The endocrinology of gestation failure in foxes (*Vulpes vulpes*)

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The endocrine basis of reproductive failure in red fox vixens was examined over two
breeding seasons in a total of 11 animals. Weekly blood samples were assayed for
progesterone, prolactin, LH and cortisol. Vaginal smears taken every 2 days over the
oestrous period indicated that all vixens had mated. Vixens that successfully gave birth to
a litter of cubs demonstrated significantly higher plasma progesterone and prolactin
concentrations but significantly lower cortisol concentrations than did females that had
ovulated, but then failed to whelp. There were no significant differences in plasma LH
concentrations. These data suggest that reproductive losses could result from lowered
plasma progesterone concentrations, possibly resulting from inadequate luteotrophic
support by prolactin. A stress-induced mechanism of reproductive failure is implicated and
is discussed in relation to social suppression of reproduction.

Introduction

The failure of vixens to conceive or reach full-term pregnancy
after conception is a widely recognized phenomenon both in
the wild red fox (*Vulpes vulpes*) (Harris and Smith, 1987;
Lindström, 1988, 1989) and in farmed red and blue fox (*Alopex
lagopus*) populations (Johansson, 1938; Wisnicky *et al.*, 1939;
Møller, 1973). Post-mortem studies have shown that up to 20%
of sexually mature females, whether wild or captive, lose their
fetuses either before or after implantation (Aamdal and
Fougner, 1973; Harris and Smith, 1987). Although the endo-
crinology of the fox reproductive cycle has been documented
and appears to be very similar to that of the dog (see
Mondain-Monval *et al.*, 1977, 1985; and Concannon, 1986
for reviews), only one study in foxes has examined the
endocrine basis of these observed reproductive failures (Møller,
1973).

This study compares the hormone profiles of mated red fox
vixens that failed to reproduce (referred to as non-breeders)
with vixens that successfully went to full term of pregnancy
(breeders), and provides evidence for a stress-related mech-
anism of reproductive failure.

Materials and Methods

Animals

During 1988 and 1989, a total of 18 (11 females and 7
males) orphaned fox cubs, obtained between the ages of 10

and 28 days, were hand-reared until maturity. Up to 21 days
of age, the cubs received a commercial milk puppy feed
(Sherley's 'Lactol': Ashe Consumer Products Ltd, Slough),
this being gradually substituted by a mixed diet of table
scraps, meat and water, *ad libitum*. All animals were handled
intensively to habituate them to human contact and manipu-
lation. At 14 weeks of age, the foxes were placed in adjacent
10 m × 40 m outdoor enclosures, located within a quiet,
high-walled garden within the vicinity of the city of Bristol
(latitude 51°25′). Each enclosure contained at least one con-
crete kennel with wooden sleeping areas, a small plywood
kennel and logs, branches and tyres to provide a source of
interest for the foxes. All foxes were kept in their natal
groups until the age of 6 months, when they were allocated
into one of seven breeding groups. This arrangement
was designed to mimic the observed situation in the wild,
in which foxes associate in family groups that typically con-
sist of one sexually mature male and one, or more, related
mature females (Hersteinsson and Macdonald, 1982;
Lindström, 1982; von Schantz, 1984). Thus, where possible,
sister or mother–daughter relationships were maintained, but
siblings of different sexes were separated.

The vulva of each vixen was examined visually and vaginal
smears taken every 2 days from January until oestrus and
mating, as determined by cellular changes in vaginal histology
and the presence of spermatozoa (Enders, 1939; Schutte, 1967;
Møller and Frøysedal, 1980) was detected. Weekly blood
samples were taken from either the cephalic or jugular vein
under mild sedation (acetyl promazine, 0.2 mg kg^{-1} body
weight, i.m.) between 14:00 and 16:00 h throughout the
breeding season. At collection, the blood was kept on ice until
centrifugation at 3200 *g* for 15 min, and the aspirated plasma
was stored at − 20°C until assayed.

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Progesterone radioimmunoassay

The assay protocol followed a standard method used in the Bristol laboratory (Follett and Potts, 1990) and used a mixed antisera (B287) raised in goats and in rabbits. The cross-reactivity with progesterone was 94%, with testosterone 9% and with oestradiol 11%. Mean extraction efficiency derived from 90 samples was 84%. The inter- and intra-assay coefficients of variation were 6.2% and 2.6%, respectively; nonspecific binding averaged 1.8%; and the assay sensitivity was 0.2 ng ml^{-1}.

Cortisol radioimmunoassay

The method used was very similar to that for progesterone. It used an anti-donkey cortisol-3-(*O*-carboxy-methyl) oxime–BSA conjugate (K4B6). Crossreactivity details supplied with the antibody were: cortisol, 100%; prednisolone, 38%; cortisone, 23%; and 11-deoxycortisol, 2.5%. Steroid extraction followed a similar procedure to that used for progesterone, except that evaporation of the organic solvent was performed under nitrogen to prevent oxidative breakdown of the cortisol molecule. Mean extraction efficiency calculated from 54 samples was 87%. After reconstituting in 120 µl of 0.05 mol phosphate buffered saline l^{-1} containing 0.1% gelatin (PBS), 10 µl was run in an assay containing 90 µl PBS, 50 µl antisera (1:6000 dilution in PBS) and 50 µl of [1,2,6,7-^{3}H]cortisol (TRK407: Amersham International plc; 8000 c.p.m. per assay tube). Standards and pools were run in triplicate, while unknown sample tubes were run in duplicate. Incubation with the charcoal–dextran mixture (10 :1) was for 15 min at room temperature, and the tubes centrifuged at 3500 *g* for 10 min. Inter- and intra-assay coefficients of variation were 13.7% and 3.1%, respectively. Mean nonspecific binding was 7.3% and the assay sensitivity was 0.5 ng ml^{-1}.

LH radioimmunoassay

LH was measured in a double-antibody system using the Niswender GDN-15 antibody raised against ovine LH (validated for the measurement of fox LH; Mondain-Monval *et al.*, 1984). Crossreactivity of the antibody at half-maximal (50%) binding compared with ovine LH was: canine LH (LER 1685-1), 35%; canine FSH (LER 1685-3A), 1.0%; and canine prolactin (6), 0.004%. Ovine LH (S23) was iodinated by the chloramine-T method (Greenwood *et al.*, 1963), and was also used as the standard. Assay protocol was a microprocedure commonly used for many protein hormone assays (Follett *et al.*, 1972). Half-maximal (50%) binding occurred at 0.39 ng ml^{-1} and the assay sensitivity was 0.03 ng ml^{-1}. Coefficients of inter- and intra-assay were 17.2% and 9.7%, respectively.

Prolactin radioimmunoassay

Prolactin was assayed according to the protocol described by Mondain-Monval *et al.* (1985) and used antisera 33-9. The sensitivity of the assay was 0.58 ng ml^{-1}, and the inter- and intra-assay coefficients of variation were 6.4% and 11.5%, respectively.

Statistical analysis

Analysis was performed on individual hormone profiles collected over two breeding seasons (except for cortisol where data from only one season were available). To examine differences between breeding and non-breeding females, and to test whether these differences were statistically significant, canonical variates (CV) analysis and multivariate analysis of variance were performed. These are powerful techniques that enable not just the significance but the form of the differences between the groups to be investigated. The procedure was as follows.

(1) The profiles were aligned in time by parturition for breeding vixens, i.e. ovulation (week 0) was considered to be 52 or 53 days before parturition (Mondain-Monval *et al.*, 1977). For non-breeding vixens, week 0 was the week immediately before the maximum rate of increase in the concentration of plasma progesterone. These times closely corresponded to visual estimates of oestrus and mating from vaginal smear data. Progesterone proved more reliable than LH as a method of hormone profile alignment, probably because it is not pulsatile in the early luteal phase (Clarke and Cummins, 1987).

(2) The dimensionality of the data was reduced by taking the first five principal components. These contain nearly all of the variance in the data, and so nearly all of the information. The remaining principal components can be regarded as noise.

(3) CV analysis was performed on the reduced data set (for each hormone separately). This gives the linear combination of the five principal components that is most different between the two groups. This is then converted back to an equation in the original time variables. Plots of the 'CV loadings', i.e. the coefficients of this equation, are given for each hormone. They are standardized so that the within groups variance is the same at each time point. This means that small but consistent differences between the groups are highlighted. Positive loadings indicate that breeders tend to have higher concentrations of the hormone at that time point, while negative loadings indicate that non-breeders exhibit higher hormone concentrations at the given time.

(4) The significance of the difference between the groups was tested in two ways. First, a multivariate analysis of variance was performed. This is analogous to the usual ANOVA, except that the correlation between time points is taken into account. The disadvantage of this technique is that it relies upon assumptions of multivariate normality and homogeneity of variance which are rarely exactly satisfied. The second test is to estimate the error rate from a discriminant analysis, which is an estimate of the probability of correctly classifying an animal as a breeder or non-breeder from its hormone profile. If this probability is greater than 0.5 then there must be a genuine difference between the groups. The 'leave-one-out' estimate of the error rate was used as it is unbiased. For a full discussion of these techniques see Krzanowski (1988).

Results

All vixens entered oestrus and mated between mid-January and the beginning of March during both breeding seasons. Oestrus
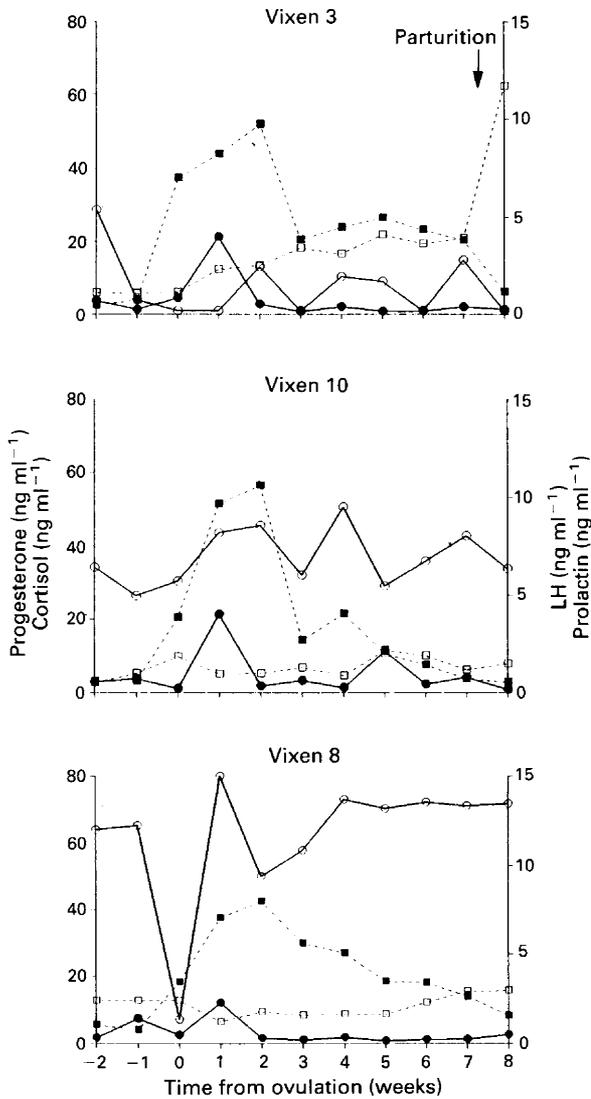
Fig. 1. Plasma hormone concentrations (ng ml^{-1}) (■, progesterone; □, prolactin; ●, LH; ○, cortisol) of three vixens throughout the period of oestrus and pregnancy/pseudopregnancy (fox gestation = 52/53 days). Vixen 3 successfully whelped, while vixen 10 and vixen 8 were both non-breeders. Vixen 10 exhibited a peak in progesterone typical of that exhibited by breeders, while vixen 8 exhibited a lower progesterone peak.

Fig. 2. (a) Mean (± SEM) concentrations of plasma progesterone for breeding (——) and non-breeding (– – –) vixens. (b) Canonical variates (CV) loading for progesterone ($n = 18$).

was detected over a 2 day or 4 day period during which time the vixen mated. The hormone profiles for three vixens, a breeder and two non-breeders, are shown (Fig. 1).

Progesterone

All breeding females exhibited a peak in progesterone concentration which exceeded 50 ng ml^{-1} within 3 weeks of the initial increase around ovulation. The non-breeding vixens demonstrated more variation in their progesterone profiles: while some exhibited a similar magnitude in progesterone concentration during the post-ovulatory rise (five animals), others barely attained concentrations above 40 ng ml^{-1} over
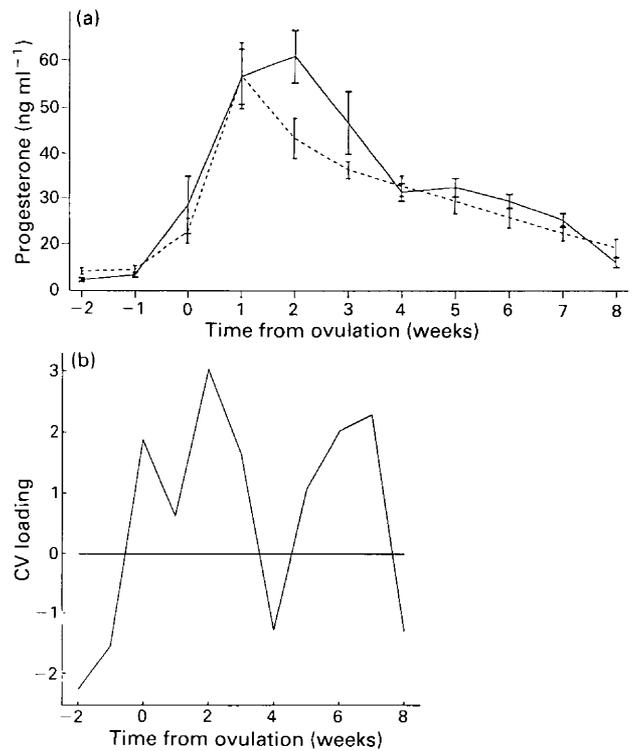
this same time period (five animals). All breeding females exhibited a secondary rise in progesterone 2–3 weeks after the initial post-ovulatory progesterone peak, but only some of the non-breeding females demonstrated this feature in their progesterone profiles. Concentrations of plasma progesterone then gradually declined in late pregnancy or pseudopregnancy.

Mean progesterone profiles for breeding and non-breeding females (Fig. 2a) show that breeding females exhibited greater concentrations throughout early and late pregnancy. The MANOVA gave a highly significant difference between the groups ($F = 5.12$, df = 5 and 12, $P < 0.01$). The estimate of correct classification was 90%, confirming the difference. The CV loadings (Fig. 2b) showed that the most important difference between the groups is that breeders tended to have a double peak around weeks 2 and 6 after ovulation, while non-breeders tended to exhibit slightly higher progesterone concentrations at the beginning and end of the period.

Prolactin

All breeding animals demonstrated a gradual rise in prolactin concentrations during pregnancy, which tended to peak around parturition. Non-breeding females exhibited a very gradual increase in prolactin, which occurred predominantly after the period indicated on the graph and which peaked during the summer anoestrus (Figs 1 and 3a). The MANOVA was highly significant ($F = 5.82$, df = 5 and 12, $P < 0.01$) and the estimate of correct classification was 83%. The CV loadings (Fig. 3b)
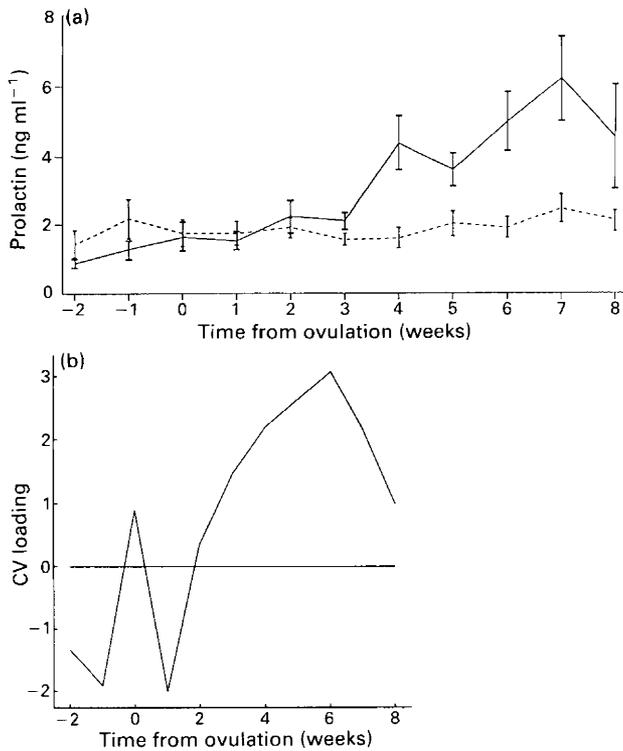
Fig. 3. (a) Mean (± SEM) concentrations of plasma prolactin for breeding (——) and non-breeding (– – –) vixens. (b) Canonical variates (CV) loading for prolactin ($n = 18$).
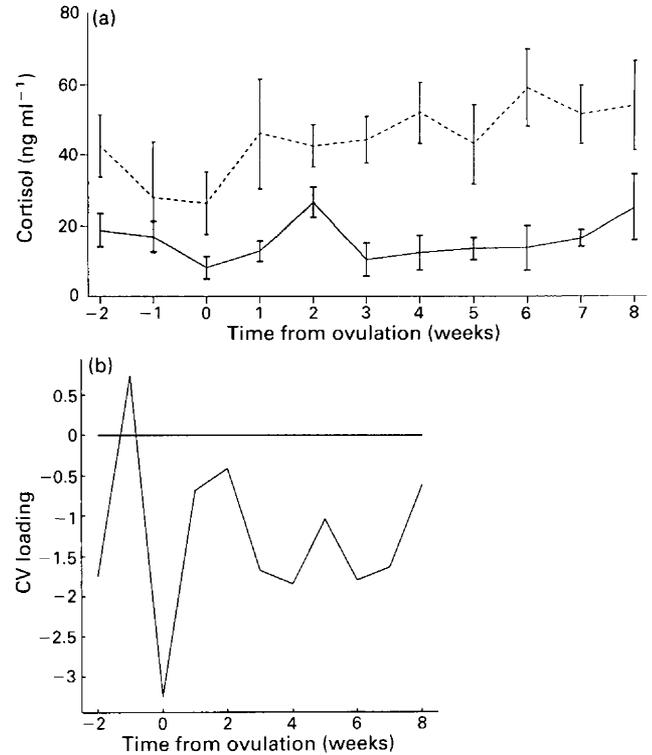

Fig. 5. (a) Mean (± SEM) plasma cortisol concentrations for breeding (——) and non-breeding (– – –) vixens. (b) Canonical variates (CV) loading for cortisol ($n = 10$).
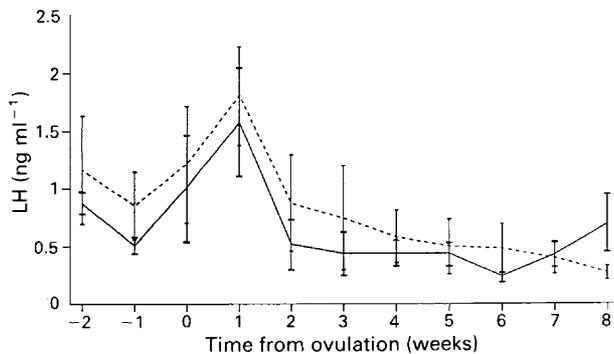

Fig. 4. Mean (± SEM) plasma LH concentrations for breeding (——) and non-breeding (– – –) vixens ($n = 18$).

confirm that the main difference between the groups was that breeders exhibited lower prolactin concentrations before week 1, but higher concentrations thereafter.

LH

The mean LH profile for breeders was slightly higher than that for non-breeders (Figs 1 and 4). The MANOVA was not significant ($F = 1.54$, df = 5 and 12, $P > 0.05$) and the estimate of correct classification was 50%, indicating no consistent difference between the groups.

Cortisol

Although there was considerable variation in cortisol concentrations both between and within individuals, non-breeding vixens generally exhibited higher cortisol concentrations throughout the breeding season (Figs 1 and 5a). The MANOVA was significant ($F = 6.70$, df = 5 and 4, $P < 0.05$) and the estimate of correct classification was 100%. The CV loadings confirmed that the main difference between the groups was the lower plasma cortisol concentrations of breeding vixens throughout the period indicated on the graph.

Discussion

In blue foxes, embryo mortality and abortion have been associated with diminished progesterone concentrations (see Møller, 1973). On commercial blue fox fur farms, where 15–20% of vixens may undergo mid- or late-term abortions (Fougner, 1972), the administration of chorionic gonadotrophins has been recommended to induce the production of an additional set of corpora lutea (Møller, 1972). In this study, non-breeding vixens exhibited significantly lower concentrations of plasma progesterone, suggesting that this may have been responsible for the observed failures. However, since Møller (1973) measured only mean maximal progesterone concentrations and used a visual discrimination of rates of decline of progesterone, no differences could be detected between pseudopregnant (non-breeding) and

pregnant vixens. The present study, however, used a more sophisticated statistical technique which clearly demonstrated that there were significant differences between the two groups of animals.

The CV loadings for progesterone revealed that breeding vixens tended to exhibit higher progesterone concentrations around weeks 2 and 6 after ovulation, which may indicate periods of susceptibility to reproductive failure. Implantation in red foxes occurs at day 12 after ovulation (Creed, 1972), coinciding with maximal progesterone concentrations (Møller, 1973; Bonnin *et al.*, 1978). Examination of individual hormone profiles revealed that all breeding vixens, but only 50% of non-breeding vixens, attained concentrations of greater than 40 ng progesterone ml^{-1} at either week 2 or 3 after ovulation. Thus, non-breeding females that exhibited a relatively small progesterone peak shortly after ovulation may have lost their blastocysts at about the time of implantation. Post-mortem studies have shown preimplantation losses to be a major cause of reproductive failure in wild foxes (Johansson, 1938; Layne and McKeon, 1956; Creed, 1972). In addition, in ewes, preimplantation embryonic mortality has been associated with lowered progesterone concentrations (Wilmut *et al.*, 1989; Kleeman *et al.*, 1991). Reproductive failure in up to 14% of wild red fox vixens has also been attributed to post-implantation losses (Lloyd, 1980; Lindström, 1982; Allen, 1984). Thus, a proportion of other non-breeding females may lose their fetuses after implantation, for instance, at about week 6 after ovulation.

Non-breeding animals were also characterized by their proportionally higher concentrations of plasma progesterone 2 weeks before ovulation. The change in the oestrogen:progesterone ratio is responsible for the onset of full oestrous behaviour in dogs and foxes (Concannon *et al.*, 1977; Wildt *et al.*, 1979; Møller *et al.*, 1980), which might suggest misalignment of behavioural with physiological oestrus in non-breeding females. However, changes in histology of the vagina, which are also indicative of the ovarian steroid ratio (Concannon, 1986), revealed that all vixens entered oestrus coincident with the rise in plasma progesterone.

Luteotrophic support in dogs, and probably in foxes, is provided by prolactin and LH (Mondain-Monval *et al.*, 1977; Rothchild, 1981; Concannon, 1986). Progesterone concentrations can be increased in pseudopregnant bitches by the administration of prolactin (Steinetz *et al.*, 1990). Thus, the increasingly marked differences in prolactin concentrations observed between breeding and non-breeding females from week 3 onwards indicate that non-breeding animals may have experienced inadequate luteotrophic support from prolactin during this time. However, during the preimplantation period there were no significant differences between the two groups of animals in either LH or prolactin concentrations, and the lower progesterone concentrations of the non-breeders at this time cannot be explained by luteotrophic factors alone.

A number of studies have reported a correlation between high cortisol concentrations and reproductive failure. Mice living at high densities tend to suffer high embryo mortality, associated with increased release of ACTH (Christian *et al.*, 1965; Christian, 1971). Injections of physiological doses of ACTH or a synthetic analogue of ACTH have been shown to induce degradation of corpora lutea in mice (Christian, 1967; Ogle, 1974a, b). Most pertinently, mid-pregnant bitches

injected with physiological doses of the synthetic cortisol analogue dexamethasone can abort or resorb their fetuses, coincident with an accelerated decline in plasma progesterone concentrations (Austad *et al.*, 1976). In this study, high cortisol concentrations, which were consistently observed in non-breeding females between weeks 3 and 7 after ovulation, may therefore have been mediary to stress-induced, reproductive failure. All vixens were tame and amenable to handling during the sampling procedure. Thus, there was no indication that cortisol concentrations were related to any stress associated with handling.

It is also possible that non-breeding vixens failed to ovulate, as it is recognized that vixens undergo pre-ovulatory luteinization and will secrete progesterone before ovulation. However, there was no evidence of significant differences in LH concentrations between non-breeding females at about the time of ovulation, and a strong body of evidence from post-mortem studies has suggested that red fox vixens are rarely anovulatory (Layne and McKeon, 1956; Creed, 1972; Lindström, 1982).

In this study, plasma hormone concentrations indicated that reproductive failure was associated with diminished progesterone concentrations, possibly the result of inadequate luteotrophic support by prolactin during the latter half of pregnancy. In all cases, a failure to reproduce was clearly associated with high plasma cortisol concentrations, pointing to a stress-related mechanism. Social suppression of reproduction is a widely recognized phenomenon in many social mammals (Christian and Davis, 1964; Wasser, 1983; Abbott, 1988), and studies of wild and captive foxes have observed that normally only one vixen from each social unit will successfully rear a litter each year (Macdonald, 1980; Lindström, 1982; von Schantz, 1984). In the present study, only one vixen from any social group whelped, although in some social groups, no vixens whelped. The nature of the captive enclosures would inevitably result in greater auditory, visual and olfactory contact between foxes than would occur in the wild, and it is likely that social stresses were high. However, behavioural studies on wild red fox populations also indicate that high levels of intergroup aggression are common during the breeding season and the results are likely to be comparable (White and Harris, in press). The failure of ovulated vixens to whelp, both in the wild and in captivity, may therefore be due to inadequate luteal production of progesterone.

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