Effects of bilateral ganglionectomy and melatonin replacement on seasonal rhythm of testicular activity in Zembra Island wild rabbits (*Oryctolagus cuniculus*)

M. M. Ben Saad¹ and D. Maurel^{2*}

¹Laboratoire de Physiologie Animale, Faculté des Sciences de Tunis, Campus Universitaire, 1060 Tunis, Tunisie; and ²Pathologie de l'Oreille interne et Réhabilitation, EPI 9902 INSERM, Faculté de Médecine Nord, Boulevard Pierre-Dramard, 13916 Marseille cedex 20, France

This study examined the effects of subcutaneous melatonin implants on testicular activity (testis volume and plasma testosterone) in wild rabbits (*Oryctolagus cuniculus*) captured on Zembra Island (North Tunisia) and maintained in natural temperature and photoperiod conditions. The period of testicular activity was 2 months longer in intact animals with melatonin implants than in intact animals without melatonin implants. After bilateral superior cervical ganglionectomy, testicular activity was inhibited and no spontaneous recrudescence in gonadal function was observed during the 13 months of the experiment, demon-

strating the absence of an endogenous circannual rhythm of reproductive function in this species. Renewed testicular activity was observed 2 months after the insertion of melatonin implants in ganglionectomized animals. These results confirm that testicular activity is stimulated by short days in late autumn and that melatonin reactivates the reproductive axis in this species. This finding is in contrast to that in continental populations of wild rabbits in which reproduction is inhibited by short days or melatonin. These results are discussed in terms of insularity and may reflect the geographical isolation of this population.

Introduction

In a previous study on the population of wild rabbits (*Oryctolagus cuniculus*) on Zembra Island, it was shown that testicular activity begins in late autumn (October–November) in short-day conditions (Ben Saad and Baylé, 1985) and that the seasonal increase in testicular activity is prevented by superior cervical ganglionectomy (Ben Saad, 1997). In other short-day mammals, such as sheep (Kennaway, 1984) and mink (Ravault *et al.*, 1986; Maurel *et al.*, 1992a), ganglionectomy, resulting in complete abolition of the diurnal melatonin rhythm, is known to inhibit the seasonal reactivation of testicular activity by short days. It is now well established that melatonin, a 5-methoxyindole synthesized by the pineal gland, plays a major role in the photoperiodic control of reproduction (that is, control of testicular function throughout the year in male mammals), showing seasonality in relation to day length in the natural environment (reviewed in Reiter, 1974, 1980; Arendt, 1986; Arendt *et al.*, 1988, Goldman, 1999). As first suggested by Hoffmann (1981), melatonin is a hormonal mediator of photoperiodic effects. It is secreted nocturnally and it is now accepted that circulating melatonin concentrations, regulated by the alternation of light and dark periods, constitute an endocrine message

providing information about daily and seasonal changes in photoperiod (Goldman and Darrow, 1983; Reiter, 1986, 1988; Goldman, 1999). The morphological and functional chain linking the retina to the pineal gland has not been completely elucidated. However, it is known that information about the photoperiod is transferred from the retina to the suprachiasmatic nucleus via the retinohypothalamic tract, and reaches the pineal gland through noradrenergic fibres via the superior cervical ganglion. Experiments involving pinealectomy or deafferentation of the pineal body by bilateral superior cervical ganglionectomy have shown that the pineal gland and melatonin play primary roles in the photoregulation of reproductive activity in various mammals (Reiter, 1972; Stetson and Watson-Withmyre, 1976; Lincoln, 1979; Carter *et al.*, 1982; Maeda *et al.*, 1985; Boissin-Agasse *et al.*, 1988; Maurel *et al.*, 1990). In long-day species, the seasonal recrudescence of sexual activity occurs when day length increases; in short-day species, the shortening of the day length triggers the reproductive season. However, in both short- and long-day species, the melatonin signal corresponding to short days (increase in duration of high melatonin concentrations during the night) is the primary photoperiodic information controlling: (i) gonad atrophy in long-day species, in which renewal occurs spontaneously, or (ii) gonad renewal in short-day species, in which sexual regression is not driven directly by photoperiod (Reiter, 1986; Boissin and Canguilhem, 1988). The present study investigated the

*Correspondence

Email: maurel.d@jean-roche.univ-mrs.fr

effects of subcutaneous melatonin pellets in intact or ganglionectomized males maintained in natural temperature and photoperiod conditions. The aim was to determine the role of melatonin in the control of reproduction in this population of wild rabbits on Zembra Island, which is considered to be a short-day species. Testicular activity was assessed by determining testis volume and plasma testosterone.

Materials and Methods

Animals

The animals were trapped on Zembra Island (30°50'N, 10°14'E) in the north of Tunisia. All the animals used in this study were adult males in good condition. The experiments were performed in accordance with the European Community Council Directive (86/609/EEC). Once caught, the animals were transferred to the laboratory where they were caged individually and exposed to natural temperature and photoperiod conditions for the duration of the experiment. The animals were fed an appropriate diet (rabbit chow, SNA Tunisie) and given water *ad libitum*.

Experimental schedule

Twenty adult male rabbits (*Oryctolagus cuniculus*) were used in this study (mean body weight, 1170 ± 60 g). The animals were randomly assigned to four treatment groups, each containing five animals: (1) intact; (2) intact with subcutaneous melatonin implants; (3) ganglionectomized; and (4) ganglionectomized with subcutaneous melatonin implants. The four groups were studied over 13 months.

Surgery and melatonin replacement

Bilateral ablation of the superior cervical ganglion was conducted under deep sodium pentobarbital anaesthesia (0.4 ml kg^{-1} body weight) between 15 June and 15 July, during the period of annual testicular quiescence.

Melatonin treatment was started in July in intact animals (group 2) and in December in ganglionectomized animals (group 4). The implants consisted of Silastic tubes (Dow Corning, medical grade, external diameter 1.96 mm, interior diameter 1.47 mm, length 50 mm) filled with 50 mg melatonin (*N*-acetyl 1-5-methoxytryptamine; Sigma Chemicals, St Louis, MO) and plugged with special glue (medical silicone, type A; Dow Corning, Midland, MI). Two tubes per animal were implanted s.c. in the dorsal region near the clavicle.

Measurement of testicular activity

Two variables were assessed for evaluation of testicular activity: testis volume (cm^3), indicating spermatogenetic activity, and plasma testosterone concentration (ng ml^{-1}). The dimensions of the testis, length (*L*), width (*W*) and thickness (*T*), were measured to the nearest 0.1 mm with callipers through the scrotum, and the formula $V = 4/3 \times \pi \times L/2 \times W/2 \times T/2$ was used to calculate testis volume (Setchell and Waites, 1964).

Testes were measured and blood was collected from the ear marginal vein between 09:00 and 11:00 h one day per month. The blood was centrifuged for 15 min at 1500 g and the plasma was stored at -20°C until assayed.

Plasma testosterone was determined by radioimmunoassay using a commercial kit (Spectria Direct Testosterone, Orion, Espoo). Each plasma sample ($50 \mu\text{l}$) was assayed in duplicate. Sensitivity, defined as the smallest quantity of hormone detectable, was 80 pg ml^{-1} . The intra- and interassay coefficients of variation were 6.5% ($n = 15$) and 7.8% ($n = 5$), respectively.

Statistical analysis

Monthly mean values are presented $\pm$ SEM. Within a given group, data were analysed by one-way ANOVA and Fisher's paired least significant difference (PLSD) test. A two-way ANOVA with repeated measures followed by a post-hoc Fisher's PLSD test were applied to compare groups. Differences between monthly values for the same group or between groups at a given time point were considered statistically significant if $P < 0.05$.

Results

Testicular activity in control animals and animals with melatonin implants

Control animals (no implants, no surgery; Fig. 1) had no testicular activity at the start of the experiment (July). The first signs of testicular activity were detected in September–October when testis volume (Fig. 1a) and plasma testosterone (Fig. 1b) increased significantly (plasma testosterone: October $2.41 \pm 0.30 \text{ ng ml}^{-1}$ versus August $1.21 \pm 0.17 \text{ ng ml}^{-1}$, $P < 0.05$). Testis volume and plasma testosterone peaked in November–December (plasma testosterone concentration $8.42 \pm 0.82 \text{ ng ml}^{-1}$) and decreased markedly in January, reaching values indicating inactivity from February ($1.87 \pm 0.14 \text{ ng ml}^{-1}$). Testis volume did not reach a minimum until March, but the annual cycle was similar.

Melatonin implants were inserted in July, during the period of sexual quiescence. Testicular activity began at the same time (September–October) in animals with implants as in those of the control group, but maximum values were recorded from December until March for animals with implants; and testicular activity reached a minimum in April (plasma testosterone: March $4.30 \pm 0.29 \text{ ng ml}^{-1}$ versus April $1.23 \pm 0.12 \text{ ng ml}^{-1}$, $P < 0.001$), 2 months later than in the control group. Plasma testosterone concentrations were significantly higher ($P < 0.001$) in animals with melatonin implants than in controls from January to March and testis volume was greater ($P < 0.001$) in animals with implants from February to April.

Testicular activity in ganglionectomized animals without and with melatonin implants

In ganglionectomized animals without melatonin implants (Fig. 2), no seasonal variation in testicular activity

Fig. 1. Effects of subcutaneous melatonin implants on testicular activity in intact wild rabbits with (○) or without (control, ●) melatonin implants. The arrow indicates the time of melatonin implantation. ** $P < 0.01$, *** $P < 0.001$, animals with melatonin implants versus controls, for the same month.

was observed during the experiment. The annual mean for plasma testosterone was 1.60 ± 0.4 ng ml⁻¹ (minimum value: 1.45 ± 0.22 ng ml⁻¹; maximum value: 1.79 ± 0.24 ng ml⁻¹) (Fig. 2a) and for testis volume was 0.54 ± 0.02 cm³ (minimum value: 0.46 ± 0.05 cm³; maximum value: 0.62 ± 0.10 cm³) (Fig. 2b), corresponding to testicular inactivity. Melatonin implants were inserted in December (when testicular activity was at a maximum in the control group). Testicular function was reactivated 2 months after melatonin implantation in ganglionectomized animals receiving implants, reaching a maximum in March and April (plasma testosterone: March 4.67 ± 0.36 ng ml⁻¹; April 3.46 ± 0.31 ng ml⁻¹) and decreasing significantly in May (1.65 ± 0.24 ng ml⁻¹, $P < 0.001$). As in intact animals with melatonin implants, plasma testosterone decreased more rapidly than testis volume in ganglionectomized animals with melatonin implants. The testis volume of ganglionectomized animals with melatonin implants reached a minimum in July (0.85 ± 0.10 cm³) and was 2.51 ± 0.19 cm³ in May and 2.02 ± 0.18 cm³ in June (June versus July, $P < 0.001$).

Fig. 2. Long-term effects of ganglionectomy and subcutaneous melatonin implants on testicular activity in wild rabbits: controls (●); ganglionectomized animals (○); ganglionectomized animals with melatonin implants (▼). The arrow indicates the time of melatonin implantation. *** $P < 0.001$, ganglionectomized animals with melatonin implants versus ganglionectomized animals without implants, for the same month.

Discussion

The results of this study confirm that in wild rabbits from Zembra Island reared under natural temperature and photoperiod conditions, testicular activity is seasonal and increases in autumn, as demonstrated for this population by Ben Saad and Baylé (1985). Experimental exposure to short days (8 h light:16 h dark) at the beginning of summer induces reactivation of gonadal activity in this species (Ben Saad, 1997), demonstrating that this island population of wild rabbits is a short-day species. After bilateral superior cervical ganglionectomy, gonadal activity was not stimulated by the natural shortening of day length after the autumn equinox and no testicular activity was observed during the 13 months of the experiment, as shown in this species after optic nerve section. The lack of a testicular cycle under constant photoperiod conditions (Ben Saad, 1998) or when the seasonal melatonin signal was abolished for more than 1 year (present study) demonstrates that in this species the seasonal rhythm in reproduction is

not self-sustained. Since Pengelley and Fisher (1957, 1963) first provided evidence of circannual rhythms in body weight, hibernation and daily food consumption in a mammal, the golden-mantled ground squirrel, numerous studies have provided evidence for circannual rhythms in body weight, locomotor activity, hibernation, moulting, food consumption and reproduction in birds and mammals (reviewed in Gwinner, 1986; Boissin and Canguilhem, 1988). In stable conditions (constant light or darkness, pinealectomy or ganglionectomy, constant short-day or long-day regimen), some species show a true circannual rhythm that may persist over many years. These endogenous annual rhythms, demonstrated in various species including ground squirrels (Pengelley and Fisher, 1963; Zucker *et al.*, 1983), ferrets (Boissin-Agasse *et al.*, 1985), sheep (Karsch *et al.*, 1991) and European hamsters (Masson-Pévet *et al.*, 1994; Saboureaux *et al.*, 1999), have been called type II annual rhythms. Rhythms that are not self-sustained have also been described and are called type I annual rhythms (Zucker *et al.*, 1991; Gorman and Zucker, 1998). The wild rabbits of Zembra Island show a type I annual rhythm, similar to that demonstrated for other species such as Siberian and Syrian hamsters, spotted skunk, white-footed mice (reviewed in Zucker *et al.*, 1991) and mink, another short-day species studied by our group, after superior cervical ganglionectomy, suprachiasmatic nucleus lesion or pinealectomy (Boissin-Agasse *et al.*, 1988; Maurel *et al.*, 1991, 1992b).

The lack of testicular stimulation and the reactivation of gonadal function after subcutaneous melatonin implantation in ganglionectomized rabbits and the greater duration of testicular activity in intact animals with melatonin implants (as also observed in another short-day mammal, mink; Maurel *et al.*, 1992b) confirm that the pineal gland and melatonin are involved in the seasonal control of reproduction in this species. It was not possible to determine plasma melatonin or urinary 6-sulfatoxymelatonin concentrations in this species in these conditions. However, similar implants used in similar conditions in mink after superior cervical ganglionectomy (three implants per animal, mean body weight 1500 g) (Maurel *et al.*, 1992a,b, 1997) and in rats after pinealectomy (one implant per animal, mean body weight 500 g) (Kosa *et al.*, in press) gave plasma melatonin (rats) and urinary 6-sulfatoxymelatonin (mink) concentrations similar to the highest nocturnal values measured in control animals. These high concentrations remained stable for more than 3 months in mink and 1 month in rats. It has been shown that the continuous release of melatonin by subcutaneous implants or intrahypothalamic microimplants, without daily rhythmicity (as achieved with an osmotic pump or daily injection or ingestion of melatonin), induces gonadal stimulation, as observed in intact animals reared under natural or experimental photoperiodic stimulation. This stimulatory effect has been measured not only at the testis (testis volume, plasma testosterone) but by the reinduction of pulsatile secretion of LH in various animals such as rams (Webster *et al.*, 1991), fallow deer (Asher and

Peterson, 1991), ewes (Malpoux *et al.*, 1993, 1997) and mink (Maurel *et al.*, 1997). The most important characteristic of the melatonin signal is not circadian rhythmicity but the duration of nightly release (that is, the peak duration of melatonin), resulting in internal coincidence between sustained high concentrations of melatonin and an internal rhythm of sensitivity of the gonadotrophic axis to melatonin (Arendt *et al.*, 1987; Bonnefond *et al.*, 1990; Karp *et al.*, 1991) through diurnal variation in melatonin binding sites. Indeed, a diurnal rhythm in melatonin binding sites has been demonstrated in the suprachiasmatic nucleus and the pars tuberalis (Laitinen *et al.*, 1989; Gauer *et al.*, 1993). Melatonin receptors may be modulated by melatonin (Gauer *et al.*, 1993), but light exposure in pinealectomized rats during the dark period induces an increase in melatonin receptor density in the suprachiasmatic nucleus through serotonergic modulation (Masson-Pévet *et al.*, 1996; Recio *et al.*, 1996).

Melatonin implants triggered testicular activity in ganglionectomized rabbits but did not cause seasonal advance in intact rabbits because the triggering of testicular activity was similar in intact rabbits with implants and in controls. Thus, significant testicular reactivation was observed in October, 3 months after melatonin implantation (July). A similar latency in testicular activity (testis volume and plasma testosterone concentration) has also been observed in mink, in which significant testicular reactivation is observed after 3 months, whereas hypothalamic GnRH concentrations increase significantly after only 2 weeks and LH pulsatility parameters 1 month after melatonin implantation in ganglionectomized animals (Maurel *et al.*, 1997). Such a cascade of events, with latencies between the different steps of the GnRH axis (increase in hypothalamic LHRH, stimulation of pituitary LH release, renewal of testicular activity) as demonstrated in mink, may account for the 3 month delay in testicular activation after subcutaneous melatonin implantation in these species. Melatonin replacement in spring (April–May) rather than summer (July) might have led, with a similar delay in reactivation, to an earlier start to the breeding season. Such an experiment was not performed and in the conditions used in this study no such advance in the breeding period was demonstrated. However, the possibility that such an advance might occur in other conditions cannot be ruled out.

In rabbits with melatonin implants, the duration of the breeding season was longer in intact than in ganglionectomized animals: 1 month on the basis of testis volume and 3 months on the basis of plasma testosterone concentrations. This may be due to the timing of melatonin implant insertion, inducing reactivation of the GnRH axis in a different season. Indeed, reactivation occurred in spring and early summer in ganglionectomized animals implanted in December and in autumn–early spring in intact animals implanted in July. This difference in response might be due to seasonal variation in the sensitivity of melatonin receptors with large decrease in the number or sensitivity of these receptors in late spring and summer.

The breeding season may be longer in intact animals with melatonin implants than in non-implanted animals for a combination of reasons: (i) high melatonin concentrations supplied by the implants in February–April, when nocturnal concentrations of endogenous melatonin are decreasing due to increasing day length; and (ii) the presence of a large number of receptors or high sensitivity of the receptors during this part of the year, assuming, as suggested above, that there is a seasonal decrease in late spring and summer.

The results of the present study show that melatonin exerts a stimulatory effect on the gonadal axis in rabbits. For the wild European rabbit (*Oryctolagus cuniculus*), studies conducted in England and in France have characterized the female and male reproductive cycles (Flux, 1965; Arthur, 1980; Boyd, 1985; Boyd and Myhill, 1987; Dubry, 1987). Maximum testicular activity occurs from January to June–July after which it decreases and there is a period of rest from sexual activity from October to December. Gravid females are observed only from February to August. Thus, the wild European rabbit appears to be a long-day breeder, the sexual activity of which is inhibited by short days. This was demonstrated experimentally by Boyd (1985), who showed that transfer from long (16 h light:8 h dark) to short (8 h light:16 h dark) days or the insertion of subcutaneous melatonin implants in rabbits maintained in long days caused testicular regression. The opposite result was obtained in the present study: the wild rabbit population of Zembra Island breeds in short-day conditions and its reproductive activation is controlled by short days and melatonin. It is difficult to explain this difference in terms of temperature and photoperiod, because these are the same for the wild rabbit populations of southern France and Zembra Island (north Tunisia). This difference in the photoperiodic control of gonadal activity in these two populations may be due to the insularity of the rabbit population of Zembra Island. Indeed, as demonstrated for other populations, the geographical isolation of a given species may lead to: (i) changes in body size, such as gigantism in small mammals (Lomolino, 1985; Libois *et al.*, 1993); (ii) changes in litter size, such as the decrease in fecundity observed in the shrew *Crocidura suaveolens* on Corsica (Fons *et al.*, 1997), *Rattus rattus* on various Mediterranean islands (Cheylan, 1986) and the multimammate rat *Mastomys erythroleucus* on La Madeleine island, Senegal, Africa (Granjon, 1987); (iii) a delay in sexual maturity, as observed for *Rattus rattus* on Corsica (Granjon and Cheylan, 1988). For a species colonizing an island, the syndrome d'insularité ('isolation syndrome'), as defined by Blondel (1995), concerns new environmental conditions including, for example, variations in population density due to changes in the diversity of predators or competitors or their disappearance, changes in nutritional conditions and changes in opportunities for burrowing. As shown by Heideman and Bronson (1991) and Heideman *et al.* (1999), even within a single population there may be individuals that range from completely non-responsive to

completely responsive to photoperiod. Arthur (1980) studied continental populations of wild rabbits and found that although testicular activity is low for most animals in winter, some males are sexually active at this time of the year. Similarly, population heterogeneity has been demonstrated in the regulation of testicular function in populations of *Arvicanthis niloticus* from Mali and Burkina Faso, Africa (Sicard *et al.*, 1992) and in the sahelian multimammate rat, *Mastomys erythroleucus*, in Mali, Africa (Sicard and Fuminier, 1996).

When the rabbits were introduced to the island of Zembra, it is possible that only animals reproducing in winter were able to reproduce successfully due to the specific conditions of isolation. This would have led to the disappearance of animals reproducing in spring, corresponding to most of the population. No underground burrows were found for the wild rabbits of Zembra. In contrast, continental populations of wild rabbits excavate underground burrows to protect their young against predators and adverse climatic conditions during the first few weeks of lactation. Although the soil of the island is suitable for burrowing, wild rabbits do not excavate (Arnould and Dollé, 1954). This specific behaviour may have played a role in the disappearance of the young of females that gave birth late in the season. Specific pressures, such as seasonal predation, may have resulted in the survival of only those litters born early. This isolated population, with a pattern of photoperiodic gonadal stimulation opposite to that observed in continental populations, may be an illustration of the process of genetic adaptation. During abrupt changes in environment, such as those that occur during colonization of an island or during the creation of an environmental islet within a biotope (for example as part of the desertification process, resulting in the creation of small islets of vegetation isolating fragments of animal populations), the survival of a population depends on individuals with reproductive characteristics that rendered them marginal in the old environmental conditions. These individuals become the principal reproducers in the new environment, enabling the species to survive. This plasticity of the species, enabling it to survive abrupt environmental changes and to colonize new biotopes, is made possible by heterogeneity within a given population.

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