Daily and seasonal variations in plasma LH and testosterone concentrations in the adult male hedgehog (*Erinaceus europaeus*)

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Summary. A double-antibody heterologous radioimmunoassay (RIA) was developed to measure plasma LH values in hedgehogs. This RIA system used anti-rat LH serum and rabbit LH (AFP-559B) for radioiodination and as standard. The accuracy of the method was evaluated and indicated the ability to detect various relative concentrations of LH in plasma. The minimum detectable dose was 0.2 ng/ml. The intra- and inter-assay coefficients of variation were 4.2 and 7.9% respectively. Biological tests, e.g. effect of castration, effect of castration + testosterone implant and GnRH administration, confirmed that this method was suitable to determine subsequent changes in pituitary gonadotrophic activity in the hedgehog. LH concentrations were determined in blood samples obtained during 1 year: (a) each month, at 4-h intervals during 24 h, from different groups of unanaesthetized animals fitted with a catheter and (b) twice a month, under a light anaesthesia, from the same group of 6 animals. During the year: (1) the range of LH change was narrow (minimum values $\approx$ 0.25 ng/ml and maximum values $\approx$ 2.00 ng/ml); (2) the 24-h LH patterns did not exhibit any daily rhythm; (3) a clear annual rhythm was observed with the highest values from February to April and the lowest values in October and November. LH decreased rapidly at the end of summer and increased progressively from December to February, during hibernation. In these experiments, it was not possible to determine the characteristics of LH release patterns in the hedgehog but individual profiles indicated clearly the episodic secretion of LH, particularly during the highest pituitary activity period. During the year, a close relationship between the seasonal cycles of plasma LH and testosterone was observed.

Keywords: hedgehog; LH; testosterone; daily variations; seasonal cycle

Introduction

The hedgehog (*Erinaceus europaeus*) is a hibernating mammal widely distributed in Europe. In the male, the reproductive cycle is well known and characterized by a resting period in autumn, a resurgence of testicular activity during the last part of the hibernation period (January–February), a period of maximum activity from mid-winter to mid-summer which corresponds to the reproductive season, and involution beginning at the end of summer (Saboureau & Dutourné, 1981; Saboureau & Boissin, 1983). The most detailed studies to date concerning this reproductive cycle have been limited to morphological or histological changes of the testes and testicular features (Courrier, 1927; Allanson, 1934; Saboureau & Dutourné, 1981; Dutourné & Saboureau, 1983) or assays of gonadal steroids (Saboureau & Boissin, 1978, 1983). In this species, except for few studies on the cellular and histological aspects of the adenohipophysis (Girod & Curé, 1965; Girod *et al.*, 1966; Girod, 1971) nothing is known about the neuroendocrine mechanisms and pituitary hormone patterns regulating the seasonal reproductive cycle.

To initiate a comprehensive study of the reproductive endocrinology in the hedgehog we first have developed a heterologous LH radioimmunoassay and have applied it to determine (1) the extent of variation in plasma LH concentrations that can be expected in the male hedgehog and (2) baseline information about seasonal changes in plasma LH values.

Materials and Methods

Animals and techniques

Animals. Male adult hedgehogs caught in western central France (Department of Deux-Sèvres) were reared in the 'Centre d'Etudes Biologiques des Animaux Sauvages' in Chizé Forest (46°09'N; 0°24'W). They were housed individually in 3 m² parks and kept under natural climatic conditions of light, temperature and rainfall throughout the year. They were fed daily with a mixture of crushed chicken meat and dog biscuits (Canina; Duquesne-Purina, St Quentin, France) and water was always available.

Blood sampling. For short-term studies (≤ 24 h) requiring serial blood samples, blood was obtained from unanaesthetized animals equipped with a catheter (polyethylene tubing, PE 50; Clay Adams Intramedic; RUA, Torcy, France) inserted in the left carotid artery, some days before the beginning of the experiment (Saboureau *et al.*, 1979). In long-term or seasonal studies, blood samples were obtained by intracardiac puncture under light halothane anaesthesia (Fluothane; Coopers Veterinaire, Meaux, France) regularly at the same time in the morning (3–5 h after sunrise). During the period of hibernation, all samples were taken from aroused animals to avoid a possible effect due to change in body temperature. In each procedure, blood samples were placed into heparinized tubes and were kept on ice until centrifugation. Plasma was divided into several aliquots and stored at -25°C until used in assays.

Experiments. Daily patterns of plasma LH and testosterone concentrations were determined monthly, during 1 year, in groups of male adult hedgehogs. Blood was sampled through an indwelling catheter, at 4-h intervals (starting at 08:00 h) for 24 h.

The seasonal changes of plasma LH and testosterone concentrations were studied: (1) from daily means (means of the values obtained over a period of 24 h for each animal) determined monthly in the previous experiment; (2) in a group of 6 male adult hedgehogs, regularly sampled twice a month, during 1.5 years. At the same time the body mass of these animals was recorded to the nearest 1 g and the testicular volume was evaluated by palpation according to an arbitrary unit scale (a.u. from 1 to 5) correlated with previous data (Saboureau, 1981). Owing to the fact that testes are in an intra-abdominal position in the hedgehog this procedure avoids injury due to laparotomy.

Hormone assays

Testosterone. Plasma testosterone concentrations were measured by a radioimmunoassay method without chromatography as described by Saboureau & Duturné (1981). Intra- and inter-assay coefficients of variation were respectively 4 and 10%, and the sensitivity, determined as the smallest detectable quantity of testosterone, was < 10 pg/tube.

LH. Since, for a wild mammal such as the hedgehog, no purified pituitary hormone was available or specific antisera, the immunochemical similarity between hedgehog LH and several available LH hormones was examined using different radioimmunoassay systems. The reliability of these preliminary tests led to development of the following double-antibody heterologous radioimmunoassay (rabbit–rat) to measure hedgehog LH.

The purified rabbit LH (RbLH, AFP-559B) used for iodination and as standard was provided by NIAMDD, NIH (Bethesda, MD, USA). The rabbit antiserum raised against rat LH and the anti-rabbit γ -globulin serum raised from sheep used in the assay as the second antiserum were kindly donated by the I.N.R.A. Reproductive Physiology Laboratory (Nouzilly, France).

Rabbit LH (AFP-559B, 4 μg) was labelled with ^{125}I (IMS 300; Amersham, Les Ulis, France) to an approximate specific activity of 85 $\mu\text{Ci}/\mu\text{g}$, following a modified procedure of the chloramine T method described by Greenwood *et al.* (1963). The labelled hormone was isolated and purified by two chromatographic procedures at 4°C on Sephadex G25 and G75 (Pharmacia Fine Chemicals, Les Ulis, France) respectively. The immunological activity of the labelled hormone obtained was controlled by incubation with excess related antibody.

All reagents were diluted in 0.025 M-BSA barbitone buffer, pH 8.6. Each assay tube contained: (1) 50 μl unlabelled antigen (standard or plasma samples or BSA barbitone buffer); (2) 200 μl of diluted anti-rat LH serum (used at a final dilution of 1:65 000 in 1% normal rabbit serum); (3) 250 μl labelled antigen (diluted to obtain $\approx 10\,000$ c.p.m. per assay tube), added 24 h after the antiserum. Incubation was carried out at 4°C for 72 h. Then the precipitating second antibody (250 μl solution containing 15 μl serum per tube) was added and the system was incubated for a further 24 h at 4°C . The precipitate was rinsed and all tubes centrifuged for 45 min at $\approx 3600 g$ at 15°C . The supernatant was carefully evacuated and the bound ^{125}I -labelled LH complex was counted in a gamma spectrophotometer (Intertech-nique CG 30). The LH concentrations in plasma samples were estimated from the calibration curve and expressed as ng standard rabbit LH (AFP-559B)/ml.

The reliability of this heterologous RIA for hedgehog LH was determined with different tests. The dose-response curves of several hedgehog plasma samples diluted serially (Fig. 1) were parallel to the standard curve and indicate the ability of this assay to detect various concentrations of LH in the hedgehog. This was confirmed by quantitative recovery of definite amounts of standard rabbit LH (AFP-559B) to a known plasma sample. The linear regression line of the correlation of expected vs detected LH quantities was $y = 0.93x + 0.06$ ($r = 0.996$). The practical sensitivity of the method determined as the minimal detectable standard dose was < 7.9 pg/assay tube, i.e. < 0.20 ng/ml plasma. The precision of the assay was determined on several hedgehog plasma pools, established as quality control samples. Intra- and inter-assay coefficients of variation were 4.7% and 7.9% respectively.

Fig. 1. Comparison of the standard LH curve (RbLH, AFP-559B) with dilution curves of several plasma pools obtained from hedgehogs in different physiological states (spring activity $\blacktriangle$, castrates $\star$ $\triangle$ $\bullet$).

The accuracy and specificity of the assay system were checked using biological tests involving modifications of plasma LH concentrations (1) after castration and restoration of high levels of testosterone or (2) after GnRH injection.

The castration, in spring (end of April), of 4 male adult hedgehogs exhibiting high plasma testosterone and LH concentrations, provoked a significant increase (intact vs castrated: $\approx 200\%$; $P < 0.05$) of plasma LH (Table 1). In castrated animals, the s.c. implantation of two capsules (Dow Corning Silastic tubing: i.d. 3.35 mm, o.d. 4.65 mm, length 40 mm; Sigma Medical, Nanterre, France) filled with crystalline testosterone (Sigma Chimie, la Verpillière, France) restored high plasma testosterone concentrations and led to LH values similar to those of intact animals.

A single injection of 2.5 μ g synthetic GnRH (C.R.B., Cambridge, UK), through an indwelling arterial catheter, in 6 male adult hedgehogs in March (Table 2) was followed by a significant increase of plasma LH concentration, as early as 15 min after the injection (LH before GnRH injection: 0.79 ± 0.10 ng/ml vs LH 15 min after GnRH injection: 1.97 ± 0.11 ng/ml; $P < 0.05$). This high value was maintained over the basal level for about 2 h. At the same time, plasma testosterone concentration was also increased significantly 30 min after the injection and stayed at plateau values.

Statistical analysis. Hormonal concentrations are given as mean $\pm$ s.e.m. Variance analysis (test F) was used for statistical comparisons of the mean values.

Results

Daily variations

The daily patterns of plasma LH obtained monthly, during 1 year, are shown in Fig. 2. During the year, the amplitude of plasma LH changes was low (minimum values about 0.25 ng/ml;

Table 1. Changes of plasma testosterone and plasma LH (means $\pm$ s.e.m.) in a group of male adult hedgehogs (N = 4) submitted successively to different treatments: (a) intact, (b) castrated, (c) castrated + testosterone

Experiment	Days	Testosterone (ng/ml)	LH (ng/ml)
Intact	0	10.06 $\pm$ 1.24	1.14 $\pm$ 0.21
Castrated†	6	0.23 $\pm$ 0.05	2.19 $\pm$ 0.21
	16	0.24 $\pm$ 0.03	2.23 $\pm$ 0.15
	22	0.29 $\pm$ 0.08	2.26 $\pm$ 0.34
Castrated + testosterone‡	29	11.52 $\pm$ 1.06	2.00 $\pm$ 0.39
	36	11.03 $\pm$ 0.94	1.61 $\pm$ 0.39
	62	9.10 $\pm$ 1.67	0.82 $\pm$ 0.18

†On Day 0.

‡Given subcutaneous implants of testosterone on Day 22.

Table 2. LH and testosterone responses to injection (time 0) of GnRH (2.5 μ g/animal) in male adult hedgehogs (N = 6) in March

Time (min)	LH (ng/ml)	Testosterone (ng/ml)
Before injection		
-30	0.85 $\pm$ 0.07	21.39 $\pm$ 1.52
-15	0.77 $\pm$ 0.06	22.67 $\pm$ 1.49
-2	0.79 $\pm$ 0.10	20.52 $\pm$ 1.18
After injection		
+15	1.97 $\pm$ 0.11	22.50 $\pm$ 1.87
+30	2.69 $\pm$ 0.13	31.83 $\pm$ 1.93
+45	3.14 $\pm$ 0.16	35.61 $\pm$ 1.58
+60	3.33 $\pm$ 0.21	34.06 $\pm$ 2.62
+80	3.36 $\pm$ 0.30	36.34 $\pm$ 3.81
+100	3.06 $\pm$ 0.31	32.79 $\pm$ 2.59
+120	2.63 $\pm$ 0.37	32.96 $\pm$ 3.19

Values are mean $\pm$ s.e.m.

maximum values about 2.00 ng/ml). The 24-h patterns of LH did not exhibit a daily rhythm at any moment of the year. The amplitude of the LH pattern was different during the year; it was largest in February, March and April, then intermediate from May to August and very low in October and November. Moreover, the comparison of the minimum and the maximum values recorded during the 24-h period showed significant differences from February to August ($P < 0.05$), but no difference in October and November. In December, a great variability was observed between the animals; some showed large-amplitude LH variations (from 0.30 to 1.12 ng/ml) while others had low-amplitude variations (0.25 to 0.45 ng/ml).

Individual patterns of plasma LH (Fig. 3) showed pulses with large amplitude in February, during the beginning of the reproductive season, and no detectable variation in November, in autumn during the resting season.

The daily patterns of plasma testosterone are shown in Fig. 2. In February and March, large variations were observed with several peaks per day. From April to July, the testosterone

Fig. 2. Daily patterns of plasma LH and testosterone concentrations in groups of male adult hedgehogs sampled each month, during 1 year. Sampling, at 4-h intervals, started at 08:00 h (G.M.T.). Values are means $\pm$ s.e.m. for the no. of animals indicated. Horizontal bars (---) represent 24-h means with a confidence limit of 0.95 (mean $\pm$ 2 s.e.m.). Dark periods are indicated by horizontal bars (—).

Fig. 3. Daily individual patterns of LH and testosterone concentrations in hedgehogs sampled in February and November. Sampling at 4-h intervals started at 08:00 h (G.M.T.).

concentrations were significantly higher during the light phase than during the dark phase ($P < 0.05$). From the end of August to November the values were very low (< 1 ng/ml) with no variation over the 24 h. In December, when the testis is redeveloping, plasma testosterone patterns showed small (at rest) or large (during rise) variations.

Like LH, the individual patterns of plasma testosterone (Fig. 3) showed large variations during the active season and no variations at rest.

Seasonal variations

In groups of animals sampled monthly during 24 h. The means of LH concentrations determined over a period of 24 h, each month, showed a clear seasonal pattern (Fig. 4). Mean plasma LH concentrations were elevated from February to April with a maximum in March (1.05 ± 0.05 ng/ml). After a significant decrease in spring (April [0.70 ± 0.03 ng/ml] vs June [0.46 ± 0.02 ng/ml]; $P < 0.01$), mean LH values stayed steady from May to the end of August (0.52 ± 0.02 ng/ml) although a non-significant increase occurred in August. At the end of summer, mean LH values decreased rapidly and the lowest concentrations were observed in October and November (August vs October [0.30 ± 0.04 ng/ml] or November [0.36 ± 0.02 ng/ml]; $P < 0.01$). LH concentrations then increased progressively in winter from December (0.52 ± 0.04 ng/ml) to February (0.67 ± 0.04 ng/ml).

Fig. 4. Seasonal variations of LH (---) and testosterone (—) concentrations determined in male hedgehogs over a period of 24 h each month. The 24-h means are given with their standard errors.

As for LH, the 24-h means of plasma testosterone also showed a clear seasonal pattern (Fig. 4). Mean concentrations were maximum in February (12.52 ± 1.19 ng/ml)–March (16.47 ± 1.07 ng/ml), decreased significantly in April (6.40 ± 0.63 ng/ml; March vs April: $P < 0.01$) and stayed at an intermediate level until July (4.59 ± 0.84 ng/ml). Mean testosterone concentrations decreased rapidly in summer and were minimal from August to November (< 1 ng/ml). An increase in testosterone occurred in December (3.24 ± 0.65 ng/ml) and the concentrations in February (11.28 ± 1.63 ng/ml) were similar to those observed the year before.

In the same group of animals sampled twice a month, during 1.5 years. The seasonal changes of plasma LH, plasma testosterone and of testicular volume, in 6 animals studied from December 1985 to June 1987, in relation to the changes of daylength and environmental temperatures, are presented in Fig. 5.

As in the first experiment, plasma LH varied significantly with season and showed similar characteristics; i.e. maximum LH concentrations from December to April ($\text{LH} \geq 0.6$ ng/ml, except in February); an intermediate plateau from May to August ($\text{LH} \geq 0.5$ ng/ml); minimum values

Fig. 5. Changes of daylength, environmental temperatures and seasonal variations of LH, testosterone and testicular volume in a group of 6 male adult hedgehogs, sampled twice a month, during 1.5 years. Values are means $\pm$ s.e.m.

from September to November (LH about 0.3–0.4 ng/ml) and increasing values beginning in late December with a great variability. The only difference from the former results was the lower levels of LH in winter and spring when pituitary gonadotrophic activity was maximal. Such a result can be explained taking into account the number of blood samples (several *vs* one) and the method of sampling (animals unanaesthetized *vs* anaesthetized).

Testicular activity determined by the variations of testicular volume and plasma testosterone concentrations also showed a clear seasonal pattern. Testicular volume was maximum from February–March to August and minimum from the beginning of September to December; reactivation took place in winter (late December) and involution at the end of summer (from August). Plasma testosterone concentrations showed a biphasic profile during the breeding season with two peaks: the first peak was higher and longer, from February (16.10 ± 2.71 ng/ml) to April (15.36 ± 2.97 ng/ml) and the second peak in late June–July (8.89 ± 2.58 ng/ml) was lower. The resting season occurred in late summer and in autumn, and reactivation of testicular endocrine activity began in December.

Discussion

The present study provides, for the first time, a description and the application of a double-antibody heterologous radioimmunoassay technique developed to study plasma LH in the hedgehog, a wild and hibernating mammal, belonging to the Order Insectivora. Even though only relative values, expressed in terms of rabbit LH, AFP-559B, the RIA developed, as indicated by dose-response curves, recovery test and biological tests (effects of castration, testosterone implantation in castrates and GnRH administration), could be determined, the assay was suitable to determine subsequent changes in pituitary gonadotrophic activity in the hedgehog.

Sensitivity, intra- and inter-assay coefficients of variation and accuracy of this LH assay were in agreement with similar assays developed for other wild mammals (Sharma *et al.*, 1978; Su *et al.*, 1980; Mann *et al.*, 1982; Maurel *et al.*, 1984; Schneyer *et al.*, 1985).

In the hedgehog, the amplitude of plasma LH changes was low and similar to that observed in various other species (white-tailed deer: Bubenik *et al.*, 1982; bottlenosed dolphin: Schneyer *et al.*, 1985; brown hare: Caillol *et al.*, 1986).

During the year, the 24-h patterns of LH fluctuations did not exhibit any daily rhythm but the mean level and the amplitude of the fluctuations varied according to season. The largest amplitude variations of LH were observed as early as December in some animals and then in all animals from February to April, i.e. the period which corresponds to the resurgence of and maximal sexual activity. These results are in agreement with those of Lincoln & Short (1980) and Pelletier *et al.* (1982) who showed in the ram that the amplitude of plasma LH peak increases when the animals pass from the non-breeding season to the breeding season. In contrast, the lowest amplitude variations of plasma LH occurred in autumn, which corresponds to the resting season and also the first part of hibernation. Considering individual patterns of LH, particularly from February to April, the secretion of LH seems to be episodic but the sampling intervals were large and further experiments are needed to determine the characteristics of LH release in the hedgehog.

The 24-h plasma testosterone changes also showed variations of large amplitude at the end of winter during the period of maximal sexual activity. Then, from April to July a regular decrease of testosterone was observed during the night. This decrease occurred during the activity phase of the hedgehog which is a nocturnal mammal (Saboureau *et al.*, 1979).

In the hedgehog, the seasonal profiles of LH and testosterone were quite parallel during the year. Such seasonal variations of LH in relation to the reproductive cycle have been documented previously for many domestic or wild mammalian species subjected to annual environmental fluctuations (ram: Schanbacher & Ford, 1976; Lincoln & Davidson, 1977; Pelletier *et al.*, 1982; pygmy goat: Muduuli *et al.*, 1979; mongoose: Soares & Hoffman, 1982a, b; roe deer: Sempéré & Lacroix, 1982; fox and badger: Maurel *et al.*, 1984). The seasonal variations of LH obtained from 24-h mean plasma LH values from different groups of animals sampled each month and those from the same group of animals sampled regularly twice a month during the year were similar despite limitations inherent in measuring hormone concentrations at different sampling intervals. The only difference observed between the two experiments was the lower levels of LH measured in the second experiment when pituitary gonadotrophic activity was higher. In this second experiment, in addition to the single blood sample, the method of sampling under anaesthesia must be taken into account because halothane has been shown to reduce LH secretion (Clarke & Doughton, 1983). Nevertheless, according to the large variations of gonadal activity in the hedgehog, throughout the year (Saboureau & Boissin, 1978; Saboureau & Dutourné, 1981), the results obtained with the second method are sufficient to establish the existence of distinct variations in pituitary and gonadal secretory activity and to measure hormone variations in groups of few animals submitted for a long time to experimental treatments.

LH rose and reached a peak during testicular redevelopment in winter and then declined to lower steady values during most of the reproductive season, as described for the golden hamster

(Berkowitz & Heindel, 1984). The annual minimum plasma LH concentration observed in autumn during the first part of hibernation is in agreement with histological studies on pituitary gonadotrophic activity in the hedgehog (Girod & Curé, 1965). Similar low LH values were also described for two species of golden-mantled ground squirrel (Licht *et al.*, 1982; Barnes, 1986) during the hibernation season. In the present study, although our purpose was not to demonstrate a relationship between body temperature and LH or testosterone secretion during the hibernation season as described in golden-mantled ground squirrels (Barnes, 1986), we did observe, during the end of hibernation (from December), several torpid animals with elevated plasma testosterone concentrations (Saboureau, 1986; El Omari, 1987). Nevertheless, in the hedgehog, a relationship between body temperature, gonadotrophin secretion and testicular endocrine responsiveness must be considered, taking into account the alternation of torpor bouts and periodic arousals in animals studied individually during the hibernation season.

At the end of the breeding season (in August), LH always decreased after testosterone, and this relationship may be due to a decrease of Leydig cell sensitivity to LH. This phenomenon may be related to internal factors involved in preparation for hibernation. At the end of summer, temperatures are still high but photoperiod, which decreases rapidly, may be involved because gonadal activity ceased rapidly in male hedgehogs exposed after the summer solstice to short artificial photoperiods (≤ 12 h light) or 24 h darkness (Saboureau, 1981, 1986).

At the end of autumn and the start of winter the effect of negative steroid feed-back or photoperiod seems to be reduced or eliminated, as increased LH secretion occurs, despite the attendant rise in plasma androgen concentrations and non-stimulatory photoperiod. Comparable results were observed in hibernating rodents (Ellis *et al.*, 1979; Licht *et al.*, 1982; Zucker & Licht, 1983; Barnes, 1986). This result may also be compared to the sudden surge of gonadotrophins seen during spontaneous testicular redevelopment in the golden hamster (Matt & Stetson, 1979).

From these preliminary results it seems that the seasonal cycle of LH in the hedgehog could be regulated by several different mechanisms. Among them (1) endogenous changes in the activity of the hypothalamo-hypophysial system or (2) changes in the sensitivity of the hypothalamo-hypophysial axis to internal (steroid feed-back) or external (e.g. photoperiod, temperature) factors may be involved.

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