Seasonal variations of plasma prolactin and LH concentrations in the female blue fox (*Alopex lagopus*)

M. Mondain-Monval, O. M. Møller*, A. J. Smith*, A. S. McNeilly† and R. Scholler

Fondation de Recherche en Hormonologie, 67–77 boulevard Pasteur, 94260 Fresnes, France;

**Department of Animal Husbandry & Genetics, Norwegian College of Veterinary Medicine, P.O. Box 8146 Dep., 0033 Oslo 1, Norway; and †M.R.C. Unit of Reproductive Biology, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, U.K.*

Summary. A heterologous radioimmunoassay system developed for the rabbit and suitable for a wide range of mammalian species has been shown to measure prolactin in the plasma of the blue fox. Evaluation of prolactin levels throughout the year showed that concentrations displayed a circannual rhythm with the highest values occurring in May and June. Prolactin concentrations remained low (~ 2.5 ng/ml plasma) from July until April with no consistent changes found around oestrus (March–April). In 8 pregnant females, the prolactin increase in late April and May coincided with the last part of gestation and lactation: concentrations (mean $\pm$ s.e.m.) increased to 6.3 ± 0.6 ng/ml at mid-gestation, 9.7 ± 2.1 ng/ml at the end of gestation and 26.7 ± 5.0 ng/ml during lactation. In 10 non-pregnant animals, the mean $\pm$ s.e.m. values were 7.2 ± 1.2 ng/ml in April, 8.8 ± 2.2 ng/ml in May and 9.8 ± 1.3 ng/ml in June. The prolactin profile in 4 ovariectomized females was similar to that observed in non-pregnant animals, but the plasma values tended to be lower during the reproductive season (April–June). In intact females, the only large LH peak (average 28 ng/ml) was observed around oestrus. During pro-oestrus, baseline LH levels were interrupted by elevations of 3.1–10.4 ng/ml. During the rest of the year, basal levels were < 3 ng/ml. In ovariectomized females, LH concentrations increased within 2 days of ovariectomy and remained high (35–55 ng/ml) at all times of year.

Introduction

The mechanisms by which the environment affects the reproductive physiology of the blue fox (*Alopex lagopus*) are poorly understood. It is well established that the seasonal pattern of reproductive activity in many species is controlled by the photoperiod. The blue fox is considered to be a long-day breeder since the reproductive season occurs when daylength is increasing. Oestrus (3–5 days) normally takes place from mid-March until the first week of May, and if conception occurs gestation length is about 52 days. In captivity, lactation lasts for 6–8 weeks. The corpora lutea that form in non-pregnant vixens have a lifespan similar to that of corpora lutea in pregnant animals (Møller, 1973). Seasonal anoestrus lasts about 9 months.

Changes in photoperiod exert a regulatory role on the gonads via the hypothalamic–pituitary axis. LH and prolactin have been implicated in the development of seasonal reproductive activity or quiescence. Prolactin concentrations are elevated during lactational anoestrus in several species

including goats (Hart, 1974), rats (Lu *et al.*, 1976) and sheep (Lamming, Moseley & McNeilly, 1974) and also during seasonal anoestrus as in the ewe (Munro, McNatty & Renshaw, 1980). Until now the pattern of secretion of LH and prolactin has not been determined in the female blue fox and the mechanisms behind the control of seasonal breeding in this species are poorly understood. The first aim of this study was to validate a heterologous radioimmunoassay for fox prolactin. Secondly, to investigate whether photoperiod is involved in the control of prolactin and LH secretion, we have studied the annual variations in plasma concentrations of these two hormones in pregnant and non-pregnant vixens and the changes in response to ovariectomy.

Materials and Methods

Animals and sampling

The 32 blue fox vixens used in this study were 3–5 years old. They were individually housed under natural conditions of daylength and temperature before and during the investigation on the Research Farm for Furbearing Animals, Heggedal, Norway. They were given a ration of standard Norwegian wet feed and water *ad libitum*.

To determine changes in plasma prolactin and LH concentrations during anoestrus, 5 intact females and 4 ovariectomized females were bled at monthly intervals from the end of June until March the following year. The ovariectomies were performed under general anaesthesia in June.

Vixens were mated during the breeding season immediately before the study (28 March–20 April), oestrus lasting 3–5 days, and matings were allowed twice at 48-h intervals. Eight pregnant vixens were bled 2–3 times weekly during pro-oestrus, gestation and lactation, and daily around oestrus. Ten non-pregnant vixens and the 4 ovariectomized animals were bled at weekly intervals between March and July.

Blood samples (5–10 ml) were collected between 09:00 and 12:00 h without anaesthesia from the cephalic vein into heparinized tubes. Plasma was separated by centrifugation and was stored at –20°C until assayed.

Prolactin assay

Fox prolactin was measured using a heterologous double-antibody radioimmunoassay as described previously (McNeilly & Friesen, 1978) with minor modifications.

Assay procedure. Ovine prolactin (LER-860-2) donated by Dr L. E. Reichert was radioiodinated using the lactoperoxidase method of Holohan, Murphy, Buchanan & Elonore (1973). Immediately after iodination, unreactive iodide was removed by gel filtration and the labelled hormone was purified using the procedure described by Hinds & Tyndale-Biscoe (1982).

The assay for fox prolactin utilized a guinea-pig anti-human prolactin (33-9) and a crude fox pituitary extract as reference preparation after comparison with a highly purified ovine prolactin (LER-860-2). Pituitaries were collected within 15 min of death from blue foxes (*Alopex lagopus*), silver foxes (*Vulpes vulpes argenteus*), red foxes (*Vulpes vulpes*) and racoon dogs (*Nyctereutes procyonoides*) and were stored at –20°C. Aqueous homogenates (10 mg tissue/ml) were then prepared in 0.05 M-sodium phosphate buffer, pH 7.4. This solution was serially diluted (1:10 to 1:12 000). All dilutions were made in 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl and 1% BSA. The incubation mixture consisted of reference preparations (100 µl) or samples (100 µl) to be assayed, buffer (300 µl), antiserum (100 µl at a final dilution of 1:36 000) and ¹²⁵I-labelled ovine prolactin (5000 c.p.m. in 100 µl). The mixture was then incubated at 4°C for 48 h. Rabbit anti-guinea-pig gamma globulin (Wellcome, Beckenham, U.K.; 100 µl at 1:10 dilution) and normal guinea-pig serum (100 µl at 1:200 dilution) were then added and incubation continued at 4°C for another 24 h before separation of bound and free hormone by centrifugation. The antibody-

bound iodinated prolactin in the precipitate was measured in an automatic gamma counter (LKB). Computation of potencies for the prolactin radioimmunoassay was performed using an LKB computer data processing program.

Assay characteristics. The antiserum was characterized by McNeilly & Friesen (1978) who showed that it cross-reacted with prolactin from a wide range of mammalian species. Since purified fox prolactin was not available, parallelism was assessed between ovine prolactin (LER-860-2), a highly purified canine prolactin (AFP-24A, provided by Dr A. F. Parlow, NIAMDD, Rat Pituitary Hormone Distribution Program), dilutions of fox plasma and crude pituitary extracts of different varieties of foxes. Parallelism in the displacement curves caused by ovine prolactin and fox pituitary extract was tested by comparing the slopes of the straight line (obtained by a logit-log transformation) using Student's *t* test. To evaluate cross-reaction with gonadotrophins, 2 male blue foxes were treated with LHRH (2 µg buserelin acetate; Hoechst, Frankfurt, West Germany) and 2 females were ovariectomized.

The accuracy of the assay was determined by the measurement of known amounts of ovine prolactin or diluted fox pituitary extracts added to fox plasma containing low levels of prolactin.

The detection limit was defined as the minimum concentration of ovine prolactin (33-9) capable of causing a significant displacement ($P < 0.05$) of radiolabelled prolactin from the antiserum. The precision was assessed by the repeated assay of 3 pools of plasma.

To evaluate the radioimmunoassay further, prolactin was measured in serum from 4 groups of 2 females and 2 males injected in February or March with thyrotrophin-releasing hormone (TRH) (Stimu-TSH: Roussel, Paris, France; 125 µg i.v.), metoclopramide (Primperan: Delagrang, Paris, France; 5 mg i.v.), bromocriptine (CB-154: Sandoz, Basel, Switzerland; 4 mg s.c.) or 0.9% (w/v) NaCl (0.5 ml s.c. or 1 ml i.v.).

Presentation of results. No fox standard was available for testing, and so serial dilutions of fox pituitary homogenates were used as reference preparations, since they gave curves that were nearly parallel to that for ovine prolactin. The prolactin values were expressed in ng LER-860-2 equivalents/ml plasma. Plasma samples and reference preparations were measured in duplicate.

Measurements of LH and progesterone

LH and progesterone were measured as previously described (Møller, Mondain-Monval, Smith, Metzger & Scholler, 1984a; Mondain-Monval, Bonnin, Canivenc & Scholler, 1984). The sensitivity of the LH assay was 0.4 ng/ml and the inter- and intra-assay coefficients of variation were 10.3% and 7.3% respectively. For the progesterone assay, the sensitivity and inter- and intra-assay coefficients of variation were 300 pg/ml, 13.4% and 9.9% respectively.

Statistical analysis

All concentrations are reported as mean $\pm$ s.e.m. For statistical evaluations, the data were analysed by Student's *t* test, the Mann-Whitney U test or the Kruskal-Wallis test (Conover, 1980).

Results

Validation of the prolactin assay

Specificity. The method proved to be specific for fox prolactin. The binding of ^{125}I -labelled prolactin to antiserum 33-9 was displaced in a parallel manner by crude pituitary homogenates of the different varieties of foxes and purified ovine prolactin (Text-fig. 1). The slopes of the straight line for the blue fox pituitary extract ($b = -0.860$) and for ovine prolactin ($b = -0.943$) were not

significantly different ($P > 0.30$). Serial dilutions of plasma from pregnant or lactating females were parallel to the inhibition curves obtained with pituitary extracts, ovine and canine prolactin (Text-fig. 2). The addition of 100 μ l plasma from a male treated with bromocriptine to the standard curve (ovine prolactin or pituitary extract) did not affect the binding (Text-fig. 1). All samples were therefore assayed against the pituitary extract which had an immunological potency of 16 ng LER-860-2/ml for a 1:640 dilution of pituitary homogenate.

Text-fig. 1. Inhibition curve for ovine prolactin (LER 860-2) prepared in assay buffer with (□) and without (◇) 100 μ l plasma from a blue fox male after administration of bromocriptine and dose-response curves for dilutions of pituitary extracts of blue fox with (▲) and without (●) plasma, of red fox (★), silver fox (○) and racoon dog (◆) without plasma.

Text-fig. 2. Dose-response curve for dilutions of blue fox plasma (★) samples in the prolactin radioimmunoassay and inhibition curves for ovine prolactin (LER 860-2) (△), canine prolactin (AFP-24A) (○) and blue fox pituitary extract (●).

Text-fig. 3. Plasma concentrations of prolactin and LH in 2 female blue foxes after ovariectomy and in 2 males after an injection of 2 µg LHRH.

Injection of LHRH or ovariectomy had no significant effect on plasma prolactin concentrations (Text-fig. 3).

Accuracy. The recoveries of known amounts of ovine prolactin (1.5–50 ng) or pituitary extract (1:80 to 1:10 240) added to plasma from a fox treated with bromocriptine and previously assayed at ≤ 1 ng/ml were $94.3 \pm 2.6\%$ ($n = 14$) and $97.6 \pm 2.5\%$ ($n = 14$) respectively.

Sensitivity and precision. Under routine conditions the assay was sensitive to 1 ng/ml plasma. The proportion of total radioactivity bound by the antiserum in the absence of hormone ranged from 30 to 65%. The intra- and inter-assay coefficients of variation were 8% ($n = 14$) and 13% ($n = 12$) respectively.

Biological validation. All the foxes showed an increase in prolactin concentrations 30 min after the i.v. injection of TRH or metoclopramide, the effect being significantly greater in the female than in the male. A decrease was seen after injection of bromocriptine (Text-fig. 4).

Text-fig. 4. Plasma concentrations of prolactin in 2 male (----) and 2 female (—) blue foxes after the injection (↓) of saline, TRH, metoclopramide or bromocriptine.

Seasonal variations of plasma prolactin and LH in intact and ovariectomized vixens

Evaluation of prolactin levels throughout the year showed that baseline levels (2.4 ± 0.2 ng/ml) followed a circannual rhythm with highest values occurring during May and June (Text-fig. 5b). No consistent changes in circulating prolactin levels were found around oestrus.

In pregnant females, the highest values coincided with gestation and lactation. Concentrations increased to 6.3 ± 0.6 ng/ml at mid-gestation, 9.7 ± 2.1 ng/ml at the end of gestation and 26.7 ± 5.0 ng/ml during lactation. In the non-pregnant females, the same variations were observed during the

Text-fig. 5. Variations of prolactin (b) and LH (c) concentrations in relation to daylength in 5 intact and 4 ovariectomized female blue foxes during anoestrus and in intact (5 non-pregnant ($\triangle$ --- $\triangle$) and 8 pregnant ($\bullet$ --- $\bullet$)) and 4 ovariectomized females ($\star$ --- $\star$, $\circ$ --- $\circ$) during the reproductive season in natural daylength (a). W = whelping.

luteal phase in April (7.2 ± 1.2 ng/ml) and May (8.8 ± 2.2 ng/ml). In June, prolactin concentrations remained high (9.8 ± 1.3 ng/ml) but were significantly different ($P < 0.05$) from those observed in lactating vixens (26.9 ± 5.0 ng/ml).

When females were ovariectomized in June, prolactin values remained low from July to April (2.2 ± 0.3 ng/ml, $n = 4$) and increased significantly ($P < 0.01$) in May and June (7.3 ± 0.3 ng/ml) but to a lesser degree than in intact females. The circannual changes in prolactin concentrations were parallel to those of daylength.

LH levels were low during anoestrus (July–February: 1.09 ± 0.3 ng/ml), during gestation or the luteal phase (April–May: 0.6 ± 0.1 ng/ml) and during lactation (May–June: 0.6 ± 0.1 ng/ml). Basal LH concentrations increased in March even when values at the time of the preovulatory LH peak were disregarded (Text-fig. 5).

LH levels increased within 2 days of ovariectomy and remained high throughout the year (range 31.4–67.5 ng/ml; Text-fig. 5c).

Prolactin and LH patterns during the reproductive season

Text-figure 6 shows the changes in hormone levels at weekly intervals from March until July in the three groups of females. In pregnant vixens, there was no variation in prolactin concentrations at the end of March and the beginning of April which corresponds to the oestrous period for intact vixens. Similar results were also observed when daily concentrations of prolactin and LH were

Text-fig. 6. Weekly variations of plasma prolactin and LH concentrations in intact (8 pregnant, 10 non-pregnant) and ovariectomized (4) female blue foxes. W, whelping. Values are mean $\pm$ s.e.m.

Text-fig. 7. Plasma concentrations of LH, prolactin and progesterone in 9 female blue foxes around oestrus and at the beginning of pregnancy. Values (mean $\pm$ s.d.) are synchronized to the day of the LH peak.

measured in the 8 vixens that had been pregnant and when hormone values were synchronized to the day of the preovulatory LH peak (Text-fig. 7).

As shown in Text-fig. 6, prolactin concentrations during the first week of April were significantly higher ($P < 0.05$) in non-pregnant than in pregnant vixens. Thereafter until the middle of May the average prolactin concentrations were similar in pregnant (7.2 ± 1.1 ng/ml) and non-pregnant females (8 ± 1.3 ng/ml). Significant differences ($P < 0.05$) between the two groups of females were observed after 10 May in the last days of gestation (14.6 ± 2.0 and 7.4 ± 0.7 ng/ml) and when lactation occurred (20.2 ± 2.0 and 7.6 ± 0.6 ng/ml). In the ovariectomized females prolactin concentrations from 20 April to 10 May were significantly lower ($P < 0.01$) than those of the two other groups (4.1 ± 1.0 ng/ml). The first significant increase was observed at the beginning of May (6.3 ± 0.6 ng/ml) and values rose progressively until June. Levels at the end of May and in June (7.3 ± 0.3 ng/ml) were not different from those of non-pregnant females (7.5 ± 0.5 ng/ml). The highest values were observed in June and levels decreased continuously thereafter.

Discussion

We have shown that the heterologous radioimmunoassay system developed by McNeilly & Friesen (1978) for the rabbit and suitable for a wide range of mammalian species (including the dog), can also be used to measure prolactin in the plasma of the blue fox. Increasing quantities of fox plasma or pituitary tissue from several varieties of fox gave dose-response curves that were parallel to the ovine or canine prolactin curves. The absence of cross-reactivity with gonadotrophins was demonstrated by injection of LHRH in the male and by ovariectomy of females. The resultant increase in LH did not affect prolactin levels. TRH or metoclopramide administration stimulated prolactin release as in other carnivores such as the dog (Knight, Hamilton & Scanes, 1977; Reimers, Phemister & Niswender, 1978) and the cat (Banks & Stabenfeldt, 1983). The response was greater in the female than in the male. A depressive effect of bromocriptine on prolactin levels

was not clearly demonstrated, possibly because studies with this compound were performed when basal prolactin concentrations are low (March). The absence of significant fluctuations of prolactin concentrations throughout these experiments (except in one male) shows that stress (handling and bleeding) does not seem to affect prolactin secretion as is the case in cattle (Raud, Kiddy & Odell, 1971).

Sensitivity, intra- and inter-assay variabilities and the accuracy of the prolactin assay were similar to those described for a similar assay (McNeilly & Friesen, 1978; McNeilly, Abbott, Lunn, Chambers & Hearn, 1981; Hinds & Tyndale-Biscoe, 1982).

Plasma prolactin concentrations in the female blue fox followed a circannual rhythm with peak values occurring in May and June in intact (pregnant and non-pregnant) and ovariectomized vixens. In contrast to other species (rat, sheep, mouse, hamster; see McNeilly (1980) for review), no consistent changes in circulating prolactin levels have been found around oestrus.

In the pregnant female, the prolactin increase coincided with the second part of gestation and lactation. Møller, Mondain-Monval, Smith & Scholler (1984b) have shown that, in the blue fox, plasma prolactin concentrations rise gradually from around 2 weeks after the LH peak until parturition while progesterone levels decline. Values were highest on the day preceding parturition and during the first 10 days of lactation. These results are in agreement with those reported by Concannon, Butler, Hansel, Knight & Hamilton (1978) for the bitch.

In the non-pregnant females the prolactin increase coincided with the middle of the luteal phase. Nevertheless, values were lower than in the pregnant female, in contrast to the findings of Reimers *et al.* (1978) for the dog.

The prolactin values recorded in ovariectomized females in May and June suggest that prolactin secretion is closely related to photoperiod. The fact that prolactin concentrations remained unchanged after ovariectomy and followed the same circannual changes as in intact females suggests that its regulation is independent of ovarian secretory feedback mechanisms. The prevailing photoperiod is probably an important factor in the regulation of prolactin secretion. Similar seasonal variations of prolactin have been described in females of wild species (white-tailed deer: Schulte *et al.*, 1980; red deer: Kelly, McNatty, Moore, Ross & Gibb, 1982; mink: Martinet, Ravault & Meunier, 1982) and also in domestic species (heifers: Koprowsky & Tucker, 1973; goats: Hart, 1975; ewes: Walton, McNeilly, McNeilly & Cunningham, 1977). Similar variations have been reported for the male red fox (Maurel, Lacroix & Boissin, 1984) and the male blue fox (Smith, Mondain-Monval, Møller, Scholler & Hansson, 1985). In the female blue fox, the decrease in prolactin in July is not followed by a resurgence of ovarian activity. The fall in prolactin precedes the onset of reproductive activity by several months in males and females. The role of prolactin therefore remains unclear. It is possible that in the blue fox prolactin has an anti-gonadotrophic effect during the transition to seasonal anoestrus, but changes in prolactin secretion are not necessary for the onset of normal ovarian function. It has been proposed that the changing prolactin concentrations in the ewe during the autumn are partly responsible for the initiation of the breeding season (Walton *et al.*, 1977). While hyperprolactinaemia may play a role in suppressing gonadotrophin secretion in the ewe, there is little evidence to support this suggestion (McNeilly & Land, 1979; Land, Carr, McNeilly & Preece, 1980; Worthy & Haresign, 1983). Our results show that in the blue fox LH remained low even with basal levels of prolactin and increased only in February–March without any concomitant changes in the plasma concentrations of prolactin.

As expected, females ovariectomized after the breeding season in June showed increased LH concentrations within 2 days of the operation and values remained high throughout the rest of the study. This suggests that the ovaries of the anoestrous vixen secrete oestrogens in sufficient quantities to suppress plasma LH concentrations as in the ewe (Bittman, Karsch & Hopkins, 1983; Worthy & Haresign, 1983).

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