Seasonal variation in the feedback of sex steroid hormones on serum LH concentrations in the male horse

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Summary. The possibility of seasonal variation in the feedback effect of testosterone or oestradiol was investigated by giving replacement treatment to geldings for 2–3 weeks during breeding and non-breeding seasons. In the non-breeding season, testosterone suppressed LH values (mean $\pm$ s.e.m., ng/ml) in all geldings (before treatment, 7.5 ± 2.3 ; final treatment week, 1.8 ± 0.2 ; $P < 0.05$), whereas early in the breeding season, testosterone caused a prolonged rise in LH (before, 6.8 ± 2.3 ; final week, 18.9 ± 6.4 ; $P < 0.05$). In all testosterone experiments, LH returned to pretreatment levels within 2 weeks after treatment. Oestradiol treatment caused a prolonged increase ($P < 0.05$) in LH concentrations (mean $\pm$ s.e.m., ng/ml) in both seasons (breeding: before 5.2 ± 1.1 ; final week, 16.2 ± 4.8 ; non-breeding: before, 10.9 ± 1.9 ; final week, 20.1 ± 5.2). We conclude that in geldings the feedback effect of testosterone varies with season and, further, that testosterone replacement may be able to restore to geldings the stallion's seasonal pattern of LH secretion. The results suggest that, in male horses, testosterone and possibly oestradiol, are important components in the neuroendocrine pathway controlling seasonal breeding and, moreover, are essential for the generation of a positive signal for LH secretion in the breeding season.

Introduction

In the stallion, serum LH concentrations vary with season being 3–5 times higher in the spring (breeding season) than early winter (non-breeding season) (Thompson, Pickett, Berndtson, Voss & Nett, 1977; Harris, Irvine & Evans, 1983). Castration abolishes this seasonal pattern of LH secretion (Irvine & Alexander, 1982). Furthermore, although castration results in an acute 5–14-fold rise in LH concentration (Thompson, Pickett, Squires & Nett, 1979; Irvine & Alexander, 1982), this elevation is transient. Within a year, LH has returned to pre-castration levels (Alexander, 1982) and annual mean LH concentrations are similar in geldings and stallions (Irvine & Alexander, 1982). In the early breeding season, therefore, the unusual situation occurs in which LH concentrations in the intact animal are more than twice those in the castrate. These observations indicate that, unlike the mare (Garcia & Ginther, 1976), in the stallion (1) gonadal hormones are necessary to maintain the normal seasonal pattern of LH secretion and (2) at the onset of the breeding season a positive signal for LH secretion must occur which depends on the presence of the testes for its generation. We report here the effects of testosterone or oestradiol replacement at various times of the year on LH concentrations in long-term (> 3 years) geldings. A preliminary account of the findings has been published in abstract form (Irvine & Turner, 1983).

Materials and Methods

Experimental procedure

Experiment 1. To determine whether geldings showed marked short-term fluctuations in serum LH concentrations, which would necessitate multiple sampling to assess accurately daily mean LH concentration, 4 geldings that had been castrated at least 3 years earlier (i.e. 'long-term' geldings) were bled by jugular venepuncture at 15-min intervals for 6 h in March (autumn in southern hemisphere). Another 4 geldings were bled through jugular venous cannulae (Angiocath: Deseret Co., Sandy, Utah, U.S.A.) at 15-min intervals for 6–8 h in September (spring in southern hemisphere). Since in the main experiments, blood would be sampled usually between 10:00 and 16:00 h, collections in this experiment began at 10:00 h. Both series of intensive bleeding were done during transitional periods between seasons, since work in other species has shown that in the male LH fluctuations are most marked at these times (Lincoln & Short, 1980). Frequent sampling of steroid-treated geldings was not done since similar experiments in stallions have produced no evidence for short-term LH fluctuations (Irvine, Alexander & Hughes, 1984, 1985).

Experiment 2. Four long-term geldings of light horse breeds, aged 5–8 years and weighing 550–700 kg were used in this experiment conducted at Lincoln College, New Zealand. The horses were maintained outdoors and fed on meadow hay and pasture. In the non-breeding season of the southern hemisphere (treatment start: 28 July, 1982) horses were given i.m. injections of 125 mg testosterone (Sigma Chemical Co., St Louis, MO, U.S.A.) in peanut oil every other day for 20 days. Testosterone propionate in oil given to this regimen has been shown to maintain serum testosterone concentrations similar to those in stallions in the breeding season (Thompson *et al.*, 1979). In castrated sheep, injection of testosterone or testosterone propionate in oil produces comparable plasma testosterone concentrations and LH suppression whether measured at 48, 72 or 96 h after administration (Garnier, Terqui & Pelletier, 1977). We found in a preliminary trial, in which 2 long-term geldings were given 2 i.m. injections of testosterone in peanut oil 48 h apart and bled at 30-min intervals for 2 h after the first injection and then every 8 h until 48 h after the second injection, that mean ($\pm$ s.e.m.) serum testosterone concentrations (ng/ml) during treatment were indistinguishable from those of stallions in the breeding season (geldings, 2.1 ± 0.7 and 2.6 ± 0.6 ; stallions ($N = 5$), 2.0 ± 0.3). In the geldings, testosterone concentrations during the first 2 h after injection were 2–3 times those of stallions but fell gradually to be 29% and 75% of values in stallions 48 h after the first and second testosterone injections, respectively. Slow elimination of the free steroid from the gelding has also been observed by Houghton & Dumasia (1979), who found that only 37% of testosterone injected in oil was excreted within 48 h by geldings compared with 90% by men.

In the main experiment, blood samples were collected by jugular venepuncture usually between 11:00 and 16:00 h once daily for 2 days before the start of treatment, before each testosterone injection, and approximately twice weekly for 3 weeks after cessation of treatment. The experiment was repeated at the beginning (treatment start: 28 October, 1982) and end (treatment start: 22 March, 1983) of the breeding season in the southern hemisphere. Although the pre- and post-treatment sampling regimen of these experiments was designed to allow each gelding to act as his own control, an associated control experiment under identical conditions was also done in which 5 long-term geldings received no steroid treatment but were bled 8 times a month for 1 year. The results of this experiment have been published (Irvine & Alexander, 1982).

Experiment 3. Four long-term geldings of light horse breeds, aged 5–10 years and weighing 500–600 kg were used in this experiment conducted at the University of California, Davis, CA, U.S.A. The horses had been maintained in outdoor yards, but for the experiment were stabled in loose boxes, under natural photoperiod, and fed on meadow hay. In the northern hemisphere non-breeding season (treatment start: 18 November, 1983), horses were given i.m. injections of 40 μ g oestradiol cypionate/kg in oil (ECP: Upjohn CO., Kalamazoo, MI, U.S.A.) every other day for 21

days. This regimen was used because it has been shown that oestradiol dibenzoate, for which dose rate is identical to the cypionate (Merck Index, 7th edition, p. 417), given to this regimen maintains serum oestradiol levels similar to those in stallions in the breeding season (Thompson *et al.*, 1979). Also, in ovariectomized mares, oestradiol cypionate at one-tenth of the dose rate used by us maintains total plasma oestrogen concentrations at 18–35 pg/ml (Ganjam *et al.*, 1982), which is one-tenth of the concentration found at the height of the breeding season in stallions (128 pg oestradiol/ml plus 132 pg oestrone/ml; Thompson, Pickett & Nett, 1978). Blood samples were collected by jugular venepuncture between 10:00 and 14:00 h once daily for 3 days before the start of treatment, before each oestradiol injection, and twice weekly for 10 days after cessation of treatment. In the second part of this experiment, 1 pony and 3 horse long-term geldings aged 8–22 years and weighing 200–500 kg were used. The horses were maintained in outdoor yards and fed on meadow hay. Early in the northern hemisphere breeding season (treatment start: 18 March, 1983), horses were given i.m. injections of 40 µg oestradiol/kg every 2nd or 3rd day for 14 days. Blood samples were collected as in the first part of this experiment except that sampling ceased 1 week after treatment.

In Exps 2 and 3, behavioural effects of steroid treatment were subjectively assessed by observing responses of geldings to oestrous mares presented to them over a teasing rail.

Blood samples were allowed to clot overnight at 4°C, and serum was harvested by centrifugation and stored at –20°C until assay.

Assays

LH was measured in all samples by radioimmunoassay (RIA) as previously described (Alexander & Irvine, 1982) but using a highly purified preparation of equine pituitary LH (HP E98A, 1.0 ng = 3.0 ng NIH LH-S1 by the ovarian ascorbic acid depletion assay (Licht *et al.*, 1979), gift from Dr H. Papkoff, University of California, San Francisco, CA, U.S.A.) as standard (Irvine *et al.*, 1985). The antibody (GDN 15; gift from Dr G. D. Niswender, Colorado State University, Fort Collins, CO, U.S.A.) is highly specific and the 9% cross-reaction reported for horse FSH in the RIA has been attributed to LH contamination of the FSH preparation used (Evans & Irvine, 1976). Minimum detectable LH concentration (i.e. 2 s.d. from zero) was 13 pg/tube. Within- and between-assay coefficients of variation were 3.7% and 10.1%, respectively. However, to eliminate the effect of between-assay variation on results, for each experiment, all samples from each individual gelding were assayed together.

Testosterone was measured in preliminary trial samples by direct RIA as described by Schanbacher & D'Occhio (1982). The antibody (gift from Dr J. E. Cox, University of Liverpool, U.K.) was raised in sheep against testosterone-3-oxime-BSA and was highly specific for testosterone, having a significant cross-reaction only with 5α-dihydrotestosterone (7.5%) (Cox, Williams, Rowe & Smith, 1973). All samples were assayed in one assay in which the coefficient of variation between duplicates was 4.1%.

Statistical analysis

In Exp. 1, pulses in serum LH concentrations were identified using criteria similar to those used by Goodman & Karsch (1980). Briefly, (1) a peak had to occur within 3 samples of a previous nadir, (2) the amplitude had to exceed the sensitivity of the assay, (3) the LH value at the peak had to exceed the 95% confidence limits at the preceding and subsequent nadirs, and (4) single point 'pulses' were not allowed. Confidence limits were determined as described by Rodbard, Rayford, Cooper & Ross (1968). Pulse amplitude was measured as percentage peak increase above the preceding nadir.

For each steroid-treated gelding, mean pretreatment LH concentrations and weekly mean values during and after steroid treatment were calculated. Data were then subjected to analysis of variance (ANOVA) using a split plot-in-time model (Gill & Hafs, 1971). Each experiment was analysed separately. When ANOVA yielded a significant F value for treatment or treatment interaction, means were compared by Duncan's new multiple range test (Steel & Torrie, 1980).

Results

Experiment 1: short-term patterns in serum LH concentrations

In geldings bled at 15-min intervals for 6–8 h in spring or autumn, serum LH concentrations were stable, and although LH fluctuations meeting the criteria for classification as pulses occurred in 5 of the 8 horses, amplitude was very low (mean $\pm$ s.e.m. $23.3 \pm 3.9\%$ increase above nadir concentrations) (Fig. 1). On the basis of these results, a frequent blood sampling regimen was not used in Exps 2 and 3.

Fig. 1. Serum LH concentrations in 4 geldings bled at 15-min intervals for 6 h in March (southern hemisphere autumn) and in another 4 geldings bled at 15-min intervals for 6–8 h in September (southern hemisphere spring). LH pulses, identified by criteria based on assay statistics (see 'Materials and Methods'), are marked by arrows.

Experiment 2: effects of testosterone on LH concentrations

In all geldings, testosterone treatment in the non-breeding season caused a decline in LH values (Fig. 2a). By the 3rd week of treatment mean LH concentrations were significantly lower than before treatment ($P < 0.05$) and remained suppressed during the 1st week after treatment ($P < 0.05$), but returned to pretreatment values during the 2nd week after treatment (Table 1). In contrast to the effect of testosterone treatment in the non-breeding season, treatment early in the breeding season caused an increase in LH concentrations in all geldings (Fig. 2b). By the 2nd week

Fig. 2. Effect of testosterone administration (T) in the non-breeding season (a), early in the breeding season (b) and during the transition between breeding and non-breeding seasons (c) on serum LH concentrations in 4 geldings.

of treatment mean LH concentrations were significantly higher than before treatment ($P < 0.05$) and remained elevated during the 1st week after treatment ($P < 0.05$), but returned to pretreatment values during the 2nd post-treatment week (Table 1). However, the effects of testosterone treatment during the transition between breeding and non-breeding seasons were variable, with 3 of the 4 geldings showing an initial increase and then a decrease in LH concentrations, and the fourth gelding showing LH suppression only (Fig. 2c). Only the LH-suppressing effect was significant, with mean LH concentrations during the 3rd treatment week being lower than pretreatment values ($P < 0.05$), remaining suppressed during the 1st week after treatment ($P < 0.05$) and returning to pretreatment values during the 2nd week (Table 1).

Table 1. Mean ($\pm$ s.e.m.) LH concentrations (ng/ml) in 4 geldings before, during and after testosterone treatment (Weeks 1–3) in the non-breeding season, and early and late in the breeding season

Week	Season		
	Non-breeding	Early breeding	Late breeding
–1	7.5 ± 2.3^{ab}	6.8 ± 2.3^a	8.7 ± 4.6^a
1	5.6 ± 1.7^{abc}	11.4 ± 4.1^{ac}	8.6 ± 2.8^a
2	3.4 ± 0.5^{bc}	16.0 ± 6.2^b	5.3 ± 0.5^{ab}
3	1.8 ± 0.2^c	18.9 ± 6.4^b	2.0 ± 0.3^b
4	2.0 ± 0.2^c	14.7 ± 4.5^{bc}	1.4 ± 0.2^b
5	5.9 ± 2.1^{abc}	7.2 ± 2.6^a	4.6 ± 1.8^{ab}
6	10.1 ± 3.8^a	6.8 ± 2.4^a	9.9 ± 3.8^a

Within each time of the year, LH means with different superscripts are significantly different ($P < 0.05$). Between-season comparisons were not made.

At all 3 times of the year, all geldings developed stallion behavioural characteristics, showing extreme aggressiveness towards one another (and people) and a keen interest in oestrous mares. Penile erections were achieved and maintained during teasing of mares.

Experiment 3: effects of oestradiol on LH concentrations

In all geldings, oestradiol treatment in the non-breeding season caused a rise in LH concentrations which was sustained for the duration of the experiment in 3 of the 4 geldings (Fig. 3a). Mean LH concentrations were significantly higher during and after treatment than before ($P < 0.05$) (Table 2). When oestradiol was given early in the breeding season, the 3 horse geldings showed a marked increase in LH concentrations, whereas the pony gelding showed a small decrease in LH concentrations (Fig. 3b). The geldings also differed in that the horses had begun to shed their winter coats, whereas the pony had not. Mean LH concentrations were significantly higher during and after treatment than before ($P < 0.05$) (Table 2). In contrast to the effects of testosterone treatment, in neither oestradiol experiment was there a significant interaction between steroid treatment and time.

Two of the 4 geldings treated during the non-breeding season developed some of the components of stallion behaviour, showing mild aggression towards one another in the presence of an oestrous mare. One of these 2 horses achieved a partial penile erection while teasing. Masculinization of the 4 geldings treated early in the breeding season was not observed.

Fig. 3. Effect of oestradiol cypionate administration (E_2) in (a) the non-breeding season on serum LH concentrations in 4 geldings and (b) early in the breeding season on serum LH concentrations in 1 pony and 3 horse geldings.

Table 2. Mean ($\pm$ s.e.m.) LH concentrations (ng/ml) before, during and after oestradiol cypionate treatment (Weeks 1–3) in 4 geldings treated during the non-breeding season and in another 4 geldings treated early in the breeding season

Week	Season	
	Non-breeding	Breeding
–1	10.9 $\pm$ 1.9	5.2 $\pm$ 1.1
1	16.2 $\pm$ 2.7	14.7 $\pm$ 4.0
2	21.5 $\pm$ 5.2	16.2 $\pm$ 4.8
3	20.1 $\pm$ 5.2	–†
4	18.9 $\pm$ 6.0	14.8 $\pm$ 4.2
5	20.1 $\pm$ 5.5	–‡

In both seasons mean LH concentrations were significantly higher during and after treatment than before ($P < 0.05$); however, the interaction between steroid treatment and time was not significant.

† Steroid treatment ended after 14 days.

‡ Blood not sampled.

Discussion

In castrates of many species, marked short-term fluctuations in serum LH concentrations occur (monkey: Plant, 1982; red deer: Lincoln & Kay, 1979; sheep: Riggs & Malven, 1974; rat: Gay & Sheth, 1972), necessitating frequent blood sampling to assess accurately LH status. By contrast, in castrated male horses, LH concentrations were stable, although extremely low-amplitude fluctuations were occasionally seen in some horses. Similar observations have been made in stallions bled at 10-min intervals for 3 h during the breeding season (Irvine *et al.*, 1984) or at 15-min intervals for 6 h during the transition between non-breeding and breeding seasons (Irvine *et al.*, 1985). The stability of LH concentrations in the horse does not necessarily mean that secretion is non-pulsatile, but probably is a result of the extraordinarily long circulatory half-life of horse LH (5 h: Irvine, 1979) compared with 43 min for sheep LH (Akbar, Nett & Niswender, 1974) which would blunt the appearance of peripheral pulses by minimizing decay between pulses. Pulse detection would therefore be difficult, particularly when frequency was high. In any case, the stability of LH concentrations in the horse means that frequent blood sampling is not needed to determine mean LH concentration.

In other species, castration results in chronically elevated LH concentrations which are suppressible by administration of testosterone (sheep: D'Occhio, Schanbacher & Kinder, 1982; rat: Steiner, Bremner & Clifton, 1982; monkey: Plant, 1982) or oestradiol (sheep: Karsch & Foster, 1975; rat: Neill, 1972). Likewise, in the horse, castration causes a 5- (Irvine & Alexander, 1982) to 14- (Thompson *et al.*, 1979) fold rise in LH, which is reversible by testosterone treatment (Thompson *et al.*, 1979). However, in contrast to other species, LH concentrations in geldings do not remain elevated, but return to annual mean stallion levels within a year of castration (Alexander, 1982). Thereafter, concentrations are stable throughout the year, as shown by pretreatment values in the 4 geldings in Exp. 2 (Table 1) and results of the associated control study in which 5 geldings received no steroid treatment but were bled 8 times monthly for 1 year (Irvine & Alexander, 1982). By contrast, stallions show a markedly seasonal pattern of LH secretion with LH rising at the onset of the breeding season to reach levels in late spring 3–4 times those in early winter (Thompson *et al.*, 1977; Harris *et al.*, 1983). Early in the breeding season, therefore, LH concentrations in stallions exceed those in geldings whereas in the non-breeding season the situation is reversed (Irvine & Alexander, 1982). The present experiments have shown that testosterone administration to geldings exerted the expected inhibitory effect on LH in the non-breeding season but during the breeding season caused a prolonged elevation in LH concentrations. At all times of the year, mean LH concentrations had returned to pretreatment values by the 2nd week after testosterone treatment, strongly suggesting that the observed LH changes were due to treatment, and not to some other factor such as handling. These observations suggest that testosterone replacement may be capable of restoring the stallion's seasonal pattern of LH secretion to the gelding.

Since the stallion testis also produces large amounts of oestrogens (Bedrak & Samuels, 1969) we studied the seasonal effects of oestradiol replacement on gelding LH concentrations. In contrast to testosterone, oestradiol treatment caused a prolonged increase in LH concentrations in both breeding and non-breeding seasons. Similar observations have been made in ovariectomized mares given oestradiol for 15 days in the breeding and non-breeding seasons (Garcia, Freedman & Ginther, 1979). In the gelding, the different results of testosterone and oestradiol treatments in the non-breeding season suggest that at this time of year testosterone does not exert its effect on LH secretion through aromatization to oestradiol. Oestradiol treatment was largely ineffective in restoring stallion behaviour to geldings, which is consistent with earlier work in which supraphysiological but not physiological oestradiol concentrations were needed to produce full stallion libido in geldings (Thompson, Pickett, Squires & Nett, 1980).

In several species, the response of the neuroendocrine axis to steroidal feedback has been found to alter with time after gonadectomy, which makes the long-term castrate a questionable model for the intact animal (monkey: Plant, Hess, Hotchkiss & Knobil, 1978; rams: Edgerton & Baile, 1977;

bulls: D'Occhio, Kinder & Schanbacher, 1982). However, in acutely castrated horses the effects of replacement of physiological levels of testosterone or oestradiol in the non-breeding season on serum LH concentrations are identical (Thompson *et al.*, 1979) to those observed by us in the chronic castrate (effects in the breeding season were not studied in the acute castrate). Furthermore, the long-term geldings in the present experiments unequivocally responded to testosterone treatment, whereas long-term castrates of other species seem to be androgen-insensitive.

Observations of other species show that in some, but not all, seasonal breeders the annual rhythm of gonadotrophin concentrations is largely dependent on the presence of the gonads. For example, gonadectomy greatly attenuates or completely abolishes seasonal LH secretion in male red deer (Lincoln & Kay, 1979), female Scottish Blackface, Finnish (Land, Wheeler & Carr, 1976) and cross-bred (Legan, Karsch & Foster, 1977) sheep and male golden hamsters (Urbanski, Simpson, Ellis & Follett, 1983). In contrast to the ewe, castrated male Soay (Lincoln & Short, 1980) and Ile-de-France (Pelletier & Ortavant, 1975) sheep retain the ability to respond to changing photoperiod with appropriate gonadotrophin secretion. Similarly, ovariectomized pony mares, unlike horse geldings, show a seasonal pattern of LH concentrations (Garcia & Ginther, 1976) similar to that in intact mares and stallions. When gonadectomy affects seasonal changes in LH levels, treatment with gonadal steroids can restore the intact pattern. For example, in ovariectomized ewes, oestradiol replacement regenerates the appropriate seasonal pattern of LH secretion, with the elevated LH concentrations of the castrate being suppressed to a markedly greater extent in the non-breeding than breeding season (Legan *et al.*, 1977). These observations suggest that in these species seasonal breeding in the intact animal may be regulated by changing sensitivity of the neuroendocrine axis to the feedback effects of gonadal steroids. Our observations of steroid-treated geldings are consistent with this concept, with the striking addition that in this species the variation in testosterone feedback seems to range from negative to positive.

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