Effects of melatonin implants on plasma concentrations of testosterone, thyroxine and prolactin in the male silver fox 
(Vulpes vulpes)

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Summary. Melatonin administration in the form of slow-release implants advanced breeding activity in silver fox males when treatment began in June. Plasma testosterone concentrations were significantly higher in treated animals than in controls from September to November, whereas in February and March they were significantly lower. Plasma prolactin concentrations were significantly reduced immediately following melatonin treatment in June but increased to greater levels than control values and 'peaked' after 7 months. This 'peak' was associated with a rapid decrease in testosterone secretion. The normal seasonal spring rise in prolactin secretion was prevented by melatonin administration. Thyroxine values decreased and were significantly lower after 2 months of melatonin treatment.

Keywords: melatonin; reproduction; silver fox; male; prolactin; thyroxine; testosterone

Introduction

Survival and successful reproduction in animals are strongly dependent on ability to predict seasonal changes in the environment. The most reliable predictor of the seasons in the temperate and polar regions is the annual changes in daylength. The physiological mechanisms by which changes in daylength regulate hypothalamic–pituitary–gonadal function and dictate the seasonal pattern of hormone secretion (and ultimately fertility) are still poorly understood. There is strong evidence that the pineal gland is involved through its secretion of melatonin (for review, see Arendt, 1986; Reiter, 1987). Recent studies show that melatonin affects hypothalamic dopamine metabolism and alters pituitary responses to dopamine (Steger et al., 1984).

The role of prolactin in the reproductive cycle of the male is unclear. It has been suggested that prolactin may not only enhance the effect of LH on testosterone production but also act synergistically with testosterone to stimulate the growth and secretion of accessory sex organs (Matthews et al., 1978; Zipf et al., 1978). In most seasonally breeding species prolactin presents a clearly defined annual cycle with peak levels in spring and summer. Although the reason why secretion increases in summer is unknown, the hormone is known to have important seasonal effects on the moultng cycle (Allain et al., 1981; Lincoln & Ebling, 1985). The pineal also appears to mediate the effects of photoperiod on hormonal functions previously not considered directly associated with reproduction. Seasonal fluctuations in thyroid hormones seem to depend on photoperiod and to be involved in the regulation of reproductive function in mink and sheep (Jacquet et al., 1986; Nicholls et al., 1988).

Earlier experiments have shown that the reproductive cycle as well as the moultng cycle of the silver fox (Vulpes vulpes) male can be influenced by changes in photoperiod (Forsberg et al., 1989) and melatonin administration (Connor, 1988; Forsberg et al., 1990). Under natural daylight conditions plasma concentrations of testosterone and thyroxine show distinct seasonal variations
(Joffre & Joffre, 1975; Joffre, 1976; Maurel & Boissin, 1981), and prolactin secretion increases during and after the mating season (Maurel et al., 1984).

The aim of the present study was to determine how long-term melatonin treatment affects the secretion of testosterone, thyroxine and prolactin in the male silver fox.

**Materials and Methods**

*Animals and experimental design.* Ten adult silver fox males were housed individually under natural daylight conditions at the Swedish University of Agricultural Sciences, Uppsala (latitude: 59°50' N; longitude: 17°50' E) from March 1987 until the end of April 1988. The animals were given water *ad libitum* and fed a standard ration wet feed. Melatonin implants (Wildlife Laboratories Inc, Fort Collins, Colorado, USA) (10 mg: length 9 mm, width 2 mm, surface area 65 mm²) were placed beneath the skin over the left chest wall while the animals were anaesthetised with ketamine (10 mg/kg) (Ketalar: Parke-Davis, Raritan, NJ, USA; 50 mg/ml). Five males were given implants of 40 µg melatonin on 1 June 1987 (when the testes were fully regressed) and again on 4 August and 5 October 1987. All implants remained in place until the end of the experiment. The other 5 males served as controls.

**Blood sampling.** Blood samples were collected monthly from the cephalic vein between 11:00 and 15:00 h. The animals were sedated with 2 mg acepromazine/kg (Plegicil: Agrivet AB, Uppsala, Sweden; 10 mg/ml). All blood samples were collected in heparinized tubes. Plasma was immediately centrifuged and stored at −20°C until assayed.

**Hormone assays.** Plasma concentrations of testosterone, melatonin, thyroxine and prolactin were determined by radioimmunooassays. The assays for testosterone and melatonin have been previously validated for use in the silver fox (Forsberg et al., 1989, 1990).

The intra-assay coefficients of variation for testosterone, calculated from the precision profiles of 5 different assays, were below 13% in the range of 3-5 to 34-7 nmol/l. The inter-assay coefficients of variation (%) for quality control samples were 8.6 (mean = 9.2 nmol/l, n = 13), 8.0 (mean = 17.4 nmol/l, n = 13) and 8.6 (mean = 25.5 nmol/l, n = 11).

The intra-assay coefficient of variation for the melatonin assay was 20% at 84 pmol/l and remained < 15% for concentrations exceeding 168 pmol/l. The coefficients of variation (%) for two pooled plasma samples of silver fox origin, estimated in 5 different assays, were 15.5 (mean = 346 pmol/l) and 11.9 (mean = 649 pmol/l).

The system to measure thyroxine in fox plasma utilized 125I-labelled L-thyroxine (Du Pont de Nemours & Co., NEN Research Products, Boston, MA, USA) as tracer, L-thyroxine (Sigma Chemical Company, St Louis, MO, USA) as standard and thyroxine antiserum (Benenden Antiser A, RAST Allergy Unit, Cranbrook, UK) at a final dilution of 1:5000. Thyroxine standards were prepared in charcoal-stripped plasma obtained from hypothyroidic dogs. To inhibit thyroxine binding to plasma proteins 8-anilino-1-naphthalene sulphonic acid was added to standards and samples as described by Larsson & Lundms (1980). After a 2.5 h incubation at room temperature, 2 ml of a second antibody suspension (PharmaMac AB, Uppsala, Sweden) were added to each tube. After an additional 30 min at room temperature the tubes were centrifuged at 1500 g and radioactivity was quantified in a gamma counter (Searle Analytic Inc., IL, USA).

The thyroxine antiserum bound 31.0% (s.d. = 2.2, n = 50) of the 125I-labelled thyroxine, and the standard curve ranged from 2 to 128 nmol/l. Dilutions of silver fox plasma were parallel to the standard curve. The intra-assay coefficients of variation for thyroxine concentrations in the range of 8.2 to 128 nmol/l were below 10%. The inter-assay coefficients of variation (%) for quality control samples, estimated in 50 assays, were 12.4 (mean = 34-4 nmol/l) and 10.3 (mean = 73.5 nmol/l). The slope of the dose–response curve after logit-log transformation was −1.003 (s.d. = 0.067, n = 50).

The prolactin assay used an antiserum against dog prolactin (cPRL) (AFP-16111778) at a final dilution of 1:87 500, radioiodinated dog prolactin (AFP-2451B), and dog prolactin (NIADDK cPRL-RP-1, AFP-2451B) as standard (A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). Cross-reactivity with dog growth hormone was 0.04%. The iodination (carrier-free 125I, Amersham International plc, Amersham, Bucks, UK) was performed by the chloramine-T method (Greenwood et al., 1963), using 7.5 µg chloramine-T/2 µg protein and an exposure time of 20 sec. Overnight incubation was carried out at room temperature. The separation of free from antibody-bound hormone followed the procedure for thyroxine described above except that 0.5 ml of the second antibody was used.

The antiserum bound 49.4% (s.d. = 5.5, n = 4) of the 125I-labelled dog prolactin and the standard curve ranged from 0.05 to 3.2 ng. Dilutions of silver fox plasma from 100 µl to 12.5 µl were parallel to the dog prolactin standard.

The sensitivity of the assay, calculated from the precision profile, was 0.02 ng/tube (s.d. = 0.01), corresponding to 0.2 µg/l for 100 µl plasma samples. The amount of dog prolactin needed to cause 50% inhibition was 0.8 ng/tube (s.d. = 0.1). The intra-assay coefficient of variation for prolactin concentrations in the range of 1-8 to 40-6 µg/l was below 10%. The coefficients of variation (%) for three pooled plasma samples of silver fox origin, estimated in 3-5 different assays, were 30.0 (mean = 1.0 µg/l), 11.3 (mean = 9.7 µg/l) and 9.6 (mean = 25.9 µg/l). The slope of the dose–response curve in 4 assays was −0.899 (s.d. = 0.012) after logit-log transformation. When known amounts of dog prolactin were added to silver fox plasma the recovery of prolactin averaged 104-9% (s.d. = 13.2, n = 12).
Examination of fur. Fur development (maturity, pigmentation) was evaluated in August 1987. Grading was done without the grader knowing which group each animal belonged to.

Statistical methods. Statistical analysis of the data was carried out with the Statgraphics (STSC, Inc., Rockville, MD, USA) statistical package using analysis of variance and the LSD test. All statistical tests having a $P$ value of $<0.05$ were considered significant. Means are expressed as ± s.e.m.

Results

Melatonin

Serum concentrations of melatonin were variable in animals treated with melatonin implants and in their corresponding controls (Fig. 1a). In the control group melatonin concentrations were significantly elevated from January to April 1988. Mean melatonin values in animals bearing implants were significantly higher than controls between the first implantation in June 1987 and the end of the study (experimental group 917 ± 56 pmol/l, control group 120 ± 15 pmol/l).

![Graphs showing seasonal variations in plasma melatonin, testosterone, thyroxine, and prolactin](image-url)
Testosterone

The seasonal changes in plasma testosterone concentrations are depicted in Fig. 1(b). In the control animals plasma concentrations of testosterone decline significantly from March until April in 1987. Basal levels (0-3–2-4 nmol/l) were observed from April until November in 1987. Mean concentrations increased gradually from December 1987, reaching significant peak values (>6 nmol/l) during the breeding season in February and March 1988, and then declining significantly to basal levels in April.

In the melatonin-treated males mean plasma concentrations of testosterone increased gradually from June to reach a peak concentration (>6 nmol/l) in November 1987. Although there was great variation between males, concentrations remained high until January 1988 and then declined to basal levels in February 1988. Thereafter they remained low until the end of the study in April 1988. Peak values in November 1987 and January 1988 were significantly higher than values in June 1987 and April 1988.

In September, October and November 1987 plasma testosterone concentrations in melatonin-treated males were significantly higher than in controls, whereas in February and March 1988 they were significantly lower.

Thyroxine

The seasonal changes in plasma thyroxine concentrations are shown in Fig. 1(c). In control animals thyroxine levels increased during spring and early summer 1987 to reach significant peak values (>40 nmol/l) in July, August and September. Values decreased gradually during the autumn and winter to a significant nadir (<25 nmol/l) in January and February 1988 before increasing again in March and April.

In the treated males thyroxine concentrations decreased between June and August 1987. During the rest of the study concentrations were variable. The lowest values were recorded in February and April 1988 (<28 nmol/l). Mean plasma concentrations in June and July 1987 were significantly higher than in February and April 1988.

In August 1987 plasma thyroxine values were significantly lower in melatonin-treated males than in control animals.

Prolactin

The seasonal changes in plasma prolactin concentrations are shown in Fig. 1(d). In control animals the clearly marked annual prolactin cycle showed a maximum (>30 µg/l) during spring/early summer and a minimum (<1-5 µg/l) during autumn/early winter.

In the melatonin-treated males plasma concentrations decreased significantly in June 1988 and remained low from July until September. Beginning in October, values gradually increased to a peak (>9 µg/l) in January 1988 and then declined until the end of the study in April.

From July until September 1987 and in April 1988 plasma concentrations of prolactin were significantly lower in melatonin-treated males than in control males. Between November 1987 and January 1988 values were significantly higher.

Moulting cycle

All treated males had completed the autumn moult and started to grow a winter coat when examined in August 1987, whereas control animals still had their summer coat.

Discussion

This study shows that administration of melatonin to silver fox males during long days influenced secretion of testosterone, prolactin and thyroxine in the same way as decreasing photoperiod does.
Melatonin on testosterone, thyroxine and prolactin in male silver foxes (Joffre & Joffre, 1975; Joffre, 1976; Maurel & Boissin, 1981; Maurel et al., 1984; Forsberg et al., 1989). These findings strengthen the hypothesis that melatonin is the hormonal signal transducing photoperiodic information in the silver fox, as described for other species.

The effects of constant exposure to high melatonin concentrations on the reproductive cycle result in an initial period of rapid testicular growth followed by involution (Forsberg et al., 1990), in the present study registered as a time shift in peak testosterone secretion.

Measurements of melatonin illustrated that the implants were capable of maintaining plasma levels at 600–1200 pmol/l during the experiment. However, it is not known whether these concentrations constitute supraphysiological levels, because the 24-h endogenous melatonin profile in the silver fox has yet to be established. The treated and control animals showed strange melatonin patterns during the last 4 months of the study. A shift to higher levels was observed in untreated males. In the treated animals, in which a gradual decrease in melatonin concentrations had been expected after the implantation of melatonin in October, values remained high. We have no obvious explanation for the melatonin patterns observed during this period but the samples have been reassayed several times with the same result. They may reflect an increase in the endogenous secretion and/or a change in the metabolic clearance of the hormone. Such an increase or change would indicate that the constant supply of melatonin from the implants did not block the endogenous secretion of melatonin. Lack of inhibitory feedback effect of exogenous melatonin has been observed in the blue fox (A. J. Smith, personal communication) and the sheep (Kennaway, 1982; Lincoln & Ebling, 1985).

In the present study melatonin was found to influence secretion of thyroxine and testosterone: thyroxine levels declined and testosterone levels increased during the period of testicular development. Maurel & Boissin (1981) also reported a negative association between thyroxine and testosterone secretion in the red fox and suggested that interactions may occur between the annual reproductive and thyroid cycles. In the mink, thyroidectomy stimulated testosterone production and prolonged the period of testicular activity, indicating an inhibitory thyroid–gonadal interaction (Jacquet et al., 1986). Nicholls et al. (1988) suggested that thyroxine is essential in the process controlling reproductive quiescence in sheep and European starlings. There are also reports supporting the view that the pineal gland, via melatonin, has a modulatory effect on both gonadal and thyroid functions. Injections of melatonin for periods of 3–10 weeks reduced plasma thyroxine and TSH concentrations in hamsters, with the effects on gonadal and thyroid function being observed simultaneously (Vriend et al., 1979, 1982; Vriend, 1983). Creighton & Rudeen (1988) observed that the effects of melatonin on the pituitary–gonadal and pituitary–thyroid axes were independently mediated via the central nervous system. In the hamster, however, the action of melatonin depends on its frequency and timing of administration; maintaining high levels of melatonin continuously by using high-dose injections or implants did not inhibit thyroxine or TSH production (Vriend et al., 1982; Vaughan et al., 1984).

In the present study, melatonin implants blocked the effects of long days, suppressed plasma prolactin concentrations and induced an earlier autumn moult. Experimental work in mink illustrates that a decrease in prolactin secretion plays a central role in controlling the autumn moult (Allain et al., 1981; Martinet et al., 1984; Rose et al., 1985, 1987). Likewise, depressed plasma concentrations of prolactin delay the spring moult in both mink and blue fox (Allain et al., 1981; Smith et al., 1987a, b), whereas prolactin injections induce an early spring moult in mink (Martinet et al., 1981).

Although the annual prolactin pattern shows clear correlations with the breeding season within a species, there is no such association between species. In the silver fox, blue fox and mink, prolactin rises during the breeding season (Martinet et al., 1981; Maurel et al., 1984; Smith et al., 1985), whereas in the sheep, goat, red deer and white-tailed deer it falls (Pelletier, 1973; Ravault, 1976; Mirarchi et al., 1978; Muduuli et al., 1979; Suttie et al., 1984). It therefore appears as if there is no fixed relationship between the timing of the breeding season and the seasonal prolactin cycle. Prolactin can therefore have a stimulatory or an inhibitory effect, depending on the species. The
lack of synchrony may be related to different control systems for prolactin and gonadotrophic hormones. Prolactin secretion is under inhibitory control of the hypothalamus and is mainly mediated by dopamine (Nelson, 1987); in contrast, the stimulatory systems for LH and FSH are mediated by GnRH.

Although initially depressed, prolactin concentrations increased in the treated males and 'peaked' after 7 months of melatonin treatment. A similar pattern in prolactin secretion was observed in the ewe after melatonin treatment (Poulton et al., 1987). This 'peak' was followed by a rapid decrease in testosterone secretion. Prolactin is believed to modify the sensitivity of the hypothalamic–pituitary axis to testosterone feedback in rats and golden hamsters (McNeilly et al., 1978; Bartke et al., 1984; Matt et al., 1984). Smith et al. (1987c) and Mondain-Monval et al. (1988) observed a decline in LH concentrations in the blue fox when plasma concentrations of testosterone were maximal and prolactin was increasing. On the other hand, Worthy & Haresolver (1983) reported that seasonal anoestrus occurred in the absence of high levels of prolactin in ewes on a short-day lighting regimen. It is unclear whether the decline in testosterone in the treated males in this study reflects a prolactin-mediated change in sensitivity of the hypothalamic–pituitary system to the negative feedback effects of testosterone. If this is the case, then prolactin may be involved in regulating the normal breeding season in this species.

Since TRH releases prolactin as well as TSH, it is also possible that thyroxine is involved in prolactin regulation through changes in hypothalamic sensitivity to thyroxine feedback (De Léan et al., 1977; Friend & Ralcewicz, 1987). Ottenweller & Hedge (1981) reported that thyroid hormones altered the daily patterns of plasma prolactin in rats. Jacquet et al. (1986) observed that thyroidectomy did not modify the general pattern of prolactin secretion in the mink, but prolactin secretion was enhanced by increasing daylength in the absence of thyroid hormones. Although major changes in prolactin secretion occur in silver fox males under natural daylight conditions and after melatonin administration, a relationship between these changes and thyroxine has yet to be established.

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References


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