Requirement of thyroid function for the expression of seasonal reproductive and related changes in red deer (*Cervus elaphus*) stags

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Summary. The effect of thyroid function on regulation of seasonal reproduction was investigated in three red deer stags thyroidectomized (THX) in summer (January 1988) in comparison with five thyroid-intact controls. Responses of luteinizing hormone (LH) and testosterone to a bolus injection of 10 µg gonadotrophin-releasing hormone (GnRH) were tested in July, October, December, February and April. Blood samples were collected at weekly intervals from December 1987 to June 1989 for measurement of testosterone, triiodothyronine (T3) and prolactin concentrations. Testis diameters were measured every 2 weeks. In October 1988 (spring), plasma LH concentrations of control stags were less responsive ($P < 0.01$) to stimulation by GnRH than those of THX stags; plasma testosterone concentrations and testis diameters were low and there was no increase in plasma testosterone concentrations after injection of GnRH in control stags during October or December (spring, early-summer). In contrast, THX stags maintained a testosterone response ($P < 0.01$) in these 2 months and did not exhibit any signs of a seasonal lack of reproductive activity at this time of year. Control stags cast antlers in spring whereas THX stags maintained hard antlers throughout the study. Concentrations of plasma T3 were not detected in THX stags from June 1988 onwards, but exhibited a seasonal pattern in control stags, with low concentrations during autumn and winter (April to July) and high concentrations in spring and summer (August to February). There was no effect of thyroidectomy on the seasonal pattern of prolactin secretion. These results show that thyroidectomy blocks the seasonal transition from the breeding to the nonbreeding season in red deer stags and support the hypothesis that thyroid hormones are required for this transition.

Keywords: thyroidectomy; seasonality; luteinizing hormone; testosterone; prolactin; red deer

Introduction

Seasonality of reproductive activity and other physiological events in mammals is presumed to arise from an interaction between endogenous circannual rhythms and a variety of environmental changes, the most important of which is daily photoperiod (Gwinner, 1986; Karsch & Wayne, 1988). It is firmly established that the link between photoperiodic signals and reproductive responses involves the pineal gland and its secretory product, melatonin (Karsch *et al.*, 1984), but it is not clear how melatonin interacts with other endocrine pathways to produce the seasonal changes. Seasonal breeding is a manifestation of changes in release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) arising from changes in secretion of gonadotrophin-releasing hormone (GnRH) (Lincoln & Short, 1980) and changes in the sensitivity of these hormones to negative feedback by gonadal steroids (Legan *et al.*, 1977; Goodman *et al.*, 1982). It is possible that other components of this system include the thyroid hormones; this is suggested by the prolongation of reproductive activity in starlings (Wieselthier & van Tienhoven, 1972;

Goldsmith & Nicholls, 1984), mink (Jacquet *et al.*, 1986) and sheep (Nicholls *et al.*, 1988; Moenter *et al.*, 1991), which occurred after these animals were thyroidectomized. These findings indicate that thyroid function may be required for termination of the annual breeding season.

Red deer stags are mammals with pronounced seasonal reproductive changes. They have a dramatic 'rutting' period, which is related to changes in gonadal function and blood concentrations of reproductive hormones and these are associated with major changes in behaviour, appetite and neck muscle mass. The annual reproductive events are linked to a spectacular cycle of growth, mineralization and casting of antlers, which are bony appendages attached to the frontal bones of the skull.

This study examined the effects of thyroidectomy on seasonal changes in red deer stags and tested whether the procedure would prevent reproductive quiescence in the spring. Some preliminary results of the study have been reported previously (Shi & Barrell, *in press*) and the present paper describes findings based on data for LH, testosterone, testis diameter, prolactin and triiodothyronine.

Materials and Methods

Animals and treatments. Ten 14-month-old red deer stags were allocated to be either thyroidectomized (THX) or nonoperated controls ($n = 5$). Thyroidectomy was performed after sedation with i.m. injection of 30–40 mg xylazine hydrochloride (Rompun, Bayer) and anaesthesia with i.v. injection of 10–12 mg sodium thiopentone/kg live weight (Intraval sodium, May & Baker) in January 1988. A 4–5 cm medial ventral incision was made through the skin and fascia overlying the trachea. Thyroid glands were located on each side of the trachea and removed following blunt dissection of the superficial muscles and fascia and tying off the thyroid blood vessels. An implant containing 20 mg thyroxine (Glaxo Laboratories (UK) Ltd) was inserted s.c. immediately after surgery. During the next 18 months, all animals grazed outdoors on pasture of predominantly ryegrass and white clover (*Lolium perenne* and *Trifolium repens*) on the research farm at Lincoln University, New Zealand. Water was provided *ad libitum* and the pasture was supplemented with lucerne hay during winter. Of the five surgically treated animals, two proved subsequently to have been only partially thyroidectomized and data from these two animals were excluded from the results.

Every week, stags were herded into a pen and a single 10 ml blood sample was collected by jugular venepuncture while each stag was held by manual restraint. Blood was collected into a glass tube containing 120 units of sodium heparin. After centrifugation at 1000 g, plasma was separated and stored at -18°C . Every second week, testicular diameter was measured as the craniocaudal distance between two lightly spring-loaded brass plates held on to the scrotum of standing stags. Response to a bolus i.v. injection of 10 μg GnRH (NIAMDD 26-306) was assessed in all three THX and four intact stags in July, October, December, February and April, corresponding to different stages of the antler cycle, i.e. hard antler after rutting, antler casting, antler growth, shedding of velvet and hard antler during rutting, respectively. To facilitate blood sampling, a polyvinylchloride cannula was inserted into a jugular vein and left in place. Samples were drawn at -30 , -15 , 0 , 10 , 20 , 30 , 50 , 70 , 90 , 120 min in relation to GnRH injection.

Hormone assays. Plasma LH concentration was determined using the method of Kelly *et al.* (1982). Values were expressed in terms of an ovine standard, NIAMDD-LH-S20. Iodinated ovine LH (NIADDK-oLH-1-3) was used as tracer and antiserum was NIADDK-anti-oLH-1 (AFP-192279). Assay sensitivity was 0.15 ng/ml and intra- and interassay coefficients of variation were 6.5 and 13.2%, respectively. Serially diluted deer plasma produced a binding curve which was parallel to that of the ovine standard.

Plasma testosterone concentration was measured by direct radioimmunoassay (Garnier *et al.*, 1978) using a nonextracted technique described by Schanbacher & D'Occhio (1982). This assay used [1,2,6,7, $-^3\text{H}$]testosterone (Amersham, TRK402) as tracer and rabbit anti-testosterone-11-bovine serum albumin serum (#250, supplied by G. D. Niswender) as antiserum. Sensitivity of the assay was 0.15 ng/ml and intra- and interassay coefficients of variation were 6.9 and 16.2%, respectively. Serially diluted deer plasma exhibited parallel binding to that of the standard curve.

Total plasma triiodothyronine (T3) was assayed by the method of Sadler & Brownlie (1975). The assay used T3 (Sigma) in deer plasma stripped by ion-exchange resin for standard, iodinated T3 (L-3,5,3'-[^{125}I]-triiodothyronine, Nex-110, Du Pont) as tracer and rabbit anti-T3-BSA conjugate (CS3-1983, Christchurch Public Hospital, NZ) as antiserum. The binding pattern of serially diluted deer samples was parallel to that of the standard curve and assay sensitivity was 0.05 nmol/l. Inter- and intra-assay coefficients of variation were 5.4 and 3.8% respectively.

Plasma prolactin was assayed using an enzyme-linked immunosorbent assay (ELISA) (L. K. Lewis, P. A. Elder & G. K. Barrell, unpublished). Reference standard for prolactin was NIADDK-o-PRL-16 and the assay used a rabbit anti-o-PRL antiserum (provided by D. F. M. van de Wiele). The second antibody was goat anti-rabbit immunoglobulins G and L peroxidase conjugate (Tago, California), the enzyme substrate contained *o*-phenylenediamine dihydrochloride, and the product absorbance was read at 492 nm with a reference wave length of 650 nm. Cross-reactivity with other pituitary hormones was less than 1% and the assay was considered specific for prolactin.

Assay sensitivity was 2.5 ng/ml with intra- and interassay coefficients of variation of 8.5 and 15%, respectively. Serially diluted deer plasma produced a change in absorbance which was parallel to the curve obtained with the ovine prolactin standards. The assay was considered to be valid for measuring prolactin in deer plasma but the values are expressed here in terms of the ovine standard.

Statistical analysis. Effects of season and thyroidectomy were examined by split-plot analysis of variance. Data for plasma concentrations of testosterone, prolactin and T3, and for peak testosterone concentration following GnRH injection were transformed to logarithms for analysis of variance. LH response to injection of GnRH was calculated as the incremental change between the mean of three preinjection samples and the peak postinjection concentration.

Results

Responses of LH and testosterone to GnRH administration

In the case of LH all animals responded to 10 µg GnRH on every occasion, but there were seasonal changes in the response (Fig. 1). In winter (July), both groups of stags exhibited similarly low responses to the stimulus, but THX stags had a greater incremental response ($P < 0.01$) than controls in the spring (October). Throughout summer and the following autumn, the mean response of LH to GnRH was equally high in THX and control stags. The pattern of response for testosterone was similar to that of LH except that control animals did not exhibit any response to GnRH during October and December (Fig. 1).

Fig. 1. Plasma concentrations of (a) luteinizing hormone (LH) and (b) testosterone in thyroidectomized (●, $n = 3$) and intact (○, $n = 4$) red deer stags showing response to a single i.v. dose of gonadotrophin-releasing hormone (GnRH) (injected at 0 min) on five occasions. **Incremental change (LH) or peak response (testosterone) following injection of GnRH was different between the two groups of stags, $P < 0.01$. Data are means $\pm$ s.e.m.

Testosterone, testis diameter and antler cycle

Plasma testosterone concentration and testis diameter of both groups of stags had a successive rise and decline associated with the rutting season (February to May, 1988). In spring (September to November) the values for these parameters decreased in control stags, but not in THX stags (Fig. 2). After midsummer (December) plasma testosterone concentration and testis diameter of

Fig. 2. Changes in the antlers of (a) thyroidectomized and (b) intact red deer stags (i.e. dead bone, casting, regrowth with velvet, cleaned antler) from December 1988 until June 1989. Fluctuations of (c) testicular diameter and (d) plasma concentration of testosterone in thyroidectomized ($\bullet$, $n = 3$) and intact ($\circ$, $n = 4$ in (c) and 5 in (d)) red deer stags recorded at weekly intervals from December 1987 until June 1989. Arrow indicates date of thyroidectomy. Asterisks indicate significant ($*P < 0.05$, $**P < 0.01$) differences between means for each group of stags. Data are means $\pm$ s.e.m.

control stags increased steeply, reaching higher values than those of THX stags by the following autumn (Fig. 2).

Antlers of control stags were cast off in September–October 1988 and replaced by new sets, whereas antlers of THX stags were not cast during the study (Fig. 2).

Triiodothyronine

Plasma T3 concentration of control stags exhibited a significant ($P < 0.01$) seasonal pattern of change (Fig. 3), with higher concentrations during spring and summer and lower in autumn and winter. In the THX animals, plasma T3 concentration decreased immediately after thyroidectomy, but rose after placement of the T4 implant. Afterwards, there was a gradual decrease in plasma T3 concentration in these animals and concentrations were undetectable from June 1988 onwards.

Fig. 3. Plasma concentrations of (a) triiodothyronine (T3) and (b) prolactin in thyroidectomized (●, $n = 3$) and intact (○, $n = 5$) red deer stags recorded at weekly intervals from December 1987 until June 1989. Arrow indicates date of thyroidectomy. In (a), means for each group of stags are different ($P < 0.01$) from date of thyroidectomy. Data are means $\pm$ s.e.m.

Prolactin

THX and control stags had similar patterns of plasma prolactin concentration throughout the experimental period (Fig. 3). Concentrations during summer (December to February) were higher ($P < 0.05$) than in winter (June to July).

Discussion

These results support the hypothesis that thyroid gland activity is necessary for the transition from the breeding to the nonbreeding state in seasonally breeding animals. In the present study, this is demonstrated by the persistence of testosterone concentrations in blood of THX stags in October and December, when they were barely detectable in the thyroid-intact controls. This study provides evidence for a seasonal pattern of T3 secretion, with lower plasma concentrations in winter, followed by an abrupt rise in spring and high concentrations in summer. Taken together, the results could indicate a regulatory role for the thyroid hormones during spring, which may account for the increase in secretion of T3 at this time of the year.

The persistence of testicular secretory activity is borne out by maintenance of post-rutting testis size and plasma testosterone concentration in the THX stags plus their retention of hard antlers. Cessation of testosterone secretion is a prerequisite for casting of antlers (Goss, 1983), but the THX stags maintained measurable plasma concentrations of testosterone, as well as detectable responses of this hormone to GnRH stimulation throughout the study, hence the continued presence of hard antlers on these animals.

The present study extends the list of mammals in which hypothyroidism has been shown to prevent the seasonal suppression of reproductive activity. This includes sheep (Nicholls *et al.*, 1988; Follett & Potts, 1990; Moenter *et al.*, 1991), golden hamster (Vriend, 1985) and mink (Jacquet *et al.*, 1986). Previously, much of the evidence for the role of thyroid hormones in the termination of seasonal breeding activity has come from avian studies. Birds under a stimulatory photoperiod (long days) eventually exhibit sexual quiescence due to photorefractoriness (Dawson & Goldsmith, 1982). Thyroidectomy abolishes photorefractoriness in European starlings (Wieselthier & van Tienhoven, 1972; Goldsmith & Nicholls, 1984) and Japanese quail (Follett & Nicholls, 1985), and this effect of thyroidectomy can be reversed in starlings by treatment with thyroxine (Goldsmith *et al.*, 1985). In mammals, evidence is accumulating to suggest that termination of the breeding season is an endogenously generated event (Malpaux *et al.*, 1988), akin to the mechanism of photorefractoriness in birds, so the underlying role of the thyroid hormones in seasonal breeding may be similar in birds and mammals.

Notwithstanding these considerations, it is possible that the cessation of testicular activity in thyroid-intact stags is a response to an inhibitory effect of the onset of long daily photoperiods in spring and it could be argued that this effect requires mediation by the thyroid hormones. In sheep, pineal gland function, as determined by monitoring blood concentrations of melatonin, showed normal responses to photoperiodic changes after thyroidectomy, even though the onset of anoestrus was blocked (Nicholls *et al.*, 1988; Moenter *et al.*, 1991). This means that, if thyroid hormones were mediators of inhibitory photoperiods, their effect on the reproductive axis would have to be at the post-pineal level of processing.

Effects of thyroidectomy in the present study were manifested by red deer stags at pituitary and testicular levels. In intact stags, pituitary and testes have very low responses to GnRH challenge in spring and summer (Fennessy *et al.*, 1988) or under long days (Suttie *et al.*, 1989). In the present study, LH response to a GnRH stimulus was greater in THX stags than in controls during October (Fig. 1). The THX stags also maintained a response of plasma testosterone to the LH elevations generated by injection of GnRH, whereas thyroid-intact controls exhibited a decrease in testicular responsiveness, especially evident in spring and early summer (October and December, Fig. 1). The effects of thyroidectomy were an enhancement of the responsiveness of pituitary and testes to endocrine stimuli, which was counter to the view that lack of thyroid hormones must lead to a depression in organ function. This point was made also by Moenter *et al.* (1991) who showed that thyroidectomized ewes maintained the negative feedback effects of oestradiol on LH secretion, which rules out general dysfunction as a universal explanation for the effects of thyroid removal.

Seasonal patterns of secretion of the thyroid hormones have been recorded in various animals, but there is some conflict in the literature regarding the timing of peak and trough blood concentrations. For instance, in some studies of deer, blood T3 concentrations increased through spring and summer, and declined during autumn and winter. This occurred with the red deer stags in the present study, in red deer and Père David's deer hinds (Loudon *et al.*, 1989), in male reindeer (Ryg & Jacobsen, 1982) and white-tailed deer (Bubenik & Leatherland, 1984). In contrast, female white-tailed deer had high serum concentrations of thyroxine in early winter and spring although the pattern for T3 was less clear (Watkins *et al.*, 1983). Likewise, plasma thyroxine concentrations in sheep showed a peak during winter in some cases (e.g. Sutherland & Irvine, 1974) and during summer in others (e.g. Fraser & McNeilly, 1982). Low environmental temperature is regarded as a major stimulus for the release of thyroid hormones (Sterling & Lazarus, 1977) and this could account for peak secretion rates in winter. Clearly, the data from the deer and some sheep studies which provide the converse pattern to this indicate that factors other than temperature have important roles in the control of circulating concentrations of thyroid hormones. Ryg & Jacobsen (1982) and Loudon *et al.* (1989) recorded significant correlations between blood T3 concentration and feed intake in deer species, the major increases in T3 accompanying the massive increase in live weight during spring.

In THX red deer stags, plasma T3 concentrations were undetectable once the effect apparently due to the thyroxine implant had disappeared (Fig. 3) and this is useful confirmation of the

effectiveness of the surgical operation. The implants were provided to prevent an abrupt loss of thyroid hormones following surgery. It is tempting to relate the onset of increased plasma T3 concentrations of control stags in spring with the concurrent demise of testicular activity, as exhibited by testis diameter and plasma testosterone concentration. This inverse relationship between thyroid and testicular function has been noted by other workers, e.g. in mink (Boissin-Agasse *et al.*, 1980) and red foxes (Maurel & Boissin, 1981), and is in keeping with the lack of reduction of testicular activity in the THX stags recorded here. However, none of this provides any evidence for causality and it may be unwise to attribute a regulatory role to the rise in thyroid hormone secretion during spring.

Changes in secretion of thyroid hormones have been correlated with changes in growth of wool by sheep (Lincoln *et al.*, 1980). In red deer stags, the spring moult was disrupted by thyroidectomy (Shi & Barrell, 1990) and the earlier spring moulting of Père David's deer was accompanied by a correspondingly early increase in plasma T3 concentrations (Loudon *et al.*, 1989). However, when the timing of coat growth in red deer hinds was delayed by treatment with bromocriptine, there was no effect on T3 concentration (Curlewis *et al.*, 1988). Clearly, the issue of thyroidal regulation of coat growth in red deer is not resolved. There is evidence for a role of prolactin in seasonal coat patterns in male mammals (Lincoln, 1989) and in female red and Père David's deer (Loudon *et al.*, 1989). The seasonal pattern of blood concentrations of prolactin in many mammals, which is associated directly with changes in daily photoperiod, has been demonstrated in red deer stags here and previously (Suttie *et al.*, 1984; Barrell *et al.*, 1985). However, this pattern was not disrupted by thyroidectomy in the present study or in sheep (Fraser & McNeilly, 1982; Moenter *et al.*, 1991). These results indicate that the effects of thyroidectomy on coat growth in red deer or sheep were not mediated by changes in prolactin secretion and that the thyroid hormones are not involved in the regulation of seasonal patterns of prolactin secretion. Secretion of thyroid stimulating hormone (TSH) and prolactin can be stimulated by a single hypothalamic factor, thyrotrophin-releasing hormone (TRH) (Fraser & McNeilly, 1982). Consequently, there may be a relationship between the regulation of prolactin and the thyroid hormones, which might account for some of the confusion that exists about this topic.

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