Comparative changes in plasma concentrations of progesterone, oestradiol and LH during the ovulatory cycle in a multiple ovulating male line and a single ovulating traditional line of turkeys

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The aim of this study was to compare the profile of circulating concentrations of LH, progesterone and oestradiol in a multiple ovulating male line with that of a single ovulating line of traditional turkeys. Plasma samples from seven traditional and 12 male-line turkeys were obtained every 3 h for 36 h. Male-line and traditional turkeys had single peaks of LH and progesterone that were of similar duration in both lines. The mean height of the plasma peaks of LH and progesterone were similar in the two lines and there was no detectable peak plasma oestrogen

concentration. Mean plasma concentrations of LH and oestrogen were higher in single compared with multiple ovulating turkeys, whereas there were no differences in mean plasma progesterone concentrations. The results indicate that the multiple ovulation state in genetically selected high-growth lines of turkey may be the result of a correlated response in the steroidogenic capacity of ovarian tissue associated with low plasma concentrations of oestrogen rather than of a disturbance in the hormone profile of the ovulatory cycle.

Introduction

The ovarian follicular hierarchy of avian species consists normally of a single series of follicles of increasing size that will be ovulated on separate days after achieving maturity. For a turkey laying an egg each day, the ovary should contain no more than nine or ten of these hierarchical follicles (Hocking, 1987), although some turkeys will have fewer follicles and will not lay on consecutive days. In contrast to traditional turkeys, modern lines that have been genetically selected for high growth rates have relatively large ovaries with many more maturing ovarian hierarchical follicles (Hocking, 1992; Melnychuk *et al.*, 1997). The presence of two or more follicles at each position in the hierarchy results in the ovulation of two or more follicles in a single day. Many of the eggs that result from multiple ovulations are not suitable for incubation because of the increased proportion of double-yolked, soft-shelled and misshapen eggs that are laid (Hocking and Bernard, 1998). A significant proportion of ova from multiple ovulations is not collected by the infundibulum and is lost into the body cavity, where the triglyceride they contain is absorbed (Hocking, 1996). It is not known whether multiple ovulations occur separately throughout the day or at the same time.

A preovulatory peak in LH occurs 4–8 h before ovulation in turkeys and is accompanied by an increase in the

concentration of progesterone in the plasma (Hammond *et al.*, 1981; Sharp *et al.*, 1981; Yang *et al.*, 1997). Opel and Arcos (1978) measured plasma oestradiol concentrations throughout the ovulatory cycle in turkeys, but found no consistent pattern of changes in relation to ovulation. The effect of a multiple follicular hierarchy on the plasma steroid hormone profile during the ovulatory cycle has not previously been investigated in turkeys. Sharp *et al.* (1976) reported that there was no increase in the number of peaks in LH concentration during the ovulatory cycle of a strain of White Leghorn chickens selected for a high incidence of multiple ovulations compared with a commercial laying strain. Sharp *et al.* (1976) concluded that the multiple ovulations were attributable to a defect in the development of the follicular hierarchy rather than to a disturbance of the profile of circulating LH concentrations.

A comparison of the hormonal profile throughout the ovulatory cycle of turkeys that produce single or multiple ovulations has not yet been conducted. Therefore, a serial sampling experiment was performed to compare plasma oestradiol, progesterone and LH concentrations throughout the ovulatory cycle in a single ovulating traditional line and a multiple ovulating male line of turkeys. The aim of the study was to detect any differences in the plasma hormone profiles between the two strains during the ovulatory cycle, and to test the hypothesis that multiple ovulations are associated with multiple pre-ovulatory LH peaks throughout the ovulatory cycle or with a prolonged increase in LH. Both of these situations would indicate that ovulations occur at different times throughout the day. Alternatively, if

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the pre-ovulatory LH peak in the male line resembled that of the single ovulating traditional line, it would imply that several follicles were ovulated together in response to a single LH peak. In spite of the excessively large quantity of ovarian tissue and hierarchical follicles in the male line, plasma oestrogen concentrations are higher in traditional-compared with male-line hens (Buchanan *et al.*, 1999, 2000). A second objective of the present study was to determine plasma oestrogen concentrations to assess the hypothesis that high concentrations at some point during the ovulatory cycle are responsible for the higher prevalence of vaginal prolapse in the male line, as discussed by Buchanan *et al.* (1999). Ideally, plasma concentrations of FSH would also have been determined but the only available avian assay (Krishnan *et al.*, 1993) does not measure turkey FSH.

Materials and Methods

Animals

The strains of turkeys (*Meleagris gallopavo*) used in these experiments were the Big 6 male line, obtained from British United Turkeys Ltd (Chester) and the Nebraska Spot, a traditional line maintained at the Roslin Institute. Thirty female turkeys from each line were obtained at 1 day old and reared in floor pens containing wood shavings. They had constant access to water and were fed a conventional turkey diet provided in suspended tubular feeders. The photoperiod was 14 h light:10 h dark (light from 02:00 to 16:00 h) for the first 18 weeks, 7 h light:17 h dark for the next 11 weeks, followed by photostimulation at 29 weeks and 4 days with 14 h light:10 h dark to stimulate sexual maturity. At 18 weeks of age, four turkeys from each strain were housed in six pens, in a randomized block design. Individual egg production was recorded by feeding the turkeys capsules containing fat-soluble dyes three times per week. Gelatin capsules (size 4, Agar Scientific Ltd, Stansted, Essex) were half filled with Sudan black or Sudan III (red) and fed by gavage three times per week. The traditional turkeys received one capsule and the male line received two capsules because of the dilution effect of their greater body weight. Within a pen, one turkey was given black, one turkey was given red, one turkey was given alternating red and black and one bird was handled but not fed a capsule. Eggs were collected daily, boiled in a waterbath for 20 min and then cut in half. The colour of the dyed rings in the yolks of the eggs enabled the egg production of each hen to be determined. Within each pen, the turkeys with the most regular egg production were selected for the experiment, as they were most likely to ovulate during the sampling period. As the laying pattern of the male line was more irregular than that of the traditional line, 12 male-line and seven traditional-line turkeys were used for the experiment.

The sampling period for plasma collection started 6 weeks after the onset of photostimulation. The turkeys were handled daily in the week before the sampling period to

allow them to become accustomed to handling, so that the interference caused by the sampling was less likely to delay oviposition. Whole blood was obtained from each turkey by superficial venepuncture of the brachial vein and transferred to blood collection tubes coated with 5 ml heparin. The samples were centrifuged at 2000 *g* for 10 min and the plasma was removed and stored in 1.5 ml Eppendorf tubes at -20°C . Each of the selected turkeys was sampled every 3 h for 36 h starting at 10:00 h. The sampling took about 1 h and the turkeys were always sampled in the same order to ensure a period of 3 h between each sample. During the hours when the lights were off, spectacle type head torches (RS Components Ltd, Corby) were used for the sampling to minimize disruption of the lighting schedule. Egg production was recorded hourly for the final 24 h of the sampling.

Turkeys were killed 12 h after the final blood sample was collected by an overdose of sodium pentobarbitone (Veterinary Drug Company, Falkirk). The abdominal cavity was opened and the ovary removed from the body cavity. Yellow ovarian follicles were cut from the residual ovary and follicles > 0.5 g were classed as hierarchical ovarian follicles, counted and weighed. The postovulatory follicles were removed and counted, and the residual ovary was weighed. The sequence of previous ovulations was estimated by arranging the postovulatory follicles according to size, with follicles of similar size grouped together by the same person as likely multiple ovulations. These data supplemented the egg production data and allowed comparison of the ovulation pattern to the hormone profile for each bird. The mass of the residual ovary was also recorded.

Radioimmunoassays

The plasma concentrations of oestradiol, progesterone and LH were measured by classical double antibody radioimmunoassays. Each assay was validated for use on turkey plasma by serial dilution of single samples to check for parallelism with the standard curve. Samples for each hormone were analysed in single assays to eliminate inter-assay variability. Oestradiol concentrations were determined in duplicate using the method of Webb *et al.* (1985). The plasma samples were extracted initially using an antibody–Sephadex extraction procedure because of the high fat content of laying turkey plasma. The efficiency of the extraction procedure was 45.7%. The mean intra-assay coefficient of variation was 15.2% and the lowest sensitivity of the assay was 1.5 pg per tube. The concentration of progesterone was measured in duplicate as described by Law *et al.* (1992). The samples were analysed in a single assay with an intra-assay coefficient of variation of 12.1% and lowest sensitivity of 25 pg per tube. The plasma concentration of LH was determined in triplicate as described by Sharp *et al.* (1987). The intra-assay coefficient of variation was 12.3% and the lowest sensitivity of the assay was 0.045 ng per tube.

Table 1. Mean ($\pm$ SEM) number of postovulatory follicles and number of postovulatory follicles of the largest size in traditional line ($n = 7$) and male line turkeys ($n = 12$)

Postovulatory follicles	Traditional line	Male line	Significance
Number	3.1 ± 0.34	7.9 ± 1.15	$P < 0.01$
Largest (similar size)	1.0 ± 0.00	2.0 ± 0.19	$P < 0.001$

Statistical analyses

Two-sample unpaired Student's *t* tests were used to compare the mean hormone concentrations over the sampling period and the ovarian characteristics for each strain. The height of the peak in hormone concentration was calculated as the difference between the highest concentration and an average baseline value for each bird. The duration of each peak was estimated as the difference in sample time from the start of the steep increase in concentration to the time when the concentration had returned to baseline values. The mean height and duration of the peaks in progesterone and LH were compared using two-sample unpaired Student's *t* tests. A one-sample *t* test was used to test whether the time difference between the peak in progesterone and the peak in LH was significantly different from zero, and a two-sample *t* test was used to compare the time difference between the two lines of turkeys.

Results

Eggs were obtained from five of the seven traditional-line and four of the 12 male-line turkeys during the sampling period. Postmortem examination of the records of eggs laid after the sampling period and the presence of ovulated follicles in the oviduct when the birds were killed indicated that all of the traditional-line and nine of the 12 male-line turkeys had ovulated during the sampling period.

The male line had significantly more postovulatory follicles than did the traditional line (Table 1). Male-line turkeys also had a significantly greater incidence of two or more postovulatory follicles at the largest position (Table 1).

Typical traces of the changes in plasma oestradiol, progesterone and LH throughout a 36 h period in a traditional-line and a male-line turkey that had ovulated during the sampling period are shown (Fig. 1a,b). Plasma LH peaks were clearly visible in ten of the male-line and all of the traditional-line turkeys. One of the male-line turkeys showed a distinct LH peak, although no ovulated follicles were present in the oviduct (Fig. 1c) and two male-line turkeys showed no evidence of a peak for any of the plasma hormones measured (Fig. 1d).

No turkey from either strain showed more than one peak in the plasma concentration of LH during the sampling period. The mean height and duration of the peak and the

Fig. 1. Changes in plasma concentration of oestradiol ($\blacktriangle$), progesterone ($\bullet$) and LH ($\circ$) at 3 h intervals during a 36 h sampling period in turkeys. (a) Traditional-line turkey that ovulated during the sampling period. (b) Male-line turkey with a recorded ovulation. (c) Male-line turkey in which no oviposition was recorded and an internal ovulation is assumed to have occurred. (d) Male-line turkey in which there was no evidence for oviposition or ovulation. Bar at the top of the figure represents the photoperiod (open) and scotoperiod (filled).

Fig. 2. Mean ($\pm$ SEM) plasma concentration, mean height of pre-ovulatory peak and mean duration of the peak plasma concentration of (a) LH and (b) progesterone in traditional-line ($\square$; $n = 7$) and male-line ($\blacksquare$; $n = 12$) turkeys sampled every 3 h for 36 h.

overall mean plasma concentration of LH for the two strains are shown (Fig. 2a). The mean plasma LH concentration was significantly greater in the traditional line than in the male line ($P < 0.001$), whereas there were no differences in the mean height or duration of the LH peaks between the two strains.

Plasma progesterone concentration showed a pre-ovulatory increase that coincided with the increase in plasma LH in both the traditional- and male-line turkeys (Fig. 1a,b). No such increase was visible in either of the male-line turkeys that did not ovulate during the sampling period (Fig. 1d). Progesterone concentrations reached a peak significantly later than LH concentrations (2.0 ± 0.5 h, $P < 0.001$) but there was no difference in this time period between the two strains. There was no significant difference in the duration of the progesterone peak between the two strains (Fig. 2b). The progesterone peak lasted slightly longer than the LH peak in both strains, but this difference was not statistically significant. The mean plasma progesterone concentration and mean height of the progesterone peak in the traditional- and male-line turkeys are also presented (Fig. 2b): neither the mean plasma concentration

Fig. 3. Plasma oestradiol concentrations, ovary mass and number of follicles in traditional-line ($\square$; $n = 7$) and male-line ($\blacksquare$; $n = 12$) turkeys. (a) Mean ($\pm$ SEM) number of hierarchical follicles and residual ovary mass 12 h after the end of a 36 h sampling period. (b) Mean ($\pm$ SEM) plasma concentration and mean maximum concentration of oestradiol sampled every 3 h for 36 h.

nor the mean plasma peak of progesterone were significantly different between the traditional and male lines.

There was no consistent pattern of changes in plasma oestradiol concentration in either the traditional- or the male-line turkeys. Some individuals showed peaks in plasma oestradiol around the same time as the LH peak (Fig. 1a,b), in others peaks occurred before or after the LH peak and in some there was no distinct oestradiol peak. The mean plasma oestradiol concentration was significantly lower in the male line compared with the traditional line ($P < 0.001$), as was the mean of the highest concentrations ($P < 0.05$) (Fig. 3b).

The male line had significantly more hierarchical follicles ($P < 0.001$) and a significantly greater residual ovary mass ($P < 0.001$) compared with the traditional line (Fig. 3a).

Discussion

The data from the 36 h sampling period showed peaks in plasma progesterone and LH associated with ovulation in both traditional- and male-line turkeys. These findings are in agreement with previous findings in turkeys (Hammond *et al.*, 1981; Sharp *et al.*, 1981) and domestic hens (Etches, 1996a). The lack of consistent patterns of oestradiol concentrations during the ovulatory cycle in either strain of turkey is also in agreement with previous results (Opel and Arcos, 1978).

The LH peaks were no more frequent in the male line

than they were in the traditional line, indicating that multiple follicles are ovulated in response to single pre-ovulatory peaks of LH and progesterone. This finding is similar to those reported by Sharp *et al.* (1976) that multiple ovulations occurred in the domestic hens after single LH peaks.

Multiple ovulations occurred in the male line during the sampling period as indicated by the presence of two or more fresh postovulatory follicles, although these were not necessarily associated with yolks in the oviduct or in laid eggs. The abdominal cavity of male-line turkeys frequently contained intact ovulated follicles or yolky fluid that was assumed to have come from ova that were being absorbed. The results indicate that a significant proportion of ovulated follicles is lost as internal ovulations into the abdominal cavity in male-line turkeys. No more than one egg was collected from each turkey during the 36 h period, confirming that the additional postovulatory follicles in the male line must represent follicles lost through internal ovulation or eggs with defective shells that were destroyed before they could be collected.

Most of the eggs laid during the sampling period contained yolks that were ovulated before the start of the sampling period and it was not possible to calculate the time between ovulation and subsequent oviposition. In general, the results did not show a clear relationship between oviposition and ovulation, such as occurs in fowl (Etches, 1996a), in which ovulation tends to occur 30–45 min after oviposition of the previous egg. A longer period of recording egg production after the sampling period would be necessary to determine the time interval between ovulation and oviposition. In the present study, it was necessary to record the presence of fresh postovulatory follicles to establish whether ovulation had occurred and a longer egg collection period was not possible.

The presence of gaps in the hierarchy corresponding to days when no follicle was recruited is more common in male-line than it is in traditional-line turkeys (Hocking and Bernard, 1998). The fact that such a gap was not observed in traditional-line turkeys in the present study may be coincidental or a consequence of prior selection for regular oviposition. The absence of peaks for LH and progesterone in ovulatory cycles without an observed or inferred ovulation is consistent with the conclusion that follicular growth and recruitment occur normally in multiple ovulating birds but that more than one follicle is likely to be recruited in any one period.

The peaks in plasma progesterone and LH were no more prolonged in the male line than they were in the traditional line, which further supports the hypothesis that several follicles are released in response to a single LH surge in the multiple ovulating strain. It seems likely that these follicles are released around the same time, relative to the time of peak plasma LH concentration. If the follicles were ovulated many hours apart, several peaks or an extended increase in LH might have been observed in a multiple ovulating line. The release of several follicles from the ovary at a similar

time during the ovulatory cycle may make it difficult for the infundibulum to capture all of the follicles that are ovulated. If the oviduct did capture more than one follicle, this is likely to lead to the formation of double-yolked, misshapen or soft-shelled eggs (van Middelkoop, 1971; Hocking *et al.*, 1987).

There was no clear pattern of changes in plasma oestradiol concentration throughout the ovulatory cycle in turkeys. The results did not provide any evidence for higher peak oestradiol concentrations in the male line than in the traditional line. When the mean plasma oestradiol concentration over the 36 h sampling period was compared between the two strains, plasma oestradiol was lower in the male line, confirming earlier reports of single-point estimates (Buchanan *et al.*, 1999, 2000).

The present results showed that plasma LH was lower in the multiple ovulating male line of turkeys, in contrast to the absence of differences in the mean plasma concentrations of LH between single and multiple ovulating groups of domestic hens (Sharp *et al.*, 1976). Williams *et al.* (1986) reported a significant difference in plasma LH concentration between fat-line and lean-line chickens, with a greater LH concentration in the lean line. It is unclear why selection for different body traits, such as increased abdominal fat or increased growth rates, should alter plasma LH concentration. It is possible that the capacities of the hypothalamus or pituitary of male-line turkeys have not increased to produce circulating plasma hormones in proportion to the increased body size of the strain. Alternatively, differential sensitivity of the hypothalamus and pituitary to stimulatory or inhibitory signals may be involved. Food restriction changes the sensitivity of the pituitary to cLH-releasing hormone I, and possibly gonadal hormones, during the laying period (Bruggeman *et al.*, 1998), and genetic changes associated with selection for growth rate and body composition might have had a similar effect.

No differences were found in the mean progesterone concentrations over the 36 h sampling period between the two strains, consistent with results obtained at a single time point (Buchanan *et al.*, 1999, 2000). In both strains, progesterone increased to two to four times its basal concentration in a preovulatory peak that occurred slightly later than the preovulatory LH peak. This finding is consistent with that of Sharp *et al.* (1981) who showed that there was a progesterone peak about 4 h after LH in the domestic fowl. A more frequent sampling regimen would be required to determine whether the duration of increased progesterone concentration is significantly longer than the duration of increased plasma LH concentration.

From studies in domestic hens, it is thought that the onset of the preovulatory surge of progesterone stimulates the release of LH from the hypothalamus in a positive feedback mechanism that eventually results in ovulation, which is followed by a decrease in the release of LH as a result of negative feedback from high plasma steroid concentrations (Etches, 1996b). The ovary is the major source of progesterone and oestradiol: the largest follicles produce

progesterone whereas the smaller follicles and residual ovary produce predominately oestrogens (Armstrong, 1984; Porter *et al.*, 1991; Buchanan *et al.*, 2001). Therefore, it was surprising that the male-line turkeys, which have many more mature follicles and a much bigger residual ovary, do not have higher plasma concentrations of progesterone and oestradiol compared with the traditional line. The results indicate that the steroidogenic capacity of the ovarian cells in the large ovary of the male line may be poor and are consistent with studies of individual ovarian follicles *in vivo* (Buchanan *et al.*, 2001). Poor synthesis of oestrogen by the ovarian cells resulting in a decrease in plasma hormone concentration may affect the sensitivity of the hypothalamus and anterior pituitary, resulting in increased FSH secretion, overstimulation of ovarian growth and increased recruitment into the follicular hierarchy. The presence of lower plasma LH concentrations in the male line is consistent with this hypothesis. Alternatively, it is possible that several follicles of the largest size are required to produce sufficient progesterone to trigger ovulation in the male line. If this were the case, then genetic selection may have inadvertently resulted in the development of the typical multiple follicular hierarchy via selective breeding from females with two or more mature follicles that collectively produced enough progesterone to cause ovulation in birds with decreased sensitivity of the hypothalamus to gonadal steroids. Genetic selection for high growth rates and meat yields in turkeys may have led to an increase in the propensity for multiple ovulation by at least two routes: firstly, by overstimulation of ovarian growth as a result of a lack of control by negative feedback of oestrogens (the peripheral, or decreased gonadal steroid hypothesis) and, secondly, by requiring the progesterone output of several follicles for ovulation to occur (the central, or decreased hypothalamus–pituitary sensitivity hypothesis). However, genetic selection may act primarily through changes in local growth factor expression in the ovary, hypothalamus and pituitary that result in altered sensitivity of these tissues to regulatory signals, as has been well documented for the insulin-like growth factor (IGF) system (Lackey *et al.*, 1999).

In summary, male-line turkeys that are characterized by multiple ovulations do not ovulate in response to multiple or extended peaks in the plasma concentrations of LH or progesterone. There was no difference in the height of the plasma peaks in LH between male-line and traditional-line turkeys, whereas the mean plasma concentration of LH was higher in traditional- than it was in male-line turkeys. The mean plasma peaks and the overall mean plasma concentrations of progesterone for the two lines were not significantly different. There was no detectable peak in plasma oestrogen concentration during the ovulatory cycle, and the mean plasma concentration of oestrogen was lower in male-line than it was in traditional-line turkeys. The multiple ovulation state in genetically selected high-growth lines of turkeys may be caused by a correlated response in the steroidogenic capacity of ovarian tissue associated with

low plasma concentrations of oestrogen and a change in the sensitivity of the hypothalamus–pituitary axis.

This work was funded by a CASE studentship from the Ministry of Agriculture, Fisheries and Food and British United Turkeys Ltd. Male-line turkey poulters were kindly donated by British United Turkeys Ltd, Chester. Thanks are due to Gerry Baxter and Richard Talbot for help with radioimmunoassays. The authors also acknowledge helpful discussions with Elaine Watson.

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Received 22 March 2001.

First decision 22 May 2001.

Revised manuscript received 31 August 2001.

Accepted 27 September 2001.