ng mg^{-1} protein.

Plasma, serum and conceptus culture media oestradiol-17β levels were measured using a ^{125}I - labelled extraction oestradiol radioimmunoassay (Mann *et al* , 1995). The sensitivity of the assay was 0.6 pg ml^{-1}. All the samples were analysed in one assay and the intra-assay coefficients of variation for QC low, medium and high were 9.6, 5.9 and 14.9% respectively.

Plasma, serum, uterine flush and luteal culture media prolactin was measured using a modified double antibody radioimmunoassay method (Hart, 1973; Gala & Hart, 1980). The guinea pig anti-ovine prolactin was obtained as a gift from I.A. Forsyth, Babraham Institute, Cambridge. The sheep anti-guinea pig IgG and normal guinea pig serum were obtained as gifts from the Scottish Antibody Production Unit, Carluke. The sensitivity of the assay was 3.0 ng ml^{-1}. Inter-assay coefficients of variation for QC low, medium and high were 11.8, 12.2 and 15.1% respectively. Intra-assay coefficients of variation for QC low, medium and high were 11.9, 12.8 and 15.1% respectively.

Determination of the duration of pregnancy and effects of alteration of photoperiod in females

A hand-reared yearling doe was placed with a hand-reared yearling buck during the mating period of late July, early August. The rut took place in a two-acre paddock and Doe (No. 001) was mated on 4 August 1996. They remained together until early May, when the clearly pregnant doe was separated into a small paddock, with inside accommodation, where she could avoid competition for food from the other animals and give birth without interference or risk of disturbance by the buck. Three two year old does (001, 002, 003) were mated during early August 1997 with a two-year-old buck in a two-acre paddock. The does were then subject to a 16 h light and 8 h dark photoperiod regimen (16L: 8D) from 17 October 1997 to 18 April 1998. All three does became pregnant. Urine was collected from each doe at a maximum of weekly intervals and the samples were frozen at -20 °C. A method of assaying these samples is currently under discussion.

Statistical analyses

The data from all the animals were expressed as one period from November through to the end of January regardless of the year in which the samples were collected. The data was separated into the period of diapause up to 31 December, blastocyst expansion (1-22 January), elongation (23-25 January) and implantation (26-29 January). Changes in conceptus and endometrial incorporation were expressed as a % of mean diapause values. The results showed a considerable degree of consistency over the three-year period.

Where an animal had two *corpora lutea* (CL) the data were expressed as a mean value after calculation of the luteal protein content from individual tissue samples and the luteal progesterone release from the equivalent individual CL. Where an animal had two conceptuses, after the calculation of the dpm/conceptus, the values were expressed as a mean.

Mean (± standard error of the mean) peripheral hormone concentration values for progesterone, oestradiol-17β, prolactin and luteal progesterone release for the periods of; 1) diapause (up to 31 December), 2) expansion (1-22 January), 3) elongation (23-25 January), and 4) implantation (26-29 January) are shown in Table 2. Analysis of the hormonal data was divided into two periods, 1) diapause/blastocyst expansion and 2) blastocyst elongation/implantation. The comparison between groups 1 & 2 were expressed as a *P*-value.

Results

Number of conceptuses and corporal lutea recovered

Nine pregnant does were used to study conceptus-endometrial interactions *in vitro.* The total numbers of *corpora lutea* and conceptuses in the pregnant does was 18 and 15 respectively. Conceptus development

Table 1. Date of collection, number of *corpora lutea* (CL), and number and size of conceptuses of pregnant roe deer.

Doe No.	Date	Stage of pregnancy	No. of CLs	No. of conceptuses	Size of conceptuses	
RD1	22-Nov	Diapausing blastocyst	2	2	0.29 X 0.27 mm	0.26 X 0.25 mm
RD2	28-Nov	" "	2	1	0.29 X 0.17 mm	
RD3	20-Dec	" "	2	2	0.37 X 0.35 mm	0.28 X 0.18 mm
RD5	2-Jan	Expanding blastocyst	2	2	0.70 X 0.60 mm	0.65 X 0.45 mm
RD8	5-Jan	" "	2	1	1.50 X 1.05 mm	
RD10	23-Jan	Elongated trophoblast	2	1	Length 90 mm	
RD11	27-Jan	Implanted fetus	2	2	*C-R 7 mm	C-R 5 mm
RD12	28-Jan	" "	2	2	*C-R 7.5 mm	C-R 7 mm
RD7	29-Jan	" "	2	2	*C-R 12 mm	C-R 9 mm

*C-R = Crown - Rump length.

Table 2. Concentrations of maternal hormones during diapause, expansion, elongation and implantation in the roe deer. Mean (± standard error of the mean).

	Diapause	Expansion	Elongation	Implantation	P-value
Plasma Progesterone (ng ml^{-1})	2.9 ± 0.8 (n = 15)	1.46 ± 0.9 (n = 3)	0.84	3.92 ± 2.1 (n = 3)	NS
Plasma oestradiol-17β (pg ml^{-1})	1.07 ± 0.4 (n = 15)	1.2 ± 0.4 (n = 3)	48.9	31.08 ± 0.3 (n = 3)	$P < 0.005$
Luteal progesterone (ng mg^{-1} protein)	7.03 ± 0.8 (n = 3)	3.7 ± 0.7 (n = 2)	7.2	10.2 ± 0.6 (n = 3)	$P < 0.05$
Plasma prolactin (ng ml^{-1})	3.05 ± 1.5 (n = 4)	4.0		<3.0	NS

P-value: comparison between diapause/expansion and elongation/implantation.
NS: Not significant $P > 0.05$.
n is the sampling size.

ranged from a 0.26 mm diapausing embryo collected on 22 November, a blastocyst starting to elongate on 5 January (Fig. 1), to a 12 mm implanted fetus on 29 January (Table 1).

Radiolabel incorporation into endometrial secretory proteins

Radiolabel incorporation into endometrial secretions was remarkably constant during the later period of diapause and the early stages of blastocyst elongation. The means radiolabel incorporation into endometrial secretory proteins for the period 22 November to 5 January was 5210 ± 88 dpm mg^{-1} (n = 5). At the elongated trophoblast stage (9 cm in length) and after implantation, during the period between 23 January and 29 January, radiolabelled secretions were again quite consistent, 12 600 ± 987 dpm mg^{-1} (n = 4). This represents an increase in secretions of 150% between diapause/early elongation and the expanded trophoblast stage and implantation (Fig. 2).

Radiolabel incorporation into conceptus secretory proteins

Incorporation of radiolabel into secreted conceptus proteins was consistent during diapause 2620 ± 926 dpm conceptus^{-1} (n = 5). The early stages of expansion, between 2 and 5 January, incorporation into secreted proteins had increased to a mean value of 10 500 ± 2550 dpm conceptus^{-1} (n = 3) which represents a 430% increase from diapause. By 23 January, when the conceptus was at the elongated trophoblast stage, incorporation was 61 590 dpm conceptus^{-1} (n = 1); an increase by a factor of 22.5 compared with diapause (Fig. 2).

Two-dimensional electrophoresis

The profile of endometrial secretory proteins was consistent during diapause and the early stages of

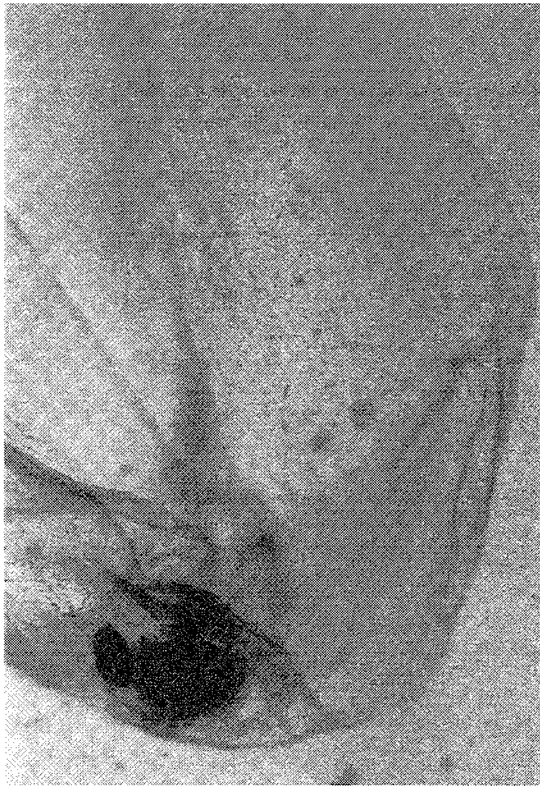

Fig. 1. Roe deer blasrocysr starting to elongate 5 January 1998. Size: 1.5 mm x 1.05 mm. The length of the short side of the photo is 0.95 mm.

elongation (Fig. 3A), but qualitative differences were evident after implantation (Fig. 3B). The majority of radiolabelled endometrial proteins secreted during diapause and early elongation had isoelectric points (*p*I) between 5.9 and 7.2. After implantation, there was a shift towards higher molecular weight basic proteins. These changes were consistent over the three-year period. Visual examination of gels produced from the uterine luminal flushings showed a consistent profile during diapause and the early stages of elongation.

Peripheral progesterone concentrations and luteal progesterone release

Data describing peripheral concentrations were available for 22 pregnant does including the 9 pregnant does used for radiolabelling and electrophoresis (Table 2). Peripheral progesterone concentrations declined from diapause (2.9 ± 0.8 ng ml^{-1}; n = 15) to 1.3 ± 0.66 ng ml^{-1} (n = 4) during expansion and elongation and did not rise until implantation (3.9 ± 3.6 ng ml^{-1}; n = 3). Values for luteal progesterone release were available from 18 minced *corpora lutea* (CL) from the above 9 animals (Table 2). Luteal progesterone decreased from 7.03 ± 0.8 ng mg^{-1} protein (n = 6 CL) during diapause to 3.73 ± 0.7 ng mg^{-1} protein (n = 4 CL) at expansion, increasing to 7.2 ± 0.3 ng mg^{-1} protein (n = 2 CL) at elongated trophoblast stage and to 10.2 ± 0.6 ng mg^{-1} protein (n = 6 CL) at implantation.

Oestradiol-17β and prolactin

Peripheral oestradiol-17β concentrations were consistently low throughout diapause and expansion (1.3 ± 0.33 pg ml^{-1}; n = 18) but increased 30-fold at the elongated trophoblast stage and implantation (37.0 ± 7.9 pg ml^{-1}; n = 4). Conceptus oestradiol-17β secretion during diapause and early reactivation were below detectable levels (< 0.6 pg ml^{-1}).

Prolactin values were available for the 9 animals used to study conceptus-endometrial interactions in vitro. Peripheral prolactin concentration was 4.8 ng ml^{-1} on the 22 November, decreased to 3.3 ng ml^{-1} on 17 December and was not detectable on 20 December (< 3.0 ng ml^{-1}). There was a rise in prolactin to 4.0 ng ml^{-1} on 2 January, which coincided with the early stages of expansion of the blastocysts, but during and after implantation, prolactin fell again below detectable levels. Prolactin in the uter-

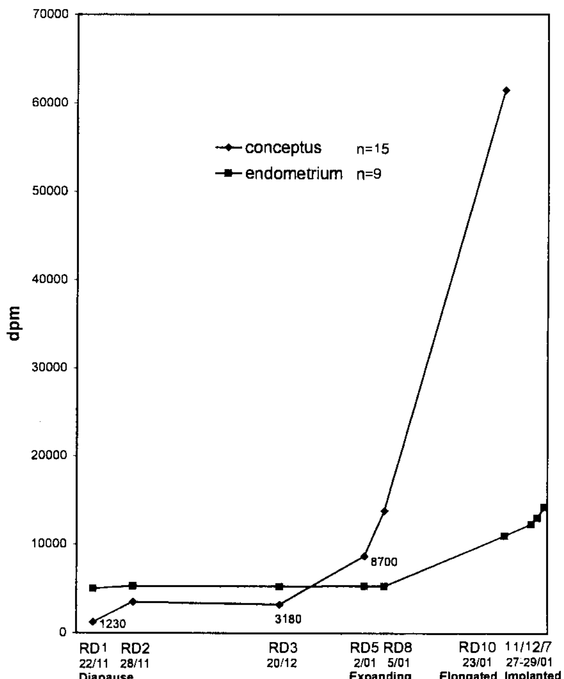

Fig. 2. Temporal increase in conceptus and endometrial ^{3}H-leucine incorporation between 24 November and 29 January. (conceptus = dpm/conceptus: endometrium = dpm/mg)

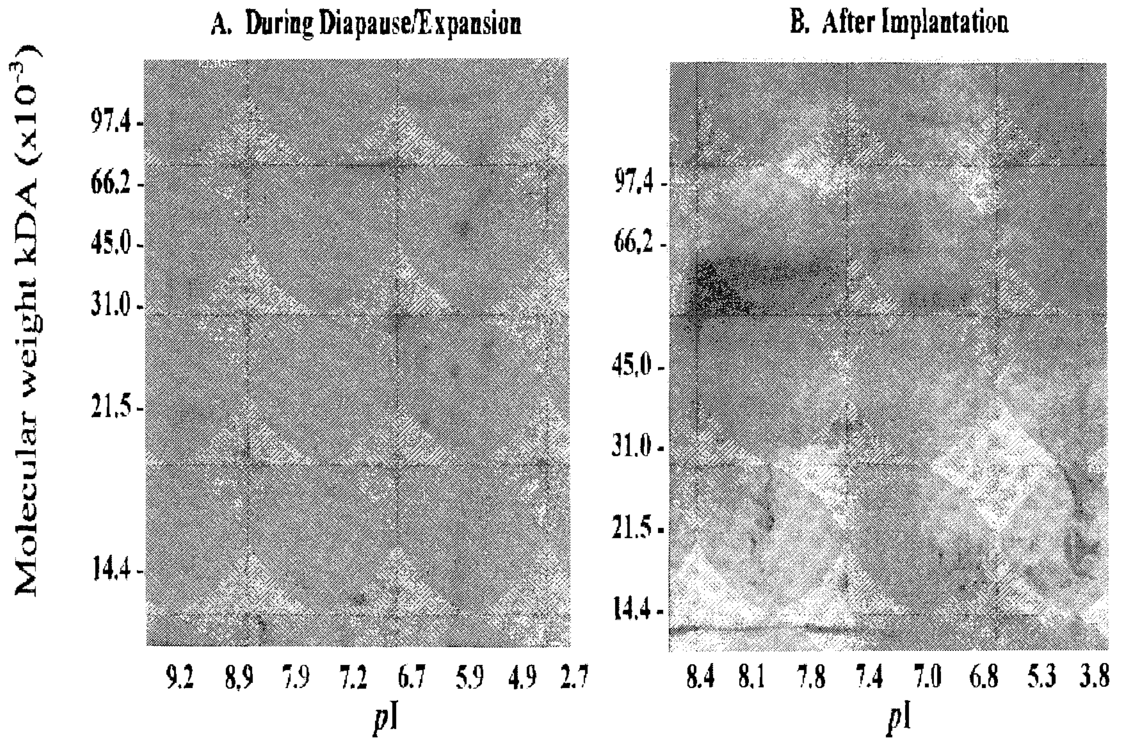

Fig 3. Fluorographs of radiolabelled endometrial protein secretions. In fluorograph A, the highest concentration of radiolabel endometrial protein secretion during diapause and early elongation was between a *p*I of 5.9 and 7.2 and a molecular weight between 22 000 and 97 400. In B, after implantation, qualitative differences are clearly evident with a shift in acidity towards higher molecular weight basic proteins between a *p*I of 7.8 and 8.4 and a molecular weight of between 60 000 and 66 200.

ine luminal flushings and luteal secretions were below detectable levels (< 3.0 ng ml^{-1}).

Determination of duration of pregnancy and effects of changes in photoperiod

A yearling doe (No. 001) mated on 4 August 1996 gave birth to twin female kids on 26 May 1997. This would establish pregnancy duration of 290 days. In August 1997 all three two year old does mated, No. 002 on 31 July 1998, No. 001 on 2 August 1997 and No. 003 on 5 August 1997. After subjection to 16L: 8D, from 17 October 1997 to 18 April 1998, parturition in all three does took place as follows; No.002 on 15 May 1998 (a single female kid), No.001 on 19 May 1998 (two female and one male kid), No. 003 on 29 May 1998 (two female kids). The mean pregnancy duration was 290.6 ± 1.56 days. However, all three animals responded to the altered photoperiod by a change in the pattern of moulting in the winter coat. The first signs of moulting were visible on 10 January 1998 in all three animals, some three months before the normal start of the moulting period in north east Scotland. The moult was erratic and slow and eventually came to a complete halt in mid March before recommencing in late May.

Discussion

This study describes temporal changes in conceptus and uterine biochemistry following *in vitro* culture of roe deer embryos and endometrium. The observation that a dramatic increase in conceptus radiolabel protein secretion precedes a more modest increase in endometrial protein secretion provides strong evidence to suggest that a signal from the embryo is the trigger for reactivation from diapause. Evidence from other species indicates that the embryonic signal for pregnancy may be oestrogen, for example the pig (Bazer, 1989) or a protein, such as hCG or interferon-τ as in cattle, sheep and related mammalian species (Roberts *et al.*, 1992). Our data showing that oestradiol-17β secretion by roe conceptuses is consistently low throughout late diapause and early reactivation suggests that the embryonic signal is

likely to be a protein. Peripheral oestradiol-17β concentrations showed a similar pattern during diapause and early reactivation, but rose dramatically at the elongated trophoblast stage.

The profile of endometrial secretory proteins showed a substantial change from diapause/early elongation when acidic proteins predominated to the time of implantation when basic proteins were more prevalent. This shift in acidity of the dominant endometrial secretory proteins between the blastocyst stage and implantation also occurs in other species such as the domestic pig (Roberts & Bazer, 1988). At this stage, we do not know the functions of the wide array of proteins secreted by the roe deer endometrium. It is possible that one of the proteins evident in the 67 000 - 80 000 molecular weight range corresponds to the uterine-specific protein, which Aitken (1974) detected in the uterine luminal flushings of roe deer at the time of embryonic elongation, and, to a lesser extent, during diapause. The amount of this protein was found to be at low levels during diapause and expansion in our 2D electrophoresis gels of the uterine luminal fluids. An increase in this protein was only observed at elongated trophoblast stage.

Our data describe a reduction in both luteal progesterone release and peripheral progesterone concentration just before and during early reactivation of the blastocyst. Sempéré (1998, pers. comm.) had also observed a decline in peripheral progesterone concentrations during late diapause, just prior to expansion of the blastocyst. Luteal progesterone release returns to pre-reactivation levels at the elongated trophoblast stage and remains elevated during early implantation. Peripheral progesterone concentrations did not rise until after implantation. Although a rise in peripheral progesterone concentration after reactivation of embryo development has been described previously (Aitken, 1979), it was not clear if this reflected an increase in luteal or placental production. Our data indicates luteal progesterone release remains at a level comparable with the levels during diapause, after implantation. A substantial rise in peripheral concentrations after implantation indicates that the rise in progesterone in roe deer is probably placental in origin. Therefore, the rise in level is as a result of reactivation and not its cause.

The question remains as to what triggers the roe deer conceptus to reactivate. The low luteal production of progesterone at early expansion suggests that the decrease in progesterone from concentrations during diapause may be an important hormonal cue. A role for intra-uterine prolactin seems unlikely, as it was undetectable in both uterine luminal fluids and luteal culture media. Also, peripheral prolactin concentrations remained at basal levels during the period studied. A role for photoperiod in the reactivation of the blastocyst would not seem to be a viable hypothesis in view of the detailed study of Sempéré *et al.* (1998) and our own study with 3 pregnant does. Though the onset of oestrous in the roe deer has shown to be photoperiod sensitive (Sempéré *et al.*, 1998), reactivation of the diapausing blastocyst, though occurring around the winter solstice suggesting a photoperiodic cue, appears to be the consequence of an endogenous annual sexual cycle (Sempéré *et al.*, 1998). The initial signs of the spring moult occurred in early January as a result of our photoperiod study, though not recorded in detail, the observations were very similar to those reported by Lincoln & Guinness (1972).

Though Aitken (1981) had discounted embryonic genetic programming as a possibility, neither of his reasons for excluding it are now tenable i.e. endometrial activity observed at the end of diapause in the absence of direct contact with an elongating blastocyst or anecdotal evidence of premature birth which could only be explained by assuming that the period of diapause was omitted. It would be possible within any population of roe deer that occasionally a doe's endocrine signals were inconsistent with the majority.

In conclusion, consistently low luteal progesterone release, peripheral progesterone, oestradiol-17β and prolactin concentrations at early expansion suggest that conceptus reactivation is not in response to a maternal endocrine trigger. The rapid increase in conceptus protein synthesis preceding that of the endometrium suggests that the conceptus rather than the mother may provide the cue for reactivation.

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