Evidence for a circannual rhythm of reproduction and prolactin secretion in a seasonally breeding macropodid marsupial, the Bennett's wallaby (*Macropus rufogriseus rufogriseus*)

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Two groups of adult female wallabies were maintained in photoperiod-controlled rooms from June 1987 until August 1988. Group SSH was held on summer solstice photoperiods throughout the experiment; group SN was subjected to weekly stepwise simulated natural changes in photoperiod. Plasma melatonin concentrations reflected photoperiod with high concentrations during the dark phase in both groups. Group SN wallabies commenced oestrous cyclicity on 21 July (± 19 days, $n = 6$) entered reproductive quiescence on 14 February (± 10 days, $n = 5$) and recommenced cycling on 8 June (± 3 days, $n = 4$). Group SSH wallabies began cycling on 27 July (± 9 days, $n = 7$) at a time that was not significantly different from that of group SN. Three out of five of group SSH exhibited a spontaneous period of reproductive quiescence of between 59 and 70 days commencing between 3 December and 25 February. There was a highly significant difference between the transient plasma prolactin response to a dopamine antagonist during cycling and quiescent periods in both groups ($P < 0.001$) such that the response was increased during periods of quiescence. Our data support the hypothesis that prolactin is involved in the control of seasonal quiescence in the female Bennett's wallaby and demonstrate that spontaneous changes in reproductive state and prolactin can occur when animals are maintained on unchanging long photoperiods.

Introduction

In some temperate zone mammals that breed for two or more years, seasonality of reproduction appears to be regulated by an endogenous circannual rhythm of reproduction, cued by seasonal changes in photoperiod. This has been extensively documented in both domestic sheep and sciurid rodents; in these animals changes in reproductive state have been shown to occur in the face of an unchanging photoperiod signal (e.g. Howles *et al.*, 1982; Karsch *et al.*, 1989; and see Davis, 1976).

Among macropodid marsupials, the females of two species exhibit photoperiodically regulated seasonality of reproduction, the tammar (*Macropus eugenii*) and Bennett's wallaby (*Macropus rufogriseus rufogriseus*). In these species the breeding season, during which unmated females exhibit repeated oestrous cycles, commences shortly after the summer solstice and terminates 2–6 weeks after the winter solstice (Andrewartha and Barker, 1969; tammar; Catt, 1977; Fleming *et al.*, 1983; Curlewis *et al.*, 1986; Loudon and Brinklow, 1990; Bennett's wallaby). Timing of the onset and termination of the breeding season can be manipulated by artificial changes in photoperiod (Curlewis *et al.*, 1987; Curlewis and Loudon, 1989; Loudon and Curlewis, 1987; Brinklow and Loudon, 1989; Loudon and Curlewis, 1989).

Pituitary LH secretion is not directly seasonally modulated by oestradiol feedback in seasonally breeding wallabies, as it is

in eutherian mammals (Goodman and Karsch, 1981). Oestrous cycling is controlled primarily at the level of the ovarian corpus luteum (Hearn, 1974; Horn *et al.*, 1985) which is held in a quiescent state under the influence of suckling or season (Berger 1966; Catt 1977; Renfree 1979; Flint and Renfree, 1982; Curlewis *et al.*, 1987; Curlewis and Loudon, 1988a). The quiescent corpus luteum inhibits ovarian follicular development (Renfree *et al.*, 1982) and, as a result of low progesterone production, an unimplanted blastocyst is held in diapause (Berger and Sharman, 1969).

There is ample evidence that pituitary prolactin, under the inhibitory control of dopamine, is responsible for maintaining the corpus luteum in lactational quiescence (Tyndale Biscoe and Hinds 1984; Curlewis *et al.*, 1986; Curlewis and Loudon 1988a). The role of prolactin in the control of seasonal quiescence is less clear. Prolactin administration blocks luteal reactivation following reduction in photoperiod or hypophysectomy (Tyndale Biscoe and Hawkins, 1977; Brinklow and Loudon, 1989; Hinds, 1989). Administration of a single 5 mg kg^{-1} dose of the dopamine agonist bromocriptine terminates lactational quiescence (Tyndale Biscoe and Hinds 1984; Curlewis and Loudon 1988a). However, in early seasonal quiescence animals are less sensitive to bromocriptine, with only a proportion exhibiting luteal reactivation, and requiring extended treatment (Tyndale-Biscoe *et al.*, 1986). Hinds (1989) demonstrated that reactivation of the corpus luteum in seasonal quiescence after bromocriptine treatment occurred only in animals in which the morning

prolactin peak was suppressed. Later in seasonal quiescence bromocriptine administration does not cause luteal reactivation (Tyndale Biscoe and Hinds 1984; Curlewis *et al.*, 1986) even with extended treatment (Brinklow and Loudon, 1989).

The present study was designed to test the hypothesis that there is an endogenous circannual rhythm of prolactin secretion and reproduction in Bennett's wallaby which persists under constant photoperiod conditions.

Materials and Methods

Animals

Sexually mature female Bennett's wallabies were obtained from the Institute's breeding colony at Whipsnade Park, Bedfordshire, UK. The animals were housed in photoperiod-controlled light-proof rooms with access to an outside enclosure during the hours of daylight. Animals were maintained with *ad libitum* access to dried grass pellets, hay and water. Details of husbandry, collection of blood samples and levels of artificial illumination were as described by Curlewis *et al.* (1986) and Loudon and Curlewis (1987).

Experimental treatments

Two groups of animals were maintained as above from June 1987 until August 1988. Group SSH ($n = 7$) was held on summer solstice photoperiods from 18 June throughout the experiment (18.25 light: 5.75 dark; lights on at 03:00 and off at 21:15 GMT). Group SN ($n = 6$) was subjected to a stepwise simulated natural photoperiod changed weekly beginning on 22 June. Photoperiods were calculated using Greenwich Royal Observatory Tables and civil twilight was added to the duration of daylength as described by Loudon and Curlewis (1987). Animals exhibiting weight loss of greater than 4 kg were removed from the study.

Collection of blood samples and the prolactin response to domperidone

Blood samples were collected twice a week throughout the experiment for monitoring oestrous cycles by plasma progesterone determination, as described by Curlewis *et al.* (1986) and Brinklow and Loudon (1989). Plasma was separated after centrifugation (10 min at 1000 g) and stored at -20°C until assayed. Tonic concentrations of prolactin determined using the heterologous radioimmunoassay detailed below do not exhibit a seasonal change in non-lactating female wallabies. The dopaminergic control of prolactin secretion throughout the experiment was investigated at intervals of approximately 4 weeks by determining the response to 1 mg dopamine antagonist domperidone (Motilium: Janssens Pharmaceuticals, Grove, Oxon) as described by Brinklow and Loudon (1989) and Pearce-Kelly *et al.* (1992). This response was determined as the net increase in plasma prolactin 30 min after a subcutaneous domperidone injection corrected for any difference after a saline control injection in a crossover design, with treatments and controls two days apart. Animals within groups were

allocated equally and randomly to either domperidone or saline treatments for each determination.

Blood samples were taken for melatonin determination every 2 h for 24 h on 14 December 1987.

Hormone assays

Progesterone was determined in *n*-hexane extracts of plasma by the double antibody radioimmunoassay described by Curlewis *et al.* (1987). The limit of detection of the assay (zero minus 2 SD) was less than the lowest standard concentration of 20 pg ml^{-1} plasma. The intra-assay coefficients of variation were 4.8% and 6.7% at 188 and 708 pg ml^{-1} , respectively, and the interassay coefficients of variation were 13.6% and 11.2% at 254 and 486 pg ml^{-1} , respectively.

Plasma prolactin concentrations were measured using a double antibody radioimmunoassay with the guinea-pig anti-human serum (33-1-8) of McNeilly and Friesen (1978) validated for the wallaby by Curlewis *et al.* (1986). The samples were assayed against standard ovine prolactin (NIADDK PRL-S15) using iodinated tracer from the same batch. The limit of detection of the assay was less than the lowest standard concentration of 0.78 ng ml^{-1} plasma. The intra-assay coefficients of variation were 1.6 and 3.8% at 3.1 and 12.4 ng ml^{-1} , respectively, and the interassay coefficients of variation were 19.8 and 8.4% at 2.6 and 17.7 ng ml^{-1} , respectively.

Plasma melatonin concentrations were determined by direct radioimmunoassay using the method of Webley *et al.* (1985) validated for the wallaby by Curlewis and Loudon (1988b). The limit of detection of the assay was 5 pg ml^{-1} plasma. The intra-assay coefficients of variation were 19.5 and 4.1% at 9 and 23 pg ml^{-1} , respectively, and the interassay coefficients of variation were 21.5 and 16.3% at 7.5 and 25 pg ml^{-1} , respectively.

Statistical analysis

Two consecutive plasma progesterone concentrations of $>300\text{ pg ml}^{-1}$ were considered to be indicative of an active corpus luteum (Curlewis *et al.*, 1987). Animals were considered to be in quiescence if more than five consecutive samples prior to the beginning of a cycle had progesterone concentrations of $<300\text{ pg ml}^{-1}$.

The effects of treatment on prolactin response to domperidone were analysed by two-way analysis of variance (ANOVA) with repeated measures and comparisons within treatments by Duncan's multiple range test as previously described (Brinklow and Loudon, 1989) using SAS (Helwig and Council, 1979) which accounts for missing values. The comparison of oestrous cycling and prolactin response was, after confirmation of homogeneity of variances, analysed by multiple ANOVA using CSS (StatSoft Inc. Tulsa).

Results

All the animals in the experiment exhibited oestrous cyclicity by 11 August (as defined by a consistent rise in plasma progesterone concentration, Fig. 1). There was no significant difference in mean date of commencement of cycling between the

Fig. 1. Concentrations of progesterone in plasma of animals held on (a) simulated natural (SN) and (b) summer solstice (SSH) photoperiods throughout the study. Periods of reproductive quiescence are indicated by stippled areas. There are no data for wallaby GL47 between 21 October and 16 November owing to a period of clinical treatment.

groups (group SN 21 July $\pm$ 19 days ($n = 6$), mean duration of photoperiod 17.2 ± 0.3 h; group SSH 27 July $\pm$ 9 days ($n = 7$), mean duration of photoperiod 17.17 ± 0.06 h). One animal in group SN (GL47) was cycling at the commencement of sampling and this date (15 June) was taken as the start of her breeding season. No samples were taken from this animal between 21 October and 16 November due to a period of veterinary treatment.

In group SN, animals entered quiescence (defined as the end of the last luteal phase of the breeding season) on 14

Fig. 2. Mean ($\pm$ SEM) net prolactin response to domperidone after 30 min (hatched bars) in animals held on (a) simulated natural (SN) and (b) summer solstice hold (SSH) photoperiods; preinjection values (open bars). Columns with different letters indicate values which are significantly different within treatments at $P < 0.05$ (Duncan's multiple range test following one-way ANOVA for repeated measures). The period of the breeding season during which animals in group SN underwent repeated oestrous cycles is shown by the horizontal bar (hatched); n = number of animals in each group.

February $\pm$ 10 days ($n = 5$) at a photoperiod 11.1 ± 0.2 h and recommenced cycling on 8 June $\pm$ 3 days ($n = 4$) at a photoperiod of 18.1 ± 0.07 h. The interval between onset of periods of cycling was between 303 and 317 days. In group SSH, three animals exhibited a period of quiescence of between 59 and 70 days commencing between 3 December and 25 February (see Fig. 1). The interval between onset of periods of cycling in these animals was between 211 and 266 days.

Although all samples from each animal were estimated in the same assay, plasma progesterone concentrations during the second period of quiescence in the SSH group were higher than those during the first period of quiescence at the beginning of the experiment.

There was no significant effect of time of year or treatment on the preinjection prolactin concentrations (Fig. 2) as previously reported (Curlewis *et al.*, 1986). There was a highly significant effect of time of year on the overall prolactin response to domperidone ($f = 8.08$, $P < 0.0001$, $d.f. = 14$) with no interaction between time of year and treatment (Fig. 2). In group SN, high concentrations of prolactin in June and July declined between September and December, rose in January to a peak in April followed by a decline by the next July. In group

Table 1. Plasma prolactin responses (mean $\pm$ SEM) to 1 mg domperidone (s.c.) during periods of oestrous cyclicity and quiescence, overall, and in animals on simulated natural (SN) and summer solstice (SSH) photoperiods

Group	Plasma prolactin concentrations (ng ml ⁻¹)	<i>n</i>	Plasma prolactin concentrations (ng ml ⁻¹)	<i>n</i>	<i>P</i> for cycling versus quiescent
SN + SSH	5.3 $\pm$ 0.5	104	14.9 $\pm$ 0.9	50	<0.001
SN	5.7 $\pm$ 0.7	46	15.5 $\pm$ 1.1	26	<0.01
SSH	5.0 $\pm$ 0.7	58	14.1 $\pm$ 1.4	23	<0.01

n = Overall number of challenges in each group; *P* values relate to differences between prolactin responses during periods of cycling and quiescence.

Fig. 3. Mean ($\pm$ SEM) concentrations of melatonin in plasma of animals held on (a) simulated natural (SN; *n* = 5) and (b) summer solstice hold (SSH; *n* = 5) photoperiods taken every 2 h for 26 h on 16–17 December 1987. The period of darkness (SN 14 h 47 min; SSH 5 h 44 min) is shown by the hatched bar.

SSH, concentrations of prolactin declined in August and rose again from January to March. There was a highly significant difference between prolactin responses during cycling and quiescent periods both overall ($P < 0.001$) and within each treatment ($P < 0.01$), with an approximately threefold increase in response to domperidone during periods of quiescence (Table 1).

Plasma melatonin measured in blood samples taken every 2 h over 24 h demonstrated that melatonin secretion occurred

only during the periods of darkness. Animals maintained on fixed summer solstitial photoperiods exhibited a compressed melatonin profile (Fig. 3), with high concentrations in only two samples. This contrasts with the profile for group SN in which concentrations were high for at least 12 h.

Discussion

The commencement of the breeding season, approximately two months after the summer solstice, in both the simulated natural and summer solstice hold photoperiod groups is comparable with earlier studies in this species (Merchant and Calaby, 1981; Curlew and Loudon, 1989). This was accompanied in both cases by reductions in prolactin response to domperidone that occurred spontaneously in group SSH with no reduction in photoperiod. These data support earlier studies that demonstrated that the onset of the breeding season occurs owing to refractoriness to a long photoperiod signal (Curlew and Loudon, 1988a). In addition, data from the tammar wallaby show that pinealectomy in seasonal quiescence does not alter the time of onset of the breeding season after the summer solstice, despite an unchanging melatonin signal (McConnell and Hinds, 1985). Here, we provide evidence that reproductive long day photorefractoriness is associated with a decline in prolactin. Further evidence that a reduction in photoperiod is not necessary for onset of the breeding season comes from the observation that one animal (GL47) had begun cycling before the summer solstice. Finally, in group SN, the onset of the second breeding season and accompanying reduction in prolactin response occurred before the time of maximum photoperiod. An explanation for this foreshortening of the annual reproductive cycle might be that we consistently overestimated the duration of the simulated natural photoperiod by adding civil twilight and thus advanced the long day photorefractory response.

In common with previous data from both the tammar and Bennett's wallaby this study demonstrates no seasonal or photoperiod-induced changes in baseline plasma prolactin values. There was, in the present study, a highly significant correlation between the transient prolactin response to domperidone and oestrous cyclicity across the whole study

with a high response during periods of quiescence and a low response during periods of oestrous cyclicity. This result agrees with observations that the transition from the breeding season to seasonal quiescence is associated with an increase in prolactin secretion induced by a dopamine antagonist (Pearce-Kelly *et al.*, 1992). In addition, tammar have been shown to exhibit a transient increase in plasma prolactin concentration at the time of lights-on in artificial long photoperiods (McConnell *et al.*, 1986; Hinds, 1989). In a study of male Bennett's wallabies culled at different times of the year, Curlew (1989) found an increase in pituitary prolactin content in summer that could provide an increased releasable pool. Taken together, these data provide strong correlative evidence for a role for prolactin in the control of seasonal quiescence, particularly as the periods of quiescence in the SSH group were not synchronous, which is reflected in the lack of clear change in the mean prolactin response after January.

We cannot explain the inability of bromocriptine to reactivate the corpus luteum in late seasonal quiescence (Tyndale-Biscoe and Hinds, 1984; Curlew *et al.*, 1986; Brinklow and Loudon, 1989). One possibility is that prolactin is regulated by stimulatory factor(s) at this time, in addition to an underlying inhibitory dopaminergic control. This possibility is supported by data from Hinds (1989), who found that only two out of four tammar in early seasonal quiescence responded to bromocriptine with an abolition of the dawn pulse of prolactin, which is observed in this species, and luteal reactivation.

Three out of four animals maintained on fixed summer solstitial photoperiods throughout the study reported here exhibited spontaneous changes in oestrous cyclicity and prolactin response to domperidone. Furthermore, these changes occurred despite an unchanging melatonin signal. Blood samples from animals from group SSH at the time of the winter solstice, 6 months after exposure to constant long photoperiods, exhibited a short period of high concentration of melatonin in plasma similar to that observed in earlier studies of wallabies maintained on long photoperiods (Loudon and Curlew, 1987; Curlew and Loudon, 1988b). In contrast, animals maintained on simulated natural photoperiods exhibited a long period of high melatonin concentration also comparable to our earlier studies (Loudon and Curlew, 1987; Curlew and Loudon, 1988b). Thus, in response to an invariant long photoperiod signal, three of the animals exhibited spontaneous changes in reproductive state with a period substantially shorter than one year.

These data are in contrast to those from wallabies held on winter solstitial photoperiods for the duration of the normal seasonal quiescent period (Loudon and Curlew, 1989), from wallabies given melatonin implants that blocked the daily changing melatonin signal (Loudon and Brinklow, 1990), or pineal denervated by superior cervical ganglionectomy during the breeding season (tammar: Renfree *et al.*, 1981). In all three cases, animals continued to cycle throughout the normal period of quiescence when a decrease in the duration of the daily melatonin signal was prevented. These studies suggest that seasonal wallabies need to experience an increase in photoperiod and associated change in melatonin signal after the winter solstice to enter seasonal quiescence. At some point after the onset of seasonal quiescence, animals develop refractoriness to a long day signal and commence the next breeding season (see Curlew

and Loudon, 1989). Spontaneous changes in reproductive state and prolactin in seasonal wallabies can apparently be expressed only when animals are exposed to long photoperiods. These spontaneous changes may be taken as evidence for an endogenous circannual rhythm. Absence of spontaneous changes of reproductive state in animals held on short photoperiods or treated with melatonin (see above) suggests an absolute photoperiodic requirement for the expression of such rhythms. It must, however, be noted that all of these studies have been conducted over relatively short periods. These data are comparable to data from red deer in which endogenous rhythms of food intake, plasma prolactin concentration and coat growth are present in hinds held on summer solstice photoperiods from birth (Brinklow and Loudon, 1990), while adult hinds treated with melatonin from mid-winter continued to exhibit repeated oestrous cycles and did not enter anoestrus (Adam *et al.*, 1989). Sheep, in contrast, have been shown to exhibit endogenous circannual rhythms in both long and short photoperiods (Howles *et al.*, 1982; Karsch *et al.*, 1989).

In conclusion, we have demonstrated a clear association between periods when the prolactin response to a dopamine antagonist is low and periods of oestrous cyclicity in non-lactating female Bennett's wallabies. This study provides further evidence that prolactin is involved in the control of seasonal quiescence. In addition we have demonstrated that spontaneous changes in reproductive state and associated changes in prolactin secretion can occur in female wallabies on constant long photoperiods.

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