Physiological basis of prolapse of the oviduct in turkeys

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described here is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

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ABSTRACT

Prolapse of the oviduct is a common problem which results in 5% mortality of female male-line breeding turkeys that have been selected for high meat yield. Prolapse is rare in unselected traditional-line turkeys. Male-line turkeys are characterised by large multiple ovulating ovaries compared to traditional-line turkeys.

It was proposed that the large ovary of the male-line resulted in a high plasma oestradiol concentration, which stimulated collagen degradation in the oviduct, impairing the structural integrity of the oviduct and predisposing the male-line to prolapse.

There was no disproportion in growth of the oviduct, ovary, uterus, vagina, *sphincter ani* muscle or muscular cord of ventral ligament in the male-line compared to the traditional-line. Histological investigation of the uterus, vagina, *sphincter ani* muscle and muscular cord of ventral ligament from traditional-, male-line and prolapsed male-line turkeys revealed no abnormalities that could be associated with prolapse.

The male-line had a lower vaginal collagen content than the traditional-line, while male-line turkeys with prolapse had an even lower vaginal collagen content. No differences were seen in the collagen content of the uterus between the different groups. It was concluded that reduced vaginal collagen in the male-line impaired the structural integrity of the vagina and predisposed the strain to prolapse.

The collagen of the prolapsed turkeys had fewer mature cross-links than the collagen of the non-prolapsed turkeys that would substantially reduce the strength of the tissue. This suggested that there may be increased collagen turnover in the prolapsed turkeys.

The male-line had a lower plasma oestradiol concentration than the traditional-line, and there was no significant difference in plasma progesterone concentration between the two strains.

Administration of oestradiol increased plasma oestradiol concentrations but had no effect on the vaginal collagen content in either strain. Progesterone administration also had no effect on vaginal collagen content in either strain although plasma progesterone was raised.

Ovulatory cycle changes in plasma concentrations of oestradiol, progesterone and luteinizing hormone in traditional and male-line turkeys were compared. It was concluded that male-line turkeys did not have higher concentrations of oestradiol or progesterone at any time during the ovulatory cycle.

Incubation of ovarian follicles from traditional- and male-line turkeys to measure the output of progesterone and oestradiol demonstrated that the multiple follicular hierarchy of the male-line had a physiological and anatomical basis. Oestradiol output was lower from male-line follicles compared to those from the traditional-line, even in the presence of luteinizing hormone. This was consistent with the lower plasma oestradiol concentration of the male-line. Aromatase activity was also lower in small follicles from the male-line than the traditional-line. The results suggest that ovarian steroidogenesis is reduced in male-line turkeys.

Food restriction of male-line turkeys to reduce body weight at sexual maturity to 0.6 of *ad libitum* fed controls inhibited the onset of lay, but had no effect on the multiple hierarchy, plasma oestradiol concentration or the vaginal collagen content of those in lay. Delaying photostimulation of male-line turkeys by 5 weeks also had no effect on the multiple hierarchy, plasma oestradiol concentration or vaginal collagen content.

It was concluded that prolapse in male-line turkeys was associated with low vaginal collagen and that this was not caused by high plasma concentrations of oestradiol.

Publications

Buchanan, S. and Hocking, P.M. (1998). Comparison of the reproductive system in turkeys with a high or a low susceptibility to uterine prolapse. *British Poultry Science,* **39**, S19-S20. (Abstract)

Buchanan, S & Hocking, P.M. (1998). The role of collagen in uterine prolapse in turkeys. *Proceedings of the 10th European Poultry Conference,* Israel. (Abstract no. 121)

Buchanan, S & Hocking, P.M. (1998). Changes in plasma oestradiol and luteinizing hormone during the ovulatory cycle in a multiple ovulating and a single ovulating strain of turkey. *Journal of Reproduction and Fertility, Abstract Series,* **22**, 24. (Abstract)

Buchanan, S. and Hocking, P.M. (1999). The relationship between vaginal collagen, plasma oestradiol and uterine prolapse in turkeys. *British Poultry Science*,(In press)(Abstract)

Buchanan, S & Hocking, P.M. (1999) Physiology and significance of multiple ovulations in turkey breeders. *Proceedings of the 22nd Technical Turkeys Conference,* Maccelsfield, UK.

Buchanan, S & Hocking, P.M. (1999) Ovarian steroidogenesis in single and multiple ovulating turkeys. *Poultry Science (Supplement 1)* **78** Abstract No 81 p19

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1. General introduction

Prolapse of the oviduct is common in female turkeys from the male-lines that have been selected for high meat yield. The prolapsed oviduct rapidly becomes torn and infected and the turkeys usually die from shock, haemorrhage or peritonitis. It is common practice on turkey breeding farms to cull birds at the first sign of prolapse to alleviate any suffering. Prolapse results in the loss of about 5% of male-line breeding flocks and is an important economic and welfare problem. The incidence of prolapse is lower in unselected traditional-lines and female-lines that have been selected for high egg production.

The work presented here was based on comparisons between male-line turkeys and a traditional unselected strain, the Nebraska Spot, with the aim of identifying factors in the male-line that could predispose it to prolapse. Figure 1.1 shows female turkeys from these two strains at 35 weeks of age.

Prolapse in turkeys is characterised by evertion of the vagina and protrusion of the everted vagina through the ventral opening. Gradually the rest of the oviduct also moves out of the body cavity through the vent. However the utero-vaginal sphincter remains intact preventing the rest of the oviduct from everting. The situation worsens over a couple of days and the intestines may follow the oviduct out of the vent. Figures 1.2, 1.3 and 1.4 shows male-line turkeys at various stages of prolapse. Prolapse of the oviduct is often referred to as uterine prolapse, which is misleading as the uterus, or shell gland as it is sometimes known, does not appear to be any more involved than the rest of the oviduct.

FIGURE 1.1 Female turkeys at 35 weeks of age from a male-line, the Big 6 male-line (left) and a traditional-line, the Nebraska spot (right)

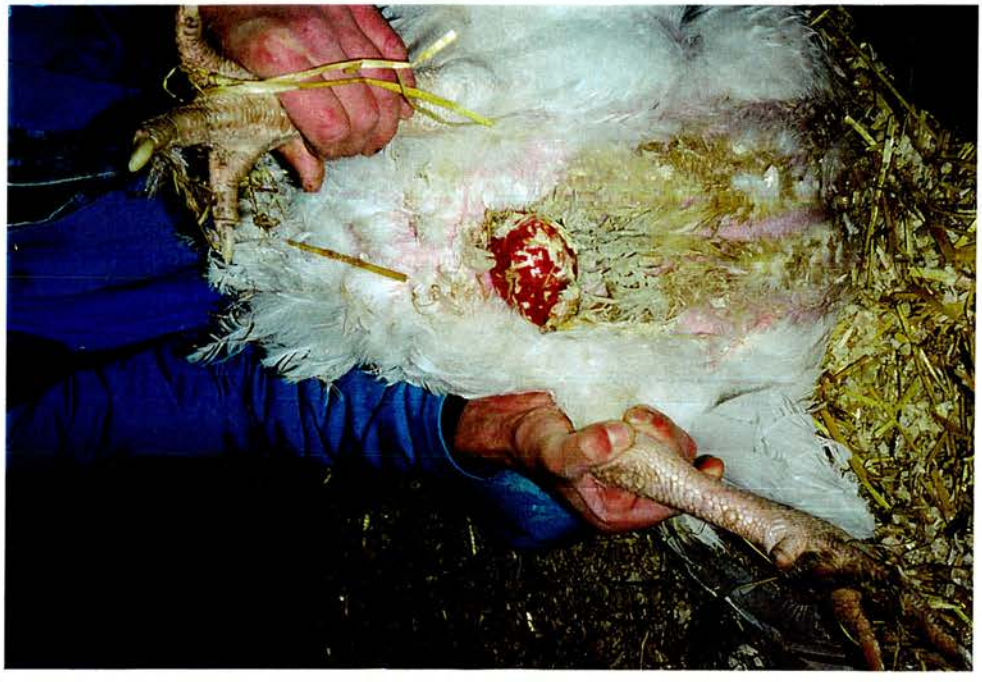


FIGURE 1.2 Female male-line turkey at 35 weeks of age with prolapse of the oviduct at the initial stage.

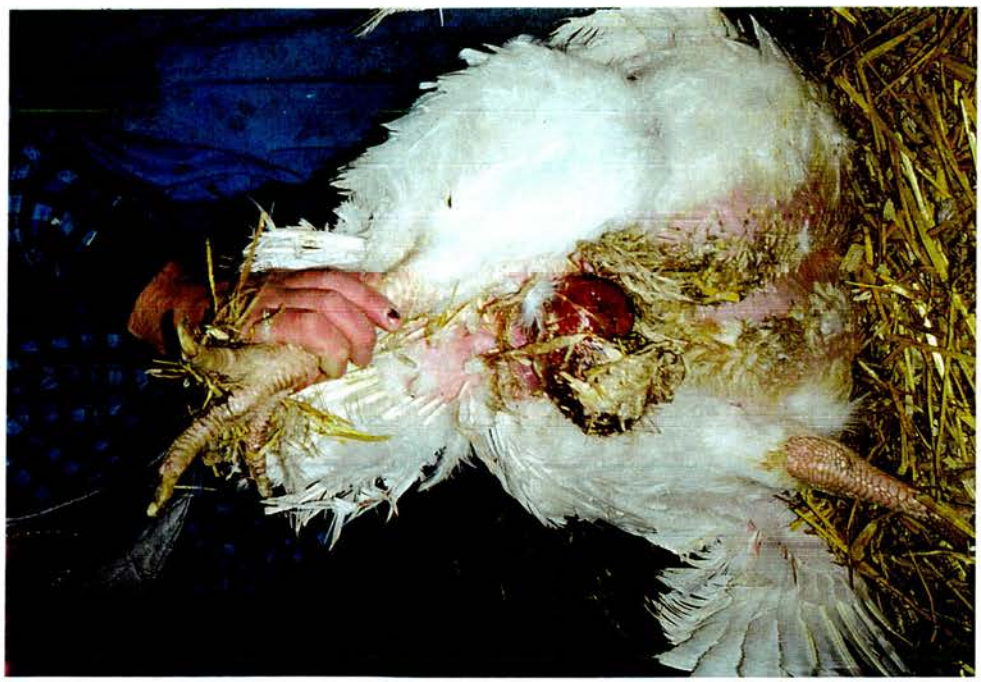


FIGURE 1.3 Female male-line turkey at 35 weeks of age with worsening prolapse of the oviduct.

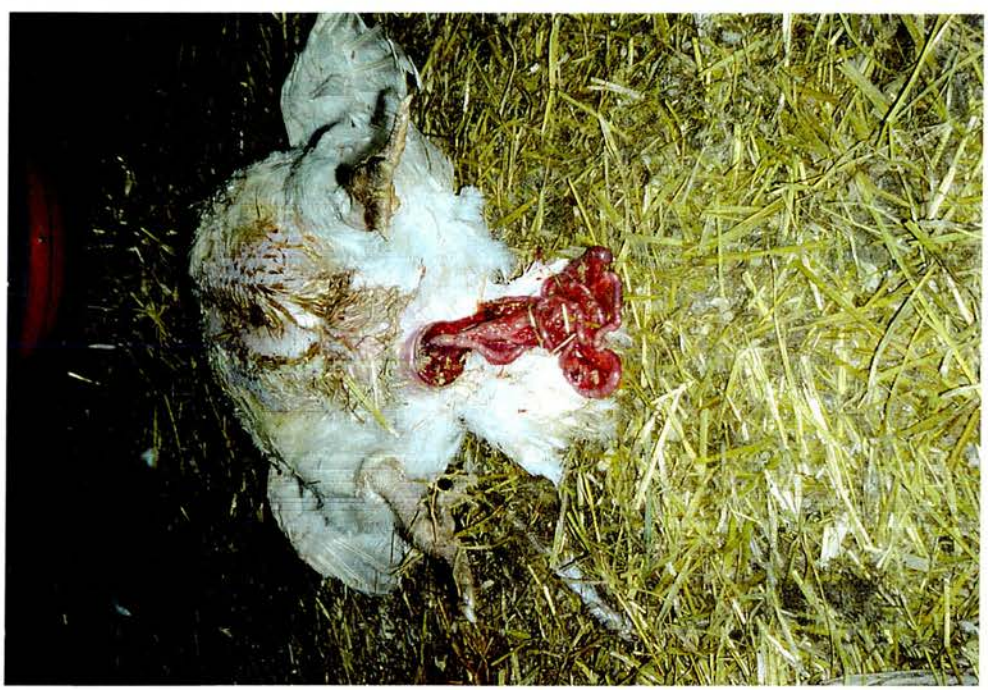


FIGURE 1.4 Female male-line turkey at 35 weeks of age that had died as a result of prolapse of the oviduct.

1.1 Prolapse in breeding turkeys

Intense selection for high breast muscle yield has taken place over the last 30 years in the turkey industry. The male-lines have been selected predominately for large breast meat yield while the female-lines have been selected mainly for egg production with some emphasis also on meat yield. Commercial meat-type turkeys are produced from parent stock by crossing male-line males and female-line females. The parent stock are supplied by turkey breeding companies such as British United Turkeys who produce separate male- and female-lines. Male-line females are grandparent stock, used by the breeding companies to produce male-line male turkeys.

Prolapse occurs at a much lower incidence (1%) in female-line turkeys and therefore is not considered to be a problem for turkey producers who only breed from female-line females and male-line males. It is the turkey breeding companies who utilise male-line females that have a considerable problem with prolapse. Figure 1.5 shows the number of turkeys culled due to prolapse in 4 strains of turkeys throughout the first 26 weeks of egg production.

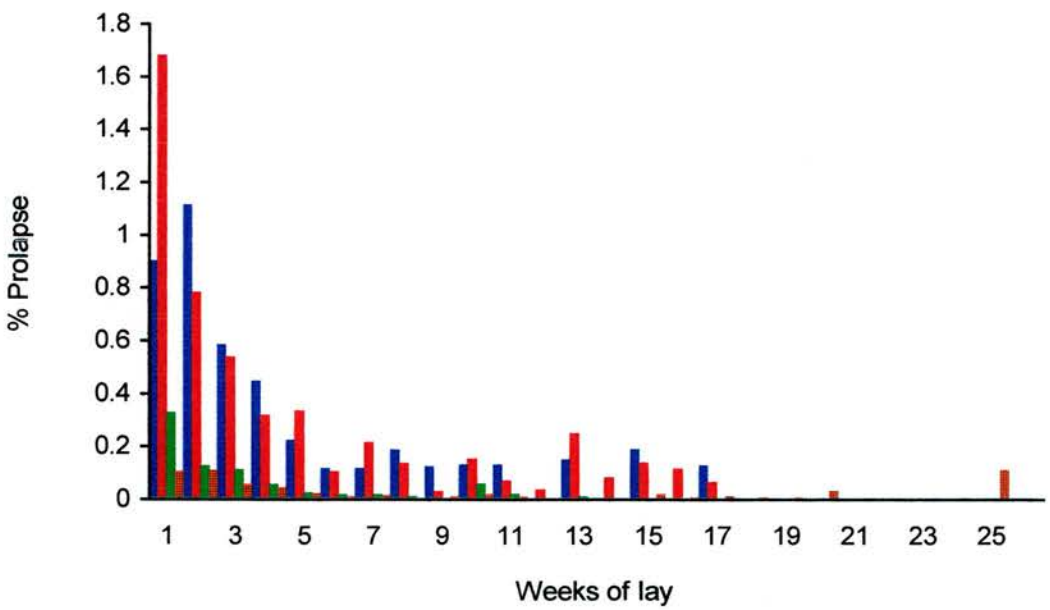


FIGURE 1.5 Weekly incidence of prolapse in four strains of turkeys throughout 26 weeks of lay. (Data supplied by Dr N French, British United Turkeys Ltd.)
Key: Big 6 male-line, Big 5 male-line, T5 female-line, B5 female-line

The Big 6 male-line and the Big 5 male-line are strains of Large White turkeys that have been selected for high breast muscle yield while the T5 female-line and B6 female-line have been selected for high rates of egg production. Clearly prolapse is more common in the male-lines than in the female-lines. Figure 1.5 also shows that prolapse is most common during the first two to three weeks of lay.

The incidence of prolapse is extremely variable and can exceed 10% of a flock, particularly if it faces a stressful challenge such as turkey rhinotracheitis or if the weather is particularly hot (Dr N French, personal communication). However other flocks can have a very low incidence of prolapse. This can make investigation into the factors involved in prolapse difficult and it would be useful to find a marker for the likelihood of prolapse. Such a marker would allow the potential benefits of treatments against prolapse to be assessed and may also allow selection against prolapse to be carried out.

There does not appear to be any obvious connection between the number of eggs laid and the likelihood of prolapse. While some turkeys prolapse having never laid others lay several eggs before prolapse occurs. Prolapse of the oviduct causes difficulties with oviposition (the laying of an egg). Some turkeys become egg bound (unable to pass an egg out of the oviduct) while other turkeys will continue to lay with a prolapse although oviposition may be prolonged.

Peritonitis (infection of the peritoneal cavity) is often seen in association with prolapse in turkeys and it was thought that this could be a factor involved in initiating prolapse (Dr N French, personal communication). A trial was carried out by British United Turkeys Ltd to assess this hypothesis. Flocks of female turkeys were injected with antibiotic (Terramycin LA) in an attempt to reduce peritonitis. However there was no difference in the incidence of prolapse between control and antibiotic injected groups. It is likely that the peritonitis observed at post mortem of prolapsed turkeys occurs as a consequence of the prolapse and is not a factor involved in causing it.

Hocking (1993b) compared the reproductive system at the onset of lay in male- and female-line turkeys. No disproportion between egg size, pelvic dimension, vent diameter or abdominal fat pad was found in the male-line, compared to the female-lines and it was concluded that these factors are not involved in the high incidence of prolapse.

Melnychuk *et al.* (1994; 1997) compared reproductive development between male- and female-line turkeys and concluded that the ovary of the male-line achieved its fully developed weight 3 days earlier than the ovary of the female-line. This led them to hypothesise that the oviduct of the male-line may be immature in comparison to the ovary at the onset of ovulations resulting in a high number of internal ovulations. The findings of Melnychuk *et al.* (1997) are investigated in Chapter 3.

Oviduct development in turkeys was investigated by Lilburn and Nestor (1993) by comparing two experimental strains; one that had been selected for high egg production and the second for high body weight. They concluded that the oviduct of the egg strain was heavier relative to body weight compared to the meat strain. This is unsurprising as the large muscle content of the meat strain greatly increases body weight in comparison to the weight of the viscera and organs. They also found that there was a significant increase in weight of the oviduct of the meat strain during the first 49 days of lay, while the body weight of the egg strain after 49 days of laying was not significantly different from that at first egg.

These studies suggested that the oviduct of strains of turkeys selected for increased meat yield may not be fully developed at the onset of egg production. An immaturity of the oviduct could impede the passage of an egg through the oviduct and predispose male-line strains to prolapse of the oviduct.

1.2 Egg production and ovarian follicles

Egg production is lower in turkeys compared to domestic hens and is lowest in male-lines compared with traditional-lines (Hocking and Bernard 1998). In laying birds

there is a hierarchy of developing ovarian follicles that will be ovulated and become the yolks of eggs. Domestic hens have a hierarchy of 4-6 follicles of decreasing size that will be ovulated over the next 6 days. The rest of the ovary consists of many small follicles and stromal tissue. The most common fate for follicles with a diameter less than 8mm is to become atretic while follicles greater than 8 mm usually develop fully and are ovulated (Gilbert *et al.* 1983)

Development of ovarian follicles takes 2 days longer in turkeys than domestic hens (Hocking 1987) and for a turkey laying one egg a day there should be 9-10 follicles greater than 8mm on the ovarian hierarchy. Male-line turkeys have larger ovaries with many more hierarchical follicles on the ovary compared to traditional- and female-lines (Hocking 1992b; Melnychuk *et al.* 1994; Melnychuk *et al.* 1997; Hocking and Bernard 1998). Figure 1.6 shows ovaries from traditional- and male-line turkeys after 5 weeks of photostimulation.

The male-line ovarian follicles are arranged as a multiple hierarchy with several follicles of a similar size at each position. Traditional-line turkeys have a much lower incidence of multiple follicles (Hocking and Bernard 1998). Figure 1.7 shows the follicular hierarchy from a traditional- and a male-line turkey.

The multiple follicular hierarchy of the male-line results in the ovulation of more than one follicle each day which reduces egg production through the production of double-yolked, soft-shelled and misshapen eggs that are not suitable for incubation. Many follicles are also lost through internal ovulations, ovulated follicles that are not captured by the oviduct and that are lost in the abdominal cavity. Female-lines also have a multiple follicular hierarchy although they have fewer follicles at each position than the male-lines (Hocking 1992b). By reducing the number of mature follicles in the hierarchy a more regular ovulation pattern of one follicle per day may be established and could result in an increased number of eggs that are suitable for incubation.

FIGURE 1.6 Ovaries from male-line (left) and traditional-line (right) turkeys at 35 weeks of age.

FIGURE 1.6 Hierarchical follicles and residual ovary from male-line (bottom) and traditional-line (top) turkeys at 35 weeks of age.

1.3 Lighting regime

Turkeys are naturally seasonal breeders and it is standard practice on turkey farms to control the amount of daylight that the birds receive to allow the timing of sexual maturity to be controlled independent of the time of year.

Day old poults are usually given 23 hours light and one hour darkness (23L:1D) for the first day to assist them with finding food and water. The photoperiod (hours of daylight) is then reduced to 14L:10D. At 18 weeks of age the photoperiod is further reduced to 7L:17D before being increased to 14L:10D at 29 weeks and 4 days. This increase in photoperiod stimulates the development of the reproductive system and the onset of sexual maturity. Egg production follows the increase in photoperiod after about 14 days in female-line turkeys and 21-28 days in traditional- and male-line turkeys.

Turkeys in the wild would be subjected to a gradual increase in photoperiod as day length increased during springtime whereas in industry the day length is suddenly changed from a short to long photoperiod. The sudden change in lighting pattern could alter the pattern of normal growth of the oviduct and ovary. Melnychuk *et al.* (1996) compared the effect of a gradual step-down: step-up lighting regime to a conventional one on the development of the reproductive system. They found that the ovary reached mature weight 10 days earlier on the gradual lighting regime and the mature ovary weigh was lower compared to the conventional lighting regime. There was no difference in the total number of eggs or settable egg number between the two regimes. The gradual lighting regime resulted in a smaller egg size and fewer double yolked eggs.

Although no experiments have been reported on the effects of step-down, step-up lighting regimes on the incidence of prolapse in poultry, Etches (1996b) reported that it was widely accepted within the industry that a step-down, step-up lighting regime does decrease the incidence of prolapse. A trial was carried out by British United Turkeys Ltd to investigate the effect of step-down, step-up lighting on the incidence

of prolapse in male-line turkeys (Dr N French, British United Turkeys Ltd. personal communication). There was no significant difference between the control and step-up: step-down groups although the incidence of prolapse was low in both treatments.

The incidence of prolapse was recorded during a trial of early and late photostimulation of Big 6 male-line female turkeys (Dr N French, personal communication). The turkeys were photostimulated either at 29 weeks and 4 days or 2 weeks early or 2 weeks late and there was no difference in the incidence of prolapse (4.5%) between the three groups.

1.4 Prolapse in domestic hens

Laying domestic hens may also suffer from prolapse of the oviduct. The prolapsed oviduct attracts attention from other hens and is subjected to pecking damage. In extreme cases this results in the loss of the oviduct and intestines through cannibalism (Savory 1995). It is difficult to establish whether the original cause of the problem was prolapse or pecking as the vent everts during oviposition and this can attract the attention of other birds. Large body weight, large egg size, hot weather, bright light and nutritional deficiencies are all factors that may be involved in predisposing a flock of hens to prolapse and vent pecking (Cobb 1986).

It is thought that there may be a connection between oestradiol and prolapse in domestic hens. Administration of diethyl-stilbestrol implants to cockerels causes lateral migration of the pubic bones and results in a more pliable vent (Burmester 1948). It has also been shown in cockerels that a single treatment with a 15mg pellet of diethyl-stilbestrol can result in prolapse of the intestines through the vent (Wheeler and Hoffman 1948). However implantation of diethyl-stilbestrol pellets to hens had no significant effect on the incidence of prolapse (Burmester 1948).

Shemesh *et al.* (1982) measured plasma oestradiol levels in laying hens with prolapse, incipient prolapse and control hens showing no signs of prolapse. They showed that the plasma oestradiol concentration was lowest in the prolapsed hens

followed by the hens with incipient prolapse, while the control hens had the highest plasma oestradiol concentration. This suggested that oestrogen deficiency may be involved in prolapse and led them to test the effect of oestrogen administration on recovery from prolapse. Injection of 100 ng oestradiol was found to aid recovery of prolapsed hens (Shemesh *et al.* 1984). However injection of between 10 and 100 μg of oestradiol hindered recovery of hens from prolapse (Shore *et al.* 1984). It was proposed by Shore *et al.* (1984) that oestradiol may have a biphasic action, aiding recovery from prolapse at low doses while hindering it at higher doses. These results suggest that oestradiol may be involved in prolapse but the nature of this involvement remains unclear.

A small trial to test the effect of oestradiol injection on turkeys with prolapse was carried out by British United Turkeys Ltd (Dr N French, personal communication). Prolapsed male-line turkeys (13) were given a series of 0.1 ml injections of oestradiol (either 10 μg per ml or 5 mg per ml). There was no improvement in the recovery from prolapse associated with the oestradiol injections. However it would be necessary to test a larger number of turkeys to define any relationship between oestradiol administration and recovery from prolapse.

Rao *et al.* (1985) investigated prolapse in a New Hampshire strain of hen and suggested that prolapse was associated with over development of the ventral ligament. They found that there was no obvious ventral ligament in the small number of control hens that were studied while the prolapsed hens had a fully developed ventral ligament that consisted of collagen and muscle fibres. They hypothesised that over-development of the ventral ligament causes increased torsion at the utero-vaginal junction of the oviduct, and that this impeded the passage of the egg resulting in prolapse. It was suggested that the over-development of the ventral ligament could arise due to a hormone imbalance in this strain of hen.

1.5 Prolapse in other species

Prolapse of the oviduct occurs in many species such as dairy and beef cattle, sheep, cats and camels (Richardson *et al.* 1981; Risco *et al.* 1984; Hosie *et al.* 1991; Ramadan and Hafez 1993; deMaaar 1996). Prolapse in these species is often associated with parturition and there is a good chance of recovery. Various methods exist to aid recovery such as repositioning the oviduct under anaesthesia and using stitches or plastic devices to hold the oviduct in place.

In human females prolapse causes great discomfort, incontinence and difficulty in defecation and surgical procedures are performed to rectify the condition. Most of the literature on prolapse in humans concentrates on the variety of surgical techniques that are used to repair the condition. This review is only concerned with the small amount of work that has been done investigating the causal factors involved with prolapse in humans that could be relevant to prolapse in turkeys.

Makinen *et al.* (1986) compared the histology of vaginal connective tissue from patients with prolapse to samples taken from patients with unrelated gynaecological problems. There was an increase in the number of slides classed as abnormal in the patients with prolapse. The abnormal slides showed decreased numbers of fibroblasts and increased amounts of collagen between cells. They went on to investigate collagen synthesis in association with prolapse and found that collagen synthesis was slightly increased in fibroblast cell cultures derived from patients with prolapse compared to controls (Makinen *et al.* 1987). They also found that procollagen mRNA levels were increased in association with prolapse which supported their finding of increased collagen synthesis. However this increased collagen synthesis could have occurred in response to prolapse rather than being a cause of it. It is possible that synthesis of vaginal collagen increases following prolapse in an attempt to improve the support of the prolapsed tissue.

Gilpin *et al.* (1989) carried out histological investigation of the *pubococcygeus* muscle in women with or without prolapse. The *pubococcygeus* muscle is a sphincter

muscle that surrounds the vaginal opening and makes up part of the pelvic floor and has a similar function to the *sphincter ani* muscle of turkeys. They found that there was hypertrophy of type II muscle fibres, increased proportions of type I muscle fibres and increased numbers of cells with centralised nuclei. Centralisation of nuclei is observed following muscle fibre denervation and subsequent re-innervation. These results suggest that there may have been previous damage to the nerve supply of the muscle, which could have occurred during parturition. However, as prolapse also occurs in nulliparous women (DeLancey 1993), damage to the nervous supply during parturition cannot be the sole factor involved in prolapse.

The relationship between joint hypermobility and the incidence of prolapse in women has been investigated (Norton *et al.* 1990). It was shown that there was an increased incidence of prolapse in women showing signs of joint hypermobility, which suggests that the two conditions share a similar aetiology. Joint hypermobility is a symptom of connective tissue disorders. This lead them to suggest that a connective tissue disorder may be involved in the aetiology of prolapse.

Norton *et al.* (1992) investigated collagen synthesis in women with prolapse compared to unaffected controls and women with stress incontinence. They found that the ratio of type I to type III collagen was reduced in the vaginal tissue of half of the patients with prolapse compared to the control patients. There was no difference in the type I:III ration in patients suffering from stress incontinence, which suggests that the aetiology of stress incontinence is different from that of prolapse. They concluded that an abnormality in the type I:III ratio of vaginal collagen could predispose individuals to prolapse by altering the mechanical properties of the vaginal tissue. Type I fibres promote tissue stability while type III fibres promote flexibility of the tissue (Kuhn and Glanville 1980) and an alteration in the ratio of these fibre types would therefore affect the mechanical properties of the tissue.

DeLancey (1993) investigated the supportive structures of the reproductive system in humans in an attempt to identify what structures fail in association with prolapse. It was found that the uterus was supported by the combined actions of the pelvic floor

muscles and the suspensory ligaments. The pelvic floor muscles are usually in a contracted state and prevent the uterus from dropping into the vagina. When the pelvic floor muscles are relaxed, such as during defecation, the suspensory ligaments support the uterus. However these ligaments cannot support the uterus indefinitely and will become stretched if the pelvic floor muscles are relaxed for a prolonged period of time. Therefore prolapse could result due to a failure of either one of these systems as both are required to support the uterus. Damage to the suspensory ligaments was highlighted as a cause of prolapse by Carey and Slack (1994) and it was suggested that this damage could be caused by childbirth.

DeLancey (1993) proposed that increased intra-abdominal pressure was a factor that could be involved in causing prolapse. Carey and Slack (1994) suggested that obesity, chronic coughing and constipation were all associated with an increased incidence of prolapse, and these factors would cause increased intra-abdominal pressure. In turkeys the incidence of prolapse is increased in association with turkey rhinotractitus, a disease of the respiratory system, which would increase intra-abdominal pressure. Prolapse is also common after stress or hot weather, both of which cause panting and increased intra-abdominal pressure.

Jackson *et al.* (1996) sampled the vaginal epithelium of women with or without prolapse and found that there were significant changes in collagen metabolism associated with prolapse. There was a 25% reduction in the total collagen content associated with prolapse and these samples also contained increased levels of the enzymes involved in collagen degradation. They also found a higher level of intermediate cross-links in the collagen of samples associated with prolapse while the solubility of collagen from these samples was reduced, which supported the hypothesis of increased turnover of collagen. They also measured the ratio of type I: type III collagen and found it was not altered in association with prolapse, which contradicted the results of Norton *et al.* (1992).

These results suggest that the overall metabolism of vaginal collagen is increased in association with prolapse with collagen degradation occurring at a greater rate than

synthesis, which results in reduced total collagen. A reduction in total collagen and a decrease in the maturity of the collagen would reduce the mechanical strength of the vaginal tissue.

Following the work of Jackson *et al.* (1996), Ayen and Noakes (1998) investigated collagen in the vagina of ewes using histological techniques to stain for collagen and image analysis to measure the amount of staining present. They compared eight ewes that had a history of prolapse in previous years to pregnant and non-pregnant ewes. They found no significant difference in the collagen content of the ewes with a history of prolapse compared to either the pregnant or non-pregnant ewes. They concluded that chemical analysis of hydroxyproline content would have been a more accurate method of determining the collagen content, and that sampling at the time of prolapse would be essential to investigate a relationship between prolapse and vaginal collagen content.

It seems likely from these investigations that an abnormality in collagen metabolism may be a factor associated with prolapse. Collagen is one of the main structural proteins and it is proposed that a reduction and/or alteration in the structure of vaginal collagen would reduce the structural integrity of the vagina and predispose the tissue to prolapse.

1.6 Reproduction in female turkeys

Reproduction in female turkeys culminates in the production of an egg suitable for incubation. It is similar to reproduction in domestic hens, and the literature is much more extensive for domestic hens than turkeys. Therefore this review will cover work carried out investigating reproduction in hens as well as turkeys.

Female turkeys can reach sexual maturity at 24-26 weeks of age although commercially it is common practice to photostimulate them at around 30 weeks resulting in the onset of sexual maturity from 32 weeks of age. Domestic hens reach sexual maturity earlier than turkeys, at 16-18 weeks of age. Control of day length is

used to control the timing of the onset of sexual maturity in turkeys and hens, using a set lighting regime as described in 1.3.

The reproductive system in turkeys and hens consists of the ovary and the oviduct. In most birds only the left ovary and oviduct fully develop at the onset of sexual maturity and become functional. The oviduct consists of five sections, the infundibulum, the magnum, the isthmus, the uterus (also known as the shell gland) and the vagina. The infundibulum is funnel shaped and captures the ovulated follicles (ovum) from the ovary. In the magnum the ovum becomes surrounded by albumin. The isthmus is where the shell membrane is laid down. In the uterus the egg becomes calcified and this is where it spends the largest proportion of time. The vagina contains sperm storage tubules and is actively involved with the uterus in oviposition.

1.6.1 Development of the reproductive system

The onset of sexual maturity follows the development of the reproductive system and the secondary sexual characteristics. In turkeys and domestic hens sexual maturity is brought about by changes in hormone levels. The avian anterior pituitary produces two hormones that resemble mammalian luteinizing hormone and follicle stimulating hormone both physically and chemically, and therefore the avian hormones are referred to by these names (Etches 1996d). However the functions of the avian hormone vary from their mammalian counterparts as luteinizing hormone does not luteinize any ovarian tissue in birds and the main function of follicle stimulating hormone is unclear.

In birds both follicle stimulating hormone and luteinizing hormone stimulate growth and maturation of the ovarian follicles, while luteinizing hormone also stimulates production of oestrogens, androgens and progesterone from the ovarian tissue. These steroid hormones initiate the development of the secondary sexual characteristics such as feathering and head furnishings, and oestrogens also stimulate the production

of yolk precursors by the liver and the deposition of medullary bone in preparation for egg production.

The release of luteinizing hormone and follicle stimulating hormone from the anterior pituitary occurs in response to stimulation by gonadotrophin releasing hormones that are secreted by the hypothalamus.

Bacon *et al.* (1980) measured plasma oestrogen levels in large white commercial turkeys from one week prior to photostimulation throughout the onset of lay. The results showed that oestrogen levels increased from photostimulation, peaked at first egg and plateaued during egg production. They found no significant increase in oestradiol levels associated with the onset of lay while oestrone levels peaked at the onset of lay. They concluded that the majority of the changes in oestrogen levels were due to an unidentified oestrogen. It is possible that there is some other oestrogen metabolite that is the main active oestrogen in turkeys although a problem with the specificity of their radioimmunoassay would be another possible explanation.

High pressure liquid chromatography (HPLC) was used to show that there were five peaks of oestrogenic activity in turkey plasma (Brown *et al.* 1979) and these have been tentatively identified as oestrone, oestradiol, 15β- and 15α-OH-oestrone, 15β-OH-oestradiol and 15α-OH-oestradiol. In a separate study it was shown that there were 4 peaks after extraction with anti-17β-oestradiol serum (Brown 1982) and it was suggested that 6-keto or 6-OH oestrogens may be present in turkey plasma. It is therefore possible that there are several different oestrogen metabolites in turkey plasma.

In domestic hens, Senior (1974) showed that plasma oestradiol rises around 7 weeks before the onset of lay and peaks 2-3 weeks before first egg. Williams and Sharp (1977) demonstrated that progesterone was low from eight weeks of age until it rose just before or at first egg and then remained elevated for at least 4 weeks after the start of lay. Itoh *et al.* (1988) also showed that plasma oestradiol concentration in

hens peaked 2 weeks before the onset of lay while plasma progesterone did not rise until 2 weeks before the onset of lay and then it remained elevated though the start of egg production.

1.6.2 Ovarian steroidogenesis

Oestrogens and progesterone are produced by the ovaries in turkeys and hens. The hiearchical follicles were found to produce progesterone in domestic hens (Yu *et al.* 1992b). Senior and Furr (1975) measured oestradiol in preovulatory follicle venous blood and in various ovarian tissues and concluded that the small follicles and ovarian stroma were likely to be the main source of oestradiol production in hens. Aromatase is the enzyme responsible for the conversion of testosterone to oestradiol and the small ovarian follicles and stromal tissue have been shown to contain about 50% of the total aromatase activity of the ovary (Armstrong 1984). Robinson and Etches (1986) investigated the output of oestradiol from the five largest follicles and from three classes of small follicles. They concluded that the small follicles are the major source of oestradiol in domestic hens. Oestradiol production by the small ovarian follicles has also been demonstrated in turkeys (Porter *et al.* 1989b; Porter *et al.* 1991b; Porter *et al.* 1991c) by incubation of follicles in medium and measurement of the oestradiol released into the incubation medium.

Ovarian follicles consist of an inner layer of granulosa cells surrounded by an outer layer of theca cells. In smaller follicles the granulosa cell layer is several cells thick. As the follicle grows and its content of yellow yolk increases the granulosa cell layer becomes single celled and the theca cell layer becomes highly vascularized (Etches 1990). Much of the work investigating ovarian steroidogenesis has been carried out on cellular preparations as this allows repetition of several treatments on a single follicle and eliminates the need for the large volumes of medium that are necessary for whole follicle incubations. The theca and granulosa cells can be separated by the method described by Gilbert (1977) and this allows their individual contributions to the ovarian hormone output to be investigated. However incubating theca and

granulosa cells separately removes the cell to cell interactions that could occur in the intact follicle.

Huang and Nalbandov (1979) separately incubated theca and granulosa cells from the three largest follicles of laying hens and demonstrated that the granulosa cells produced large amounts of progesterone and a small amount of testosterone while the theca cells produced no progesterone or testosterone. Neither cell type was shown to produce any oestradiol. Huang *et al.* (1979) showed that the addition of testosterone to the incubation system resulted in oestradiol production by theca cells from the second and third largest follicles while the addition of progesterone increased testosterone production by theca cells. They concluded that the granulosa cells produced progesterone while the theca cells produced oestradiol and testosterone. Theca cells require the presence of the precursors progesterone or testosterone to produce testosterone or oestradiol respectively. Both granulosa cells and theca cells are therefore required by the follicle for testosterone and subsequent oestradiol production. This resulted in the two cell model for ovarian steroidogenesis, where the granulosa cells produce progesterone that can be converted to testosterone and subsequently oestradiol by the theca cells (Huang *et al.* 1979).

Marrone and Hertelendy (1983b) collected ovarian follicles from domestic hens and incubated granulosa and theca cells together and separately. They demonstrated that progesterone production by granulosa cells was reduced when theca cells were present which supported the two cell model of ovarian steroidogenesis. In another study they incubated cells in the presence of radiolabelled precursors to allow the metabolites produced to be traced (Marrone and Hertelendy 1983a). This confirmed that progesterone is the major steroid produced by granulosa cells while theca cells produced a wider variety of metabolites including androgen, oestrogens and 17-hydroxoprogesterone.

The two cell model was modified by Porter *et al.* (1989a) who investigated steroidogenesis in the granulosa, theca externa and theca interna cell layers from turkey ovarian follicles. It was shown that the theca interna produced progesterone

and testosterone while the theca externa produced progesterone, testosterone and oestradiol. They concluded that aromatase activity was limited exclusively to the theca externa. A three cell model for avian ovarian steroidogenesis was proposed with the granulosa cells predominately producing progesterone, the theca interna mainly produced testosterone while oestradiol was produced by the theca externa.

Rodriguez-Maldonado *et al.* (1996) demonstrated that the theca externa of domestic hens have the capacity to secrete oestrogens and contained several associated enzymatic activities. They proposed that interaction between the theca externa and interna occurs to facilitate steroid production. They concluded that their results suggested different steroidogenic capacities of the theca externa and interna and their results supported the three cell model for avian steroidogenesis.

As the ovarian follicles mature their steroid hormone output changes. In domestic hens the largest pre-ovulatory follicle produces the greatest amount of progesterone (Yu *et al.* 1992b). Furthermore the progesterone produced in response to gonadotrophin stimulation increased while the oestradiol production decreased with the increasing size of follicles (Huang *et al.* 1979). Porter *et al.* (1991a) compared the production of progesterone, androgens and oestradiol from the granulosa, theca externa and theca interna cells of the largest and fifth largest follicles and the small white ovarian follicles of turkey. It was concluded that as follicles mature they shift from predominately producing oestradiol to androgen production and subsequently to progesterone production.

Luteinizing hormone stimulates steroidogenesis in ovarian tissue of turkeys and hens. The response of the follicle to luteinizing hormone stimulation changes as the follicles mature. Shahabi *et al.* (1975) found that the increase in progesterone concentration of the follicular wall following luteinizing hormone injection was greatest in the largest follicle. Porter *et al.* (1991a) showed in turkeys that progesterone production in response to luteinizing hormone was greater in granulosa cells from the largest follicle compared to the fifth largest follicle. Robinson and Etches (1986) investigated steroidogenesis in domestic hens and found that the

oestradiol output of theca cells in response to luteinizing hormone decreased as the follicles matured. The response of the various ovarian cells to luteinizing hormone clearly alters as the follicles mature and is involved in the alterations in steroid hormone output associated with follicular maturation.

The function of follicle stimulating hormone in ovarian steroidogenesis has not yet been identified in avian species (Johnson 1990). Any increase in hormone output in the presence of follicle stimulating hormone can be attributed to the presence of luteinizing hormone in the follicle stimulating hormone preparation (Porter *et al.* 1991a).

Other factors may also involved in controlling ovarian steroidogenesis. Vasoactive intestinal peptide stimulates production of progesterone and androgens from granulosa cells and it has been suggested that this neurohormone may have a role in the development of the ovarian cells (Johnson 1990). Growth factors may also influence ovarian steroidogenesis in avian species. Epidermal growth factor and transforming growth factor have been found to inhibit the stimulatory effect of luteinizing hormone on granulosa cell progesterone production (Johnson 1990).

Prolactin may also alter ovarian steroidogenesis and is thought to be involved in stimulating broodiness. Injection of turkeys with prolactin for 8 days resulted in depression of the oestrogen secretion from small white follicles and a reduction of the stimulatory response of small white follicles to luteinizing hormone (Lien *et al.* 1989).

As previously discussed in section 1.2 male-line turkeys have large ovaries with a multiple hierarchy of follicles. The multiple hierarchy consists of groups of follicles of a similar size, which are therefore considered to be at a similar anatomical stage. However their physiological maturation state has not previously been investigated. It seems likely that follicles of a similar size would be at a similar stage of maturation and would therefore have similar steroid hormone outputs.

The effect of the large ovary and multiple hierarchy on the plasma hormone profile of the turkey has not previously been investigated. The larger ovary contains many more small ovarian follicles which could result in greater oestradiol production while the greater number of hierarchical follicles could result in greater progesterone production. Daily injection of laying hens with pregnant mare serum gonadotrophin for 3-4 days disrupts the control of follicular maturation and promotes the development of large follicles, resulting in significantly greater concentrations of progesterone and oestradiol in the plasma (Bahr and Johnson 1984). It was therefore postulated that male-line turkeys would have higher plasma concentrations of oestradiol and progesterone due to steroidogenesis by their large ovaries and high number of hierarchical follicles.

1.6.3 The ovulatory cycle

In avian species the ovulatory cycle is much shorter than in mammals and results in the production of an egg. In several species such as chickens, turkeys and ducks, eggs are laid in sequences of an egg a day followed by a pause day when no egg is laid. The sequence varies in length between species and individual birds. Some individual birds will only lay two eggs in each sequence while some laying hens can lay an egg a day for several weeks (Etches 1990).

The ovulatory cycle is characterised by ovulation of a mature follicle from the ovary, capture of this ovum into the oviduct, passage of the ovum down the oviduct to the uterus, where it is formed into an egg, and finally expulsion of the egg from the oviduct, known as oviposition. In turkeys this process takes around 30 hours, which is 3-4 hours longer than domestic hens (Sharp *et al.* 1981).

Ovulation and oviposition are believed to be under hormonal control as they are accompanied by changes in plasma hormone concentrations and can be induced through the injection of some hormones. In turkeys plasma progesterone and luteinizing hormone concentrations peak 4-8 hours before ovulation (Mashaly *et al.* 1976; Opel and Arcos 1978; Hammond *et al.* 1981b; Sharp *et al.* 1981; Yang *et al.*

1997). Similar changes have been observed in the ovulatory cycle of domestic hens, with progesterone and luteinizing hormone rising to a preovulatory peak 7-2 hours before ovulation and oviposition (Furr *et al.* 1973; Wilson and Sharp 1973; Hammond *et al.* 1980; Etches 1990).

In domestic hens injection of luteinizing hormone stimulates an increase in plasma progesterone and testosterone, while plasma oestradiol concentration did not increase (Shahabi *et al.* 1975). Injection of luteinizing hormone releasing hormone will result in premature ovulation if a mature follicle is present (Etches and Cunningham 1976). If no mature follicle is present then ovulation does not occur and there is no increase in plasma progesterone following luteinizing hormone injection. This suggests that the surge in progesterone comes from the luteinizing hormone stimulating progesterone production by the largest preovulatory follicle.

Progesterone injections will also stimulate ovulation of a mature follicle in domestic hens and is accompanied by an increase in plasma concentrations of luteinizing hormone and progesterone (Etches and Cunningham 1976; Johnson and Tienhoven 1980). The increase in luteinizing hormone in response to progesterone injection is dependent on the ovulatory cycle, as the response is reduced immediately following the preovulatory surge in luteinizing hormone (Wilson and Sharp 1975). The increase in luteinizing hormone in response to progesterone is independent of the presence of a mature ovarian follicle (Etches and Cunningham 1976).

These experiments suggest that progesterone is a likely candidate for stimulating the preovulatory surge in luteinizing hormone, via a positive feedback mechanism. It has been proposed that a small increase in plasma progesterone concentration due to progesterone production by the largest post ovulatory follicle stimulates release of luteinizing hormone from the hypothalamus, which in turn stimulates increased progesterone secretion from the follicles (Sharp 1983). The positive feedback between the follicles and the hypothalamus results in the preovulatory peaks in plasma progesterone and luteinizing hormone concentration and stimulates ovulation.

The timing of ovulation is thought to depend on two things, the presence of a mature follicle and the 'open period' during which the events resulting in preovulatory peaks in luteinizing hormone and progesterone can occur. It has been suggested that these two systems are independently controlled, with the open period being influenced by day length (Etches and Schoch 1984). The mechanism that restricts the open period to a certain time of day has not been identified although alterations in hypothalamic or pituitary sensitivity, and an involvement of the adrenal glands and corticosterone have been suggested (Etches *et al.* 1984). The observation that the preovulatory luteinizing surge starts either at the same time or after the rise in progesterone supports the hypothesis that it is a rise in progesterone that initiates these events (Furr *et al.* 1973; Etches and Cunningham 1976).

The involvement of oestradiol in ovulation has not been demonstrated. No consistent pattern of oestradiol changes during the ovulatory cycle has been identified in turkeys (Opel and Arcos 1978). Senior and Cunningham (1974) observed a preovulatory increase in the plasma oestradiol concentration of domestic hens, which started to rise 2 hours before the start of the luteinizing hormone surge. An increase in plasma oestradiol 6 hours before ovulation was also reported by Etches and Cheng (1981) while Graber and Nalbandov (1976) showed 4 peaks in plasma oestradiol concentration during the ovulatory cycle of domestic hens.

A role for oestradiol in directly stimulating ovulation in turkeys or hens appears unlikely as luteinizing hormone injection does not result in increased plasma oestradiol (Shahabi *et al.* 1975) and injection of oestradiol does not stimulate an increase in plasma luteinizing hormone concentration (Wilson and Sharp 1976). However a requirement of oestradiol in the response of plasma luteinizing hormone to progesterone has been proposed. Ovariectomised hens do not show an increase in plasma luteinizing hormone concentration unless they have been primed with injections of both progesterone and oestradiol (Wilson and Sharp 1976) which suggests that oestradiol and progesterone are required to prime the positive feedback mechanism.

The effect of the multiple follicular hierarchy on the hormonal changes during the ovulatory cycle has not previously been investigated in turkeys. The multiple hierarchy results in the ovulation of more than one follicle each day. It is not known whether these multiple ovulations occur at the one time or are spaced out throughout the day. Sharp *et al.* (1976) compared the changes in plasma luteinizing hormone concentration throughout the ovulatory cycle in single and multiple ovulating hens. They found that the preovulatory peaks in luteinizing hormone were no more frequent in the multiple ovulating hens than the single ovulating hens. There was no difference in frequency or duration of peaks or in the maximum or baseline levels of plasma luteinizing hormone between the single and multiple ovulating hens. They concluded that the multiple ovulations occurred in response to single preovulatory luteinizing hormone peaks.

1.6.4 Oviposition

Oviposition, the expulsion of the egg from the uterus, is brought about by a series of rhythmical contractions of the uterus accompanied by relaxation of the vagina and utero-vaginal sphincter. In hens the increase in uterine contractility has been demonstrated by the measurement of increased electrical activity of the uterus in association with oviposition (Shimada and Asai 1978). Prostaglandins and arginine vasotocin are through to be involved in stimulating the oviduct for oviposition.

In turkeys, it has been shown that premature oviposition could be induced by single injections of prostaglandins E_1 (PGE_1), E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) while injection of indomethacin, a prostaglandin-synthetase inhibitor delayed natural oviposition by several hours (Hammond *et al.* 1981b). The plasma concentrations of PGE and PGF were found to increase around spontaneous oviposition, even with the terminal oviposition in a sequence, which is not accompanied by ovulation. These finding suggested that prostaglandins were involved in facilitating oviposition.

PGE_2 and $PGF_{2\alpha}$ injections also stimulate oviposition in domestic hens (Shimada and Asai 1979; Shimada *et al.* 1987) and the sensitivity of this response increases as the time of expected oviposition is approached (Goto *et al.* 1985).

Day and Nalbandov (1977) showed that PGFs were present in the largest preovulatory follicle of domestic hens and there was an increase in the concentration 4-6 hours before ovulation. They also found large amounts of PGFs in the post ovulatory follicle which increased around 24 hours after ovulation. They concluded that prostaglandin release from the largest preovulatory and post ovulatory follicles is involved in stimulating oviposition.

The effect of $PGF_{2\alpha}$ and PGE_2 injection on oviduct pressure has been investigated in domestic hens (Wechsung and Houvenaghel 1978). $PGF_{2\alpha}$ increased pressure throughout the oviduct while PGE_2 increased pressure in the infundibulum, magnum and isthmus and decreased pressure in the vagina. The effect of PGE_2 on the uterus varied between individual birds, with pressure increasing in some birds and decreasing in other.

$PGF_{2\alpha}$ increases uterine contractility in hens and this effect is blocked by indomethacin (Shimada and Asai 1979). It has been shown that the increase in uterine contractility at oviposition is accompanied by an increase in plasma $PGF_{2\alpha}$ (Shimada *et al.* 1984). It seems likely that prostaglandins produced by the preovulatory follicle and largest post ovulatory follicle play a major part in stimulating the contractions and relaxation of the oviduct to facilitate oviposition.

Arginine vasotocin (AVT) is an avian neurohypophyseal hormone, synthesised in the hypothalamus and secreted from the neurohypophysis. AVT is similar to the mammalian hormone oxytocin, which stimulates contraction of the uterus during parturition. AVT stimulates uterine contractions in domestic hens and the sensitivity of the uterus to AVT changes during the ovulatory cycle (Saito and Koike 1992). Plasma concentrations of AVT are increased in association with both spontaneous and $PGF_{2\alpha}$ induced oviposition (Shimada *et al.* 1986). Manual stimulation of the

uterus was found to result in AVT release and it has been suggested that PGFs stimulate contraction of the uterus, which in turn stimulates AVT release (Shimada *et al.* 1987).

It is possible that prolapse of the oviduct in turkeys is a result of over contraction of the uterus or over relaxation of the vagina or a combination of these two effects. If that were the case then it is possible that increased concentrations of prostaglandins, secreted by the largest follicles and largest post ovulatory follicles are involved in predisposing the male-line to prolapse. If uterine contractility was increased by high prostaglandin secretion, it would stimulate further release of AVT resulting in further stimulation of uterine contractions. It is possible that such hyperactivity of the oviduct may be involved in prolapse.

1.7 Collagen

Collagen is the most abundant protein in the body, accounting for nearly one third of total body protein. Located in the extracellular matrix it is present in most tissues and is abundant in bone, teeth, tendon and skin. The principle function of collagen is that of providing support and strength to tissue as a structural scaffolding, although it also plays an active part in tissue formation. Collagen consists of long thin fibres that are highly resistant to stretch. A 1mm wide collagen fibre can withstand loads of up to 10 kg before breaking (Stryer 1988).

1.7.1 Structure of collagen

Tropocollagen is the basic structural unit of collagen. It is one of the longest known proteins and has a very small diameter. Tropocollagen consists of three polypeptide chains that wind together in a triple helix formation. Each individual polypeptide chain has a helical formation and the three chains form a super-helix held together by hydrogen bonds.

The amino acid sequence of tropocollagen largely consists of glycine, lysine, hydroxylysine, proline and hydroxyproline. Every third amino acid residue of tropocollagen is glycine, which is essential as its small size is crucial to the formation of the helix, as there is not enough room to accommodate any other amino acid at this position in the triple helix (Robins 1988). Hydoxyproline and hydroxylysine are initially transcribed as proline and lysine and are subsequently converted by post translational modifications. These conversions are activated by the hydroxylase enzymes.

The hydroxyl groups of the hydroxyproline residues are involved in formation of the hydrogen bonds that hold the three polypeptide chains together. Ascorbic acid (vitamin C) is required for the maintenance of prolyl hydroxylase in its active form. Collagen formed in the absence of ascorbic acid is insufficiently hydroxylated and cannot form fibres properly. This results in the skin lesions and blood vessel fragility that are characteristics of scurvy (Stryer 1988).

The rod shaped tropocollagen molecules are arranged parallel to one another in a staggered pattern to form the collagen fibres, with small gaps between tropocollagen molecules. The tropocollagen molecules are arranged at opposite ends, with the amino terminal region of one molecule adjacent to the carboxyl terminal of the next molecule. Cross-links are formed between the tropocollagen molecules to stabilise the collagen. Initial cross-link formation is activated by the enzyme lysyl oxidase, which converts lysine and hydroxylysine residues to aldehyde residues, allysine and hydroxyallysine (Robins 1988). These intermediate aldehyde cross-links respectively form hydroxylysinonorleucine (HLNL) or dihydroxylysinorleucine (DHLNL) upon reduction by borohydride (Robins 1976).

With increasing age of tissue, the concentration of these reducible cross-links decreases and there is an increase in the concentration of mature cross-links. The mature cross-links are thought to be formed through the bonding of two hydroxyallysine and one allysine residues resulting in a pyridinoline cross-link, or if derived from lysine residues instead of hydroxylysine a deoxypyridinoline cross-link.

The formation of these mature cross-links increases the stability of the collagen. It is believed that the strength of the collagen comes predominately from the cross-linking of the fibrils although the role of the different types of cross-links had not yet been identified (Robins, 1999). The pyridinoline cross-link is thought to be the main mature cross-link of cartilage while the deoxypyridinoline cross-link has been identified in bone and dentine (Robins 1988).

The extent of cross-linking in collagen depends also on the function of the tissue. This can be demonstrated by the comparison of two different types of tissue from adult rats. Collagen from the Achilles' tendon is extensively cross-linked while collagen from the tail tendon has much fewer cross-links, reflecting the greater flexibility of the tail tendon in comparison to the Achilles' (Stryer 1988).

1.7.2 Types of collagen

Currently there are 19 known types of collagen (Jackson *et al.* 1996) and they differ in structure and distribution. The main types of collagen found in vaginal tissue are type I and III (Kleissl *et al.* 1978). Type I collagen consists of two $\alpha 1$ (I) chains and one $\alpha 2$ (I) chain while type III collagen consists of three $\alpha 1$ (III) chains (Stryer 1988).

The different structures of collagen types I and III allows them to lend different mechanical properties to tissue. The different properties of the collagen types can be seen in patients with connective tissue disorders. Connective tissue of patients with Ehlers-Danlos syndrome type IV does not contain type III collagen. Rupture of the aorta is a common occurrence with this condition, as the tissue has lost its elasticity and flexibility and therefore is less able to stretch and contract with the variations in blood flow. Marfan's syndrome, on the other hand, is characterised by a decrease in type I collagen. Patients with Marfan's syndrome often suffer from aortic aneurysms due to hyperelasticity of the aortic wall. From these observations it was suggested that type I collagen promotes tissue stability while type III collagen promotes flexibility of tissue (Kuhn and Glanville 1980). However more recently it has been

suggested that Marfan's syndrome does not involve a defect of either type I or III collagen (Robins 1988) and the basis of this hypothesis may be questioned.

1.7.3 Collagen metabolism

Collagen is synthesised as procollagen and post translational modifications such as hydroxylation and glycosylation occur before the procollagen molecule is transported from the cell via golgi apparatus. The procollagen molecule has additional peptides at each end that prevent formation of the collagen fibres until the molecule is transported from the cell. It is thought that they are also involved in assisting exportation of the molecule from the cell and aligning the rods for the formation of the triple helix. Once the procollagen reaches the extracellular compartment the end sections are cleaved by specific proteases called procollagen peptidases, which are located extracellularly in close proximity to the surface of the cell. Cleavage of the end sections results in tropocollagen and allows the triple helix to form (Stryer 1988).

Collagen turnover is an ongoing process in tissue, with collagen continually being synthesised and degraded. Type I collagen is synthesised by fibroblasts, osteoblasts and smooth muscle cells while collagen type III is synthesised by fibroblasts and smooth muscle cells (Kuhn and Glanville 1980). Degradation of collagen is achieved by specific proteases. These break down the peptide bonds that are located within the triple helical region of the tropocollagen molecules. Metalloproteinases play a major role in collagen breakdown, with one specific metalloproteinase, collagenase, responsible for cleavage of the α polypeptide chains. There are 12 members of the matrix metalloproteinase family identified so far (McIntush and Smith 1998). The susceptibility of collagen to degradation is dependant on the extent of cross-linking, and it is likely that degradation of more stable collagen is achieved by the combined actions of several proteases (Weiss *et al.* 1980).

Tissue inhibitors of metalloproteinases (TIMPs) are produced by most cells, particularly those located in connective tissue. At present four different TIMPs have

been identified (McIntush and Smith 1998). TIMPs bind with high affinity to matrix metalloproteinases in a 1:1 relationship and inhibit their degradative actions on collagen (Woessner 1989).

Regulation of collagen degradation can be achieved by a variety of substances that act on collagenase production, such as growth factors like platelet derived growth factor and interleukin 1, and some prostaglandins. Regulation of TIMP is also involved in controlling collagen degradation and is brought about by various substances such as growth factors, cytokines and lymphokines (Robins 1988).

As previously mentioned hydroxyproline is one of the amino acids that makes up a large proportion of the collagen polypeptide. Hydroxyproline is rarely found in other molecules and therefore measurement of the hydroxyproline content of a tissue gives an accurate measure of the relative amount of collagen that is present. One mg of hydroxyproline is considered to represent 7.14 mg of collagen (Jackson *et al.* 1996).

Collagen is essential to the structure of tissue and the type and maturity of the collagen influences the mechanical properties of the tissue. It is therefore conceivable that if the collagen of the oviduct is altered in some way in male-line turkeys this could impair the structural integrity of the oviduct and predispose the strain to prolapse.

1.8 Effects of oestradiol and progesterone on collagen

A considerable amount of work has been carried out on cervical and uterine collagen in rats and guinea-pigs in an attempt to model the changes in collagen associated with cervical ripening in mammals. Cervical ripening, the dilation of the cervix at the end of pregnancy to facilitate parturition, is thought to occur through the breakdown of cervical collagen. The degradation of this collagen is likely to be under hormonal control and therefore the influence of the sex steroid hormones on the reproductive tract collagen has been investigated.

Administration of oestrogens to pregnant rats has been shown to stimulate a decrease in uterine collagen. Pastore (1989) administered a single injection of oestradiol to rats and showed a subsequent breakdown of uterine collagen. Two hours after the injection, electron microscopy showed that the collagen fibres were fragmented, less densely packed, separated from the plasma membrane and spaces were visible in the collagen matrix. After 24 hours there were large clear spaces, many fragmented fibres and the bundles were loosely packed. The collagen structure had returned to normal 48 hours after the injection, although total collagen was still lower than in control, saline injected rats.

In cervical cell cultures from pregnant guinea-pigs, oestrogens increase the production of procollagenase, which is the precursor of collagenase, one of the collagen degrading enzymes (Rajabi *et al.* 1991a). This response was thought to be prostaglandin mediated as it can be mimicked by $PGF_{2\alpha}$ and blocked by indomethacin, a cycloxygenase inhibitor. They also showed that at low concentrations progesterone stimulates procollagen production while inhibiting it at higher concentrations.

Immunohistochemical techniques were used to demonstrate an increase in type I collagen degradation at parturition (Rajabi *et al.* 1991b) and it was shown that oestradiol stimulates degradation of type I collagen in cervical cells cultured from non-pregnant guinea-pigs. The degradation of collagen stimulated by oestradiol was completely blocked by progesterone which supported the hypothesis that oestradiol and progesterone are involved in the control of cervical relaxation.

It was hypothesised that the actions of progesterone and oestrogens on cervical collagen control cervical relaxation during pregnancy and parturition. The high progesterone concentration in the circulation towards the end of pregnancy inhibits collagenase production preventing cervical relaxation until the start of parturition, when progesterone falls and oestrogen rises, stimulating collagenase to degrade the collagen of the cervix and facilitate cervical relaxation (Rajabi *et al.* 1991b).

Rajabi *et al.* (1991c) also showed *in vivo* that there was a 2 fold net increase in procollagenase activity in the guinea-pig cervix at parturition which corresponded to a decrease in collagen staining and a loss of the collagen fibre network. These results further support an involvement of collagen degradation in facilitating cervical ripening.

The oestradiol stimulated increase in collagenase production by uterine cell cultures from pregnant guinea-pigs is likely to be mediated via activation of protein kinase C, as treatment with a protein kinase C activator elicited a similar response to oestradiol, while the oestradiol response was blocked by a protein kinase C inhibitor (Rajabi *et al.* 1992).

Bienkienwicz (1996) investigated the effect of hormones *in vivo* on rat uterine collagen. They injected pregnant, near term, rats with hormones and their antagonists and then looked at the properties of the uterine collagen. Administration of oestradiol was found to decrease the collagen content of the uterus while progesterone and tamoxifen, an oestrogen antagonist, had no effect on the collagen content. Both tamoxifen and progesterone caused a decrease in the proportion of collagen that was soluble, suggesting that the collagen was more mature, which could reflect a decrease in turnover.

The production of collagenase mRNA by rat uterine smooth muscle cell cultures was investigated by Wilcox *et al.* (1992). They found that progesterone caused a decrease in collagenase mRNA from these cells, which supports the hypothesis that progesterone inhibits collagen degradation.

From these investigations it appears likely that oestradiol and progesterone play an important role in controlling cervical ripening at parturition in rats and guinea-pigs. The high levels of progesterone in the plasma throughout pregnancy inhibit degradation of the collagen and maintain the closure of the cervix. At the onset of parturition plasma progesterone decreases while plasma oestradiol increases. This stimulates degradation of the cervical collagen allowing the cervix to dilate. The

effect of oestradiol and progesterone on the collagen of the oviduct in turkeys has not previously been investigated. It is possible that oestradiol and progesterone could be involved in controlling collagen turnover in the oviduct of turkeys.

1.9 Effects of food restriction on reproduction in turkeys

In the wild, poultry species would not have continuous access to an unlimited supply of food. Domestic poultry are often reared with constant access to food and this results in birds that could be considered obese. Food restriction is attractive to the poultry breeding industry as large savings can be made on rearing costs if food consumption is reduced. However breeding turkeys are selected at several points throughout rearing for body conformation. Food restriction limits growth and therefore may mask the true potential growth of individual birds that would have been desirable for selection. Another drawback is that food restriction can have adverse effects on reproduction, as few animals will reproduce effectively if they are in poor body condition.

There are various methods by which food restriction can be achieved, such as only supplying a set quantity of food, limiting the amount of time each day that the food is accessible, skip-a-day feeding regimes or supplying food with a low protein or metabolizable energy content or a high fibre content. Some of these methods can be used in combination.

Most of the research into the potential benefits of food restriction on breeding turkeys was carried out many years ago and selection has altered the male-lines so much over this time that the relevance of such work to the current day male-lines is questionable.

McCartney *et al.* (1977) significantly increased egg production of female breeding turkeys through feeding a low energy diet without any significant effects on other reproductive traits. Hocking (1992a) found that food restriction increased hatchability, reduced the proportion of non-settable eggs and improved poult quality,

although overall egg production was reduced. However other work has produced opposing results. Andrews and Morrow (1978) found that food restriction, either during rearing or laying, resulted in fewer eggs being produced while Krueger *et al.* (1978) found that restricted feeding from 22 to 30 weeks of age reduced subsequent egg production. Low protein diets have also been shown to have an adverse effect on reproductive performance of female breeding turkeys, decreasing egg weight, hatchability and day old poult weight (Menge *et al.* 1979). The number of eggs produced has been shown to decrease with decreasing dietary protein level (Meyer *et al.* 1980). Other work has shown no significant effects of food restriction on reproductive performance of female breeding turkeys (Borron *et al.* 1974; Potter *et al.* 1977; Owings and Sell 1980).

Food restriction has been practised for many years in the broiler chicken industry to improve the reproductive performance of the broiler breeder flocks. In 1987 it was shown that *ad libitum* fed broiler breeder hens have a multiple hierarchy of ovarian follicles and the improvement in reproductive performance associated with restricted feeding was due to a reduction in this multiple hierarchy (Hocking *et al.* 1987). The multiple follicular hierarchy results in several ovulations per day, which reduces viable egg production through the production of malformed eggs not suitable for incubation and the loss of follicles through internal ovulations. It has since been shown that food restriction after 14 weeks of age is necessary to significantly decrease the number of hierarchical follicles (Hocking *et al.* 1989) and continued restriction until first egg significantly reduced the over development of the ovarian follicular hierarchy (Hocking 1993a; Hocking 1996).

It seemed likely that if restricted feeding could be used to control the over development of the follicular hierarchy in broilers it would have the same effect in turkeys. However Nestor *et al.* (1981) found no significant effect of feeding a high fibre diet on the number of hierarchical follicles in turkeys that had been selected for increased meat yield. The degree of food restriction used in the experiment only decreased body weight to about 90% of the controls. It is possible that this degree of

restriction was insufficient to significantly effect the development of the multiple follicular hierarchy.

Hocking (1992b) restricted four strains of turkeys and compared the hierarchical follicle number at the onset of lay. There was a significant reduction in the number of hierarchical follicles with the decreased body weight of the restricted birds. However there was little effect of restricted feeding on the number of hierarchical follicles in the male-line compared to the lighter body weight strains that were investigated. The restricted turkeys of the male-line had a body weight 70% of the *ad libitum* fed turkeys and it is possible that this reduction was not sufficient to control the overproduction of the ovarian follicles.

The effect of more severe food restriction was investigated in male-line turkeys (Hocking and Bernard 1998) that were restricted to 50% of *ad libitum* body weight gain during rearing. A small reduction in follicle number (16.5 to 14.6) was observed at first egg. However the restricted turkeys were fed about 66% of the *ad libitum* allowance from 18 weeks of age, which was then increased to 80% during lay. As the body weight of the restricted turkeys was lower than the *ad libitum* fed turkeys, the food intakes relative to body weight was actually greater in the restricted birds at the onset of photostimulation. It is possible that the degree of food restriction during the onset of sexual maturity was insufficient to control ovarian follicle production.

If there is any potential benefit to be obtained by restricted feeding of breeding male-line turkeys to control the development of the multiple follicular hierarchy it is clear that the food restriction would have to be severe and should be continued throughout the onset of lay to maximise the potential effect on hierarchical follicle number. However due to the requirements of the selection programme it would not be practical to restrict turkeys throughout the rearing period. It is possible that severe restriction from 18 weeks of age throughout the onset of lay would result in a decrease in the number of hierarchical follicles in male-line turkeys.

1.10 Effect of age at photostimulation on the number of hierarchical follicles

Breeding turkeys are usually photostimulated at 29 weeks and 4 days. Early photostimulation has been shown to increase the number of ovarian follicles in the hierarchy. Turkeys photostimulated at 24 weeks had significantly more hierarchical follicles at first egg compared to those photostimulated at 30 weeks (Hocking *et al.* 1988). Photostimulation of a medium body weight strain of turkey at 18 or 24 weeks resulted in increased numbers of hierarchical follicles at 6 weeks after photostimulation compared to those photostimulated at 30 weeks of age (Hocking *et al.* 1992) with the greatest number of hierarchical follicles present in the birds photostimulated at the youngest age.

The increased numbers of hierarchical follicles in the turkeys photostimulated early could reflect an immaturity in the control of follicular development leading to increased recruitment of follicles into the hierarchy. If that is true then the multiple hierarchy of follicles present in turkeys photostimulated at around 30 weeks of age could show that the control system is not sufficiently developed at this age. It is therefore possible that delaying the onset of photostimulation would decrease the number of follicles in the ovarian hierarchy of male-line turkeys.

1.11 Hypothesis on the physiological basis of prolapse in male-line turkeys

The working hypothesis was that the large ovaries of the male-line turkeys resulted in high concentrations of oestrogens in the plasma, which stimulated degradation of collagen in the oviduct and that the decrease in tissue collagen content was associated with prolapse. If this hypothesis was substantiated then low collagen in the oviduct could be used as a marker for prolapse, allowing the likely effect of treatments on the incidence of prolapse to be assessed using a continuous rather than an all or nothing trait.

Alternatively the male-line turkeys could have had low plasma progesterone concentrations and therefore reduced inhibition of collagen degradation, which

would have reduced collagen in the oviduct. The relationships between vaginal collagen, prolapse of the oviduct and plasma oestradiol and progesterone concentration were investigated in Chapter 4.

Increased turnover of collagen in the oviduct could result in an increased proportion of intermediate cross-links and a decreased proportion of mature cross-links, which would reduce the structural strength of the tissue. The proportion of intermediate and mature cross-links in the vaginal collagen in association with prolapse was also investigated (Chapter 4).

It was proposed that food restriction or delayed photostimulation could be used to decrease the number of hierarchical follicles in the male-line and this would result in lower plasma oestradiol concentrations and increase the vaginal collagen content. This hypothesis was tested in Chapter 7.

It has been suggested that the oviduct of the male-line turkeys is not fully mature at the onset of lay and an immaturity of the oviduct could compromise the structure of the oviduct and predispose it to prolapse. The growth and development of the reproductive system was compared between traditional- and male-line turkeys to test this hypothesis (Chapter 3).

From the results of experiments described in Chapters 3 and 4 it was subsequently hypothesised that male-line turkeys had higher peak concentrations of oestradiol throughout the ovulatory cycle, compared to the traditional-line. This hypothesis was tested in Chapter 5. The results of Chapter 5 led to the proposal that ovarian steroidogenesis was reduced in the male-line, which was investigated in Chapter 6.

The general methods used throughout the experiments are described in Chapter 2. Chapter 8 discusses the conclusions from each chapter and suggests directions for future research.

2. General methods

2.1 Turkeys

The turkeys used in these experiments were from 3 strains, the Big 5 male-line, the Big 6 male-line and the Nebraska Spot. The Big 5 and Big 6 male-lines are Large White turkeys that have been specifically selected for rapid growth and increased meat yield. They were obtained from British United Turkeys Limited (Hockenhull Hall, Tarvin, Cheshire, UK). The Nebraska Spot is a traditional line that has been maintained without selection. The strain is kept at the Roslin Institute for research purposes. The experiments were carried out under UK Home Office Licence regulations (Project Licence Number 60/01668).

Unless otherwise stated the turkeys were obtained as day old poults and reared at the Roslin Institute. They were housed in 1.5 × 2.4 m floor pens covered in wood shavings. Initial stocking densities were not more than 40 poults in one pen. This was reduced as the birds grew, and after 18 weeks of age no more than 6 birds were kept in each pen. From 30 weeks of age the stocking density was a maximum of 5 birds per pen.

The turkeys were fed on conventional turkey diets based on the dietary regime used in industry for rearing female breeding turkeys. Table 2.1 gives the types of diets fed at different ages and their crude protein content. The starter diet was prepared as crumb while the other diets were all used in pellet formation. The diets were all prepared at the Roslin Institute (Roslin Nutrition Limited, Midlothian, UK).

Initially the food was available from floor trays and metal food hoppers resting on the floor of the pen. After 2-3 weeks, when the turkeys had grown sufficiently, the food hoppers were suspended above the floor of the pen and the food trays removed. They had constant access to water from bell shaped drinkers. The height of the drinkers and food hoppers was increased as the turkeys grew.

TABLE 2.1 Type of diet, age when fed and crude protein content of diets fed

Diets	Starter	Grower 1	Grower 2	Rearer	Pre-lay	Breeder
Age (weeks)	0-4	4-8	4-12	12-16	14-29	From 29
Crude protein g/kg	260-285	230-250	180-205	170-185	125-130	169-181

The lighting schedule was based on a commercial lighting regime. On the first night they were given 1 hour of darkness. After the first night the lighting was 14 hours light:10 hours dark (long photoperiod) until 18 weeks of age. It was then reduced to 7 hours light:17 hours dark (short photoperiod). At 29 weeks and four days the lighting was increased again to 14 hours light and 10 hours dark (long photoperiod). The extension of the light period was used to initiate the onset of sexual maturity and was referred to as photostimulation.

During the short photoperiod the lights came on at 07.00 hours and went off at 14.00 hours. During the long photoperiods the lights were on from 06.00 to 20.00 hours, with the exception of the experiment in Chapter 5 when they were set to come on at 02.00 and go off at 16.00 hours.

Once laying had commenced the pens were checked several times a day and any eggs were removed to minimise broodiness.

2.2 Killing and Blood Sampling

All turkeys were killed by overdose of sodium pentobarbitone (Euthatal, Veterinary Drug Company Limited, Falkirk, UK). This was administered by intravenous injection into the brachial vein. Cessation of movement of the third eyelid was used as an indication of death.

Blood samples were taken by superficial venepuncture of the brachial vein using 5ml syringes and 23G × 1" needles. Prior to sampling the syringes were rinsed with heparinised saline to minimise clotting. The blood samples were transferred into heparin coated 5ml blood collection tubes (Inverclyde Biologicals, Bellshill, UK) and placed on blood tube rollers. The samples were centrifuged at 2000 *g* for 10 minutes and the plasma was removed and stored in 1.5 ml eppendorf tubes at -20°C. Multiple eppendorf tubes were prepared from each sample to avoid having to repeatedly thaw samples for each radioimmunoassay.

2.3 Identification of eggs laid to individual turkeys by feeding of fat soluble dyes

In some experiments it was necessary to record the individual egg production of the turkeys. As turkeys were usually housed several to a pen, a technique based on feeding fat soluble dyes was used to identify which turkey had laid each egg. The dyes were Sudan Black and Sudan III (red) obtained from Sigma Chemical Company (Poole, UK). Gelatin capsules (Size 4, Agar Scientific Ltd, Essex, UK) were half filled with dye. Dye filled capsules were force fed three times a week, by pushing the capsule to the back of the throat and then gently massaging the oesophagus to ensure that the capsule was swallowed. The traditional-line received one capsule at a time while the male-line turkeys were given two capsules each time as their body weight was much greater. Within a pen each turkey received either black dye capsules, red dye capsules, alternating red and black capsules or no capsules every two to three days.

The eggs collected were boiled in a water bath for 20 minutes and then cut in half. The rings of dye were clearly visible in the yolk of the eggs and therefore allowed identification of the egg to an individual turkey. Figure 2.1 shows a cross-section of a hard boiled turkey egg, laid by a turkey fed red and black capsules alternately

2.4 Dissection of the reproductive system

With the turkey positioned on its back, the abdomen was cut open around the rib cage with a scalpel and the skin deflected back over the breast muscle. The legs were pushed backwards, dislocating the hips to increase access to the body cavity. The abdominal fat pad, gizzard and intestines were pulled to one side to reveal the underlying reproductive system. Figure 2.2 shows the reproductive system as it lies in the abdomen. The muscular cord of the ventral ligament is located on the surface of the oviduct. When required it was dissected out by cutting the thin parts of the ventral ligament that attach the muscular cord to the oviduct. The oviduct was removed by cutting at the lowest part of the vagina accessible from within the body cavity, and cutting the dorsal ligament. The uterine and vaginal sections of the oviduct, shown in Figure 2.3, were dissected from the rest of oviduct.

The mature follicles were removed by cutting their stalks with scissors, and the rest of the ovary was cut from the body cavity wall. Figure 2.4 shows a turkey ovary with the mature yellow follicles and post ovulatory follicles still attached. The post ovulatory follicles and adrenal tissue were removed from the residual ovary with scissors.

Hocking (1987) used daily feeding of fat soluble dye capsules to show that, in the turkey, there should be a maximum of 9-10 follicles greater than 8mm making up the hierarchy for an ovulation a day, compared to 6-7 hierarchy positions in the chicken. Follicles were classed as part of the hierarchy if they weighed more than 0.5g or had a diameter greater than 8mm, as once follicles reach this size they are considered to be part of the hierarchy that will proceed to ovulation (Gilbert *et al.* 1983). Hierarchical follicles were counted and their individual weights recorded. The presence of grossly atretic mature follicles was also recorded. Grossly atretic follicles were flaccid and characterised by the presence of white, milky fluid just under the follicular surface.

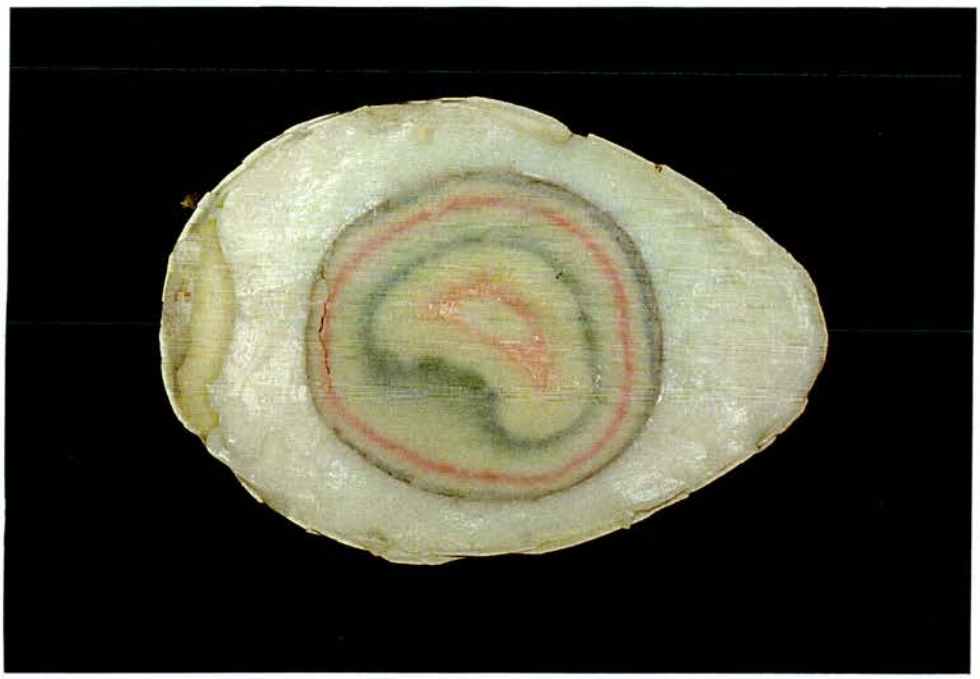


FIGURE 2.1 Cross section of a boiled egg laid by a turkey that had been fed fat soluble dye capsules, alternating between Sudan Black and Sudan III (red) every two to three days.

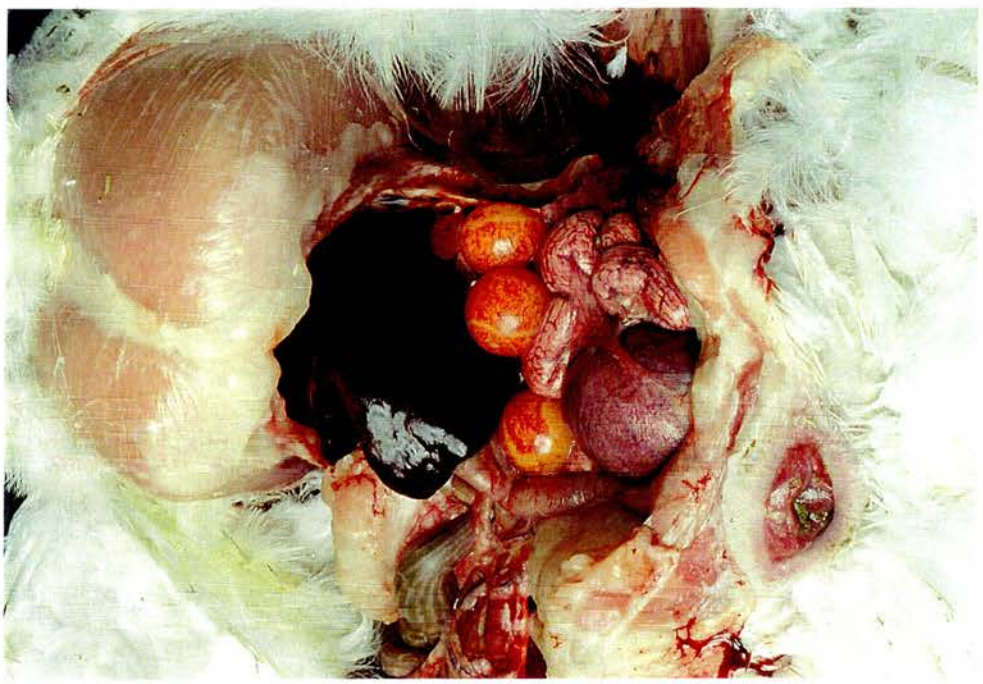


FIGURE 2.2 The reproductive system of the turkey (35 week male-line) as it is positioned in the body cavity.

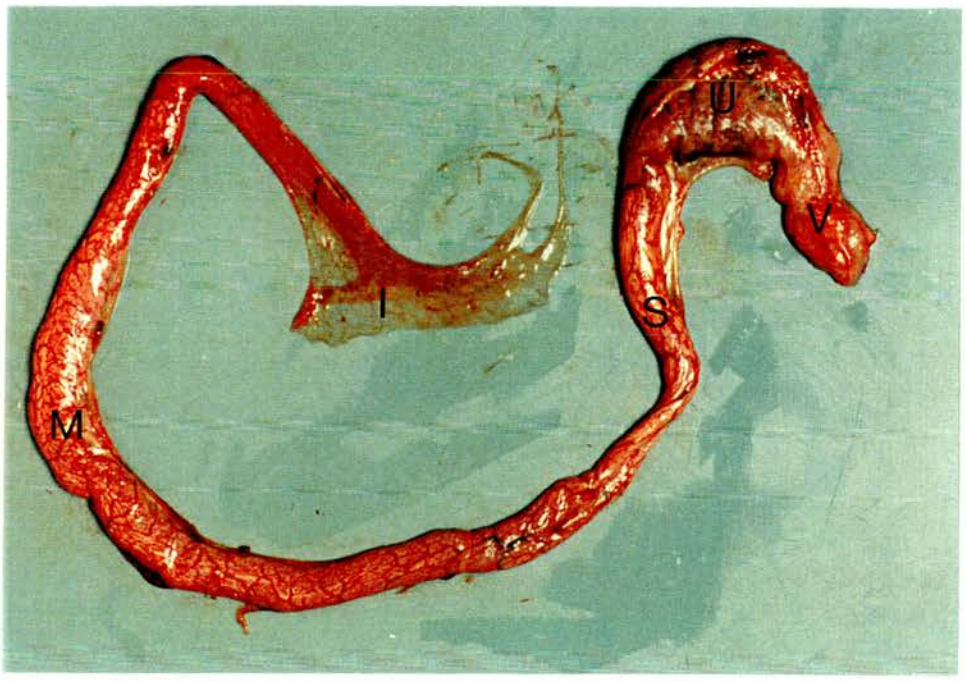


FIGURE 2.3 The oviduct of the turkey (35 weeks male-line) with the infundibulum (I), magnum (M), isthmus (S), uterine (U) and vaginal (V) sections indicated.

FIGURE 2.4 The ovary of the turkey (35 week male-line) with hierarchical follicles (H) and post ovulatory follicles (P) indicated.

2.5 Analysis of collagen content by colorimetic microassay for hydroxyproline

Tissue samples were collected at the time of killing and stored at -20°C in plastic self-sealing bags.

2.5.1 Acid hydrolysis of tissue

Tissue was thawed and 100-150g samples were weighed out in duplicate into 1.5ml screw cap tubes (Greiner Labortechnik Ltd, Gloucestershire, UK). 1ml of 6M hydrochloric acid was added to each tube and they were tightly closed and left at 107°C for 18-24 hours. Samples were dried in a vacuum oven at 40° (1-2 days). The samples were resuspended by adding 0.5 ml distilled water and vortexing until all debris was moving freely in the solution. Each sample was then spun at 1000*g* for 1 minute in a centrifuge to separate the collagen in solution from the other solid residues.

Ten µl of supernatant was removed from each sample and made up to 2ml distilled water for hydroxyproline measurement.

2.5.2 Hydroxyproline assay

This assay was based on the method described by Creemers *et al.* (1997). The ingredients for the solutions used in the hydroxyproline assay are given in Appendix 2.

Standards were made up by diluting stock hydroxyproline (65.5 µg/ml in 0.4ml) up to 4 mls and then double diluting with distilled water until 6 standards from 6.55µg/ml to 0.205 µg/ml were obtained.

60µl aliquots of each sample or standard, in duplicate, were dispensed onto a 96 well micro-plate. Each plate contained a blank and the six standards. To each well 20 µl of assay buffer and 40 µl of chloramine-T reagent were added (A.2). After a 15 minute incubation at room temperature 80 µl of DMBA reagent (A.2) was added and the contents of each well were mixed using a multichannel pipette. The plates were covered with Nescofilm (Merck Ltd, Leicestershire, UK) and placed in a 60 °C water bath for 20 minutes, such that the water was in contact with the underside of the plate as the bath was gently shaken.

The plates were cooled on ice for five minutes and read at 560 nm in a micro-plate reader (Dynatech, MR5000, Dynatech Laboratories Ltd, West Sussex, UK). The hydroxyproline content of each sample was determined from the standard curves, and the tissue hydroxyproline content was calculated taking the original sample weight into account. The collagen content of the tissue was estimated by multiplying the hydroxyproline content by 7.14 (Jackson *et al.* 1996).

2.6 Radioimmunoassay

The assays used to measure oestradiol, progesterone and luteinizing hormone were all classical double antibody radioimmunoassays. These assays were based on competition between the hormone in the sample and radiolabelled hormone for binding to the primary antibody. A second antibody was subsequently added and the binding complex was precipitated by centrifugation. After disposal of the supernatant the amount of radioactivity present represented the amount of radiolabelled hormone bound to the primary antibody, which was inversely proportional to the amount of hormone in the sample.

The radioimmunoassays were validated for use in turkey plasma by serial dilution of single samples to check for parallelism with the standard curve. The components of the buffering solutions used in the radioimmunoassays are given in Appendix 3.

The assays were analysed using Assay Zap software (Zaristow Software, Haddington, East Lothian, UK) on a Macintosh computer.

2.6.1 Oestradiol radioimmunoassay

The radioimmunoassay used to measure oestradiol concentration was an in-house radioimmunoassay maintained at the Roslin Institute (Webb *et al.* 1985).

2.6.1.1 Antibody-sepharose extraction

An initial affinity chromatography extraction procedure is necessary for measuring oestradiol in avian plasma due to the high lipid content of the plasma. This was achieved by adding [^{3}H]-oestradiol recovery label and anti-oestradiol-sepharose to the samples, which were passed through glass columns to extract the sepharose beads and bound oestradiol. The oestradiol sepharose binding was reversed with ethanol and the proportion of oestradiol recovered was calculated from the amount of tritium in each sample.

2.6.1.2. Coupling of anti-oestradiol antibody with CnBr-activated sepharose 4B

Oestradiol antiserum (150 µl, supplied by Mr G Baxter, Roslin Institute) was diluted into 18 ml coupling buffer (A.3). Nine g of Cn-Br activated sepharose 4B (Amersham Pharmacia Biotech UK Ltd., Bucks, UK) was placed onto a Grade 3 sintered funnel and washed with 9 × 200 ml aliquots of 1 mM HCl. The washed sepharose was immediately added to the antiserum/coupling buffer mixture and mixed in a 50ml ground glass stoppered tube overnight at 4°C.

The solution was transferred to a 200ml bottle and 65 ml 1M ethanolamine (adjusted to pH 9 with glacial acetic acid) was added. After mixing end over end for 2 hours at room temperature the ethanolamine was removed by washing the slurry in a Grade 3 sintered funnel with three cycles of 300 ml acetate buffer (A.3) followed by 300 ml

coupling buffer. The slurry was made up to 75ml with distilled water containing NaN_3 and stored at 4°C. After use the antibody-sepharose complex was recycled by washing with distilled water and methanol in a Grade 3 sintered funnel.

2.6.1.3 Oestradiol extraction efficiency check

Glass boiling tubes (16) were labelled and 10µl [^{3}H]-oestradiol recovery label (Amersham Pharmacia Biotech UK Ltd., Bucks, UK) was added to each tube. Half of the tubes received 3ml ovarectomised heifer plasma (supplied by Mr G Baxter, Roslin Institute) while the other half received 3ml distilled water. A further 7ml distilled water was added to every tube. Each tube received either 200, 300, 400 or 500µl sepharose mixture and was mixed end over end overnight at room temperature. The samples were treated as the assay extractions (2.6.1.4) and the amount of sepharose mixture used in the assay was determined from the amount that resulted in roughly 60% of the tritiated oestradiol being recovered.

2.6.1.4 Extraction procedure

Plasma samples (250 µl) were added to glass boiling tubes containing 10 µl recovery label ([^{3}H]-oestradiol). The tubes were vortexed and left at room temperature for 30 minutes.

Anti-oestradiol-sepharose was added to each tube as determined by the oestradiol extraction efficacy check. The amount of anti-oestradiol-sepharose used in the assays was 300 µl as determined in May 1998. Distilled water (9.25 ml) was added to each tube before end over end mixing overnight at room temperature.

The samples were poured into sintered glass columns. The columns were manufactured by Scotia Glass Technology (Kaimes Farm, Stirling, UK) according to the design supplied by Mr G Baxter, Roslin Institute. The columns consisted of a

glass tube (1cm diameter×12cm length) with a sintered disk separating the column from a tapered end section.

Each column was washed 3 times with 7 ml distilled water and residual water was removed by applying positive pressure to the column. The coupling between the oestradiol and the anti-oestradiol sepharose was reversed by adding 3 ml of 90% methanol (Fisher Scientific, Loughborough, UK) to each column to elute the extracted oestradiol into glass tubes (16 × 125 mm). Positive pressure was again applied to ensure maximum recovery of the oestradiol.

The methanol was removed from the samples by drying down in a Buchler Vortex Evaporator at 35°C and 1.8 ml of phos-gel assay buffer (A.3) was added. The tubes were mixed for 20 minutes. The recovery of oestradiol was measured by removing 500 µl sample to a scintillation vial containing 3 ml scintillation cocktail (Optiphase Hisafe 3, Fisher Scientific, Leicestershire, UK) and counting the amount of tritium present with a scintillation counter (Wallac 1410 Liquid Scintillation Counter, EG&G Wallac, Milton Keynes, UK). The total amount of tritium in 10 µl recovery label was also measured to allow the amount of tritium that would have been present had the extraction been 100% efficient to be determined. From this information the % recovery was calculated for each sample.

After use the anti-oestradiol sepharose was rinsed from the glass columns onto a large sintered glass filter funnel and washed with distilled water and 90% methanol. It was then transferred to the original container and reconstituted up to 75 ml with distilled water for future use. The columns were recycled with 3 cycles of 3 washes with 7ml distilled water and one wash with 3ml 90% methanol before re-use.

2.6.1.5 Oestradiol radioimmunoassay procedure

Assays were carried out in disposable 12 × 75 mm soda glass tubes (LIP Equipment and Services Ltd. West Yorkshire, UK) which had been washed and baked overnight.

Each sample was dispensed in duplicate (500 µl) and standards ranging from 0.5-48 pg/tube (supplied by Mr G Baxter, Roslin Institute) were dispensed in triplicate and made up to 500 µl with phos-gel (A.3). 100µl of [^{125}I]-oestradiol (12000 counts per minute, supplied by Mr G Baxter, Roslin Institute) was added to each tube followed by 200 µl of primary antibody diluted 1:40000 in phos-gel (R48, 22/3/83, supplied by Mr G Baxter, Roslin Institute). Totals, blanks, zero standards and quality controls were also included in each assay. The totals and blanks did not receive any primary antibody. A minimum of 2 standard curves were included in each single assay. The tubes were vortexed and incubated for at least 2 hours at room temperature.

Normal rabbit serum (100 µl diluted 1:400 in phos-gel, Scottish Antibody Production Unit (SAPU), Law Hospital, Lanarkshire, UK) was added to each tube except the total tubes. The second antibody, donkey anti-rabbit serum (SAPU) was diluted 1:40 in Phos-gel containing 10% EDTA (0.1M in PBS) and 100 µl was added to each tube, except the totals. The tubes were again vortexed and left overnight at 4°C.

Cold (4°C) phos-gel (1 ml) was added to each tube (except totals). The tubes were centrifuged for 25 minutes at 1800*g* and the supernatants were discarded (except totals). The amount of ^{125}I present in each tube was counted on an automatic gamma counter (1277 Gammamaster, EG&G Wallac, Milton Keynes, UK).

When possible the samples from each experiment were processed in a single assay to eliminate the effects of inter-assay variability. The mean intra-assay coefficient of variation was 15.1% while the inter-assay coefficient of variation was 19.5%.

The oestradiol concentration in Medium 199 (Chapter 6) was measured without the initial anti-oestradiol-sepharose extraction as there were no lipids present in the medium.

2.6.2 Progesterone radioimmunoassay

The progesterone concentration was measured using an in-house assay maintained at the Roslin Institute by Mr G Baxter. No extraction procedure was necessary as 8-anilino-1-naphthalenesulfonic acid (ANS) was added to inhibit plasma binding proteins (Law *et al.* 1992). This was not necessary when measuring the progesterone concentration of Medium 199 samples.

Duplicate samples (500 µl) of plasma or Medium 199 were dispensed into disposable 12 × 75 mm soda glass tubes (LIP Equipment and Services Ltd. West Yorkshire, England) which had been washed and baked overnight. Standards ranging from 5-1000pg/tube were made up to 500 µl with phos-gel assay buffer (A.3). Totals, blanks, zero standards and quality controls were also included.

Rabbit anti-progesterone (SAPU) was diluted 1:50000 in phos-gel and 200 µl was added to each tube (except totals and blanks). [^{125}I]-progesterone (Amersham Pharmacia Biotech UK Ltd., Bucks, UK) was diluted in phos-gel to approximately 14000 counts per minute /100 µl and contained 1mg/ml ANS. Each tube received 100 µl of [^{125}I]-progesterone and was vortexed before overnight incubation at 4°C.

Donkey anti-rabbit IgG (SAPU) was diluted 1:35 in phos-gel with 10% EDTA (0.1M) and 100 µl was added to each tube (except totals). Normal rabbit serum (SAPU) was diluted 1:300 with phos-gel and each tube received 100 µl (except totals). The tubes were vortexed and incubated at 4°C overnight.

One ml of phos-gel (4°C) was added to each tube before centrifugation at 1800*g* for 30 minutes and discarding the supernatant (except totals). The precipitates were counted on a gamma counter (1277 Gammamaster, EG&G Wallac, Milton Keynes, UK).

The progesterone samples from individual experiments were analysed in single assays when possible to eliminate the effect of inter-assay variability. The mean intra-assay coefficient of variation was 11.3% and the inter-assay coefficient of variation was 26.5%.

2.6.3 Luteinizing hormone radioimmunoassay

The radioimmunoassay used to measure plasma luteinizing hormone was developed by Sharp *et al.* (1987), using the assay procedure described by Follett *et al.* (1972).

Triplicate 50 µl samples were diluted to 200 µl with LH assay buffer (A.3) in 7 × 51 mm polystyrene tubes. Standards ranging from 0.045-2 ng/tube were dispensed in triplicate. Totals, blanks and quality controls were also included in the standard curves. The primary antibody (Anti-LH 3/3, supplied by Mr R Talbot, Roslin Institute) was diluted 1:152000 in assay buffer and 50 µl were added to each tube except the totals and blanks. The samples were vortexed and incubated overnight at 4°C. [^{125}I]-luteinizing hormone (50 µl containing 12000 counts per minute, supplied by Mr R Talbot, Roslin Institute) was added to each tube and samples were vortexed and left overnight at 4°C. The secondary antibody, donkey anti-rabbit IgG diluted 1:20, and normal rabbit serum diluted 1:200 were added (50µl of each, both supplied by SAPU) to each sample except the totals and the tubes were vortexed and incubated overnight at 4°C. After centrifugation at 2200*g* for 30 minutes, 50 µl of 6% starch (Sigma Chemical Company, Poole, UK) solution was added and the tubes were spun for a further 10 minutes and the supernatant was aspirated from each tube (except totals). The pellets were counted on a gamma counter (1277 Gammamaster, EG&G Wallac, Milton Keynes, UK). All samples were measured in a single radioimmunoassay with an intra-assay coefficient of variation of 12.3%.

2.7 Immunohistochemistry

Sections of uterine and vaginal tissue were dropped into isopentane that had been cooled by standing the beaker in an alcohol and dry ice slurry. After 20 seconds in the isopentane the samples were removed, placed in pre-chilled plastic self seal bags and stored at -70°C. At a later date the samples were transferred to a Shandon Model OT Cryostat (Shandon Scientific Ltd, Runcorn, UK) at -40°C and attached to metal chucks using OCT compound (Merck Ltd., Leicestershire, UK). The samples were then cut at 10 μm and placed on polysine microscope slides (Merck Ltd.). The slides were placed in -20° acetone for 5 minutes, wrapped in tin foil and stored at -70°C for immunostaining at a future date.

On the day of immunostaining the samples were removed from the -70°C freezer and left for 30 minutes to thaw. They were treated according to the staining protocols described in Appendix 4 for collagen types I and III staining and Appendix 1 for oestradiol receptor staining.

2.8 Statistics

Statistical analysis of data was carried out using Genstat 5 (Release 4.1 Third Edition, Lawes Agricultural Trust, IACR Rothamsted) or Microsoft Excel 97, with significance levels represented as follows; $P<0.05$ *, $P<0.01$ **, $P<0.001$ ***.

3. Development of the reproductive system in turkeys with a high or low susceptibility to oviduct prolapse

INTRODUCTION

Hocking (1993b) found no disproportion in egg size, pelvic dimension, vent diameter and abdominal fat pad between male-line and female-line turkeys at the onset of lay. Lilburn and Nester (1993) compared oviduct development in two strains of turkeys, one selected for body weight and therefore a male-line, and the other selected for egg production, therefore a female-line. Their results led them to suggest that the oviduct of the male-line might not be as reproductively mature as the oviduct of the female-line at the start of lay. In a similar experiment it was hypothesised that ovulation occurred in the male-line strain before the oviduct was fully mature (Melnychuk *et al.* 1994; Melnychuk *et al.* 1997). They concluded that the ovary of the male-line was fully developed 3 days before that of the female-line, but this was not substantiated by any statistical analysis. It is possible that an immaturity of the oviduct at the onset of lay could predispose the male-line to prolapse.

Rao *et al.* (1985) hypothesised that prolapse in hens was caused by over-development of the ventral ligament resulting in increased torsion at the utero-vaginal junction that impeded the passage of an egg.

In hens there is a pre-lay peak in plasma oestradiol (Senior 1974), while in turkeys there is a peak in total oestrogens at the onset of lay but no visible oestradiol peak (Bacon *et al.*. 1980). Five weeks after photostimulation, when prolapse is most common, plasma oestradiol was lower in the male-line than the traditional-line (Chapter 4). This suggested that oestradiol may not be involved in predisposing the male-line to prolapse, but high oestradiol levels before or at the onset of lay could stimulate vaginal collagen degradation and predispose the strain to prolapse of the oviduct. No peak in plasma progesterone concentration has been found between 8

weeks of age and the onset of lay in domestic hens although there is a sharp increase in progesterone at the onset of lay (Williams and Sharp 1977).

The aim of this work was to investigate the development of the reproductive system to identify any differences between traditional and male-line strains that could predispose the male-line to prolapse of the oviduct. In particular, was the oviduct of the male-line fully mature at the onset of lay and was there any difference in the weight of the ventral ligaments of the two strains?

The plasma hormone profile during sexual maturity was also investigated. Specifically plasma oestradiol and progesterone concentrations were measured to determine whether turkeys have pre-lay peaks in oestradiol or progesterone, and to compare the plasma hormone profile of the two strains from photostimulation throughout the early period of lay.

Two experiments were carried out. The first experiment compared the anatomy and histology of the reproductive system at first egg, and thrice weekly blood samples were taken from photostimulation to first egg to compare the changes in plasma oestradiol and progesterone in traditional- and male-line turkeys. In the second experiment the anatomical and histological development of the reproductive system and the plasma oestradiol and progesterone concentrations were compared in the two lines weekly for seven weeks following photostimulation.

Data on growth of the ovary and oviduct in male-line and female-line turkeys was extracted from the paper by Melnychuk *et al.* (1997) and re-analysed to evaluate the validity of the conclusions drawn from their work.

METHODS

The strains used for this experiment were the Big 5 male-line and the traditional, unselected Nebraska Spot. Female turkeys were reared as described in 2.1 and photostimulated at 29 weeks and 4 days.

In the first experiment six turkeys from each strain were housed in six pens; each pen contained one turkey from each strain. Turkeys were blood sampled on the first day of photostimulation and then three times a week until first egg. Extra care was taken when blood sampling to reduce haematoma formation by applying pressure on the sampling site immediately after the sample had been taken. In this experiment the blood sampling was carried out between 12 and 13 hours after lights on to try to minimise the effects of ovulation and oviposition on the plasma steroid hormone profile. The turkeys were fed fat soluble dye capsules twice a week from photostimulation to identify the onset of egg production in each bird, as described in 2.3. The pens were checked for eggs several times a day and turkeys were killed the day after laying their first egg.

In the second experiment 32 traditional and 32 male-line turkeys were used. Starting from photostimulation 4 birds were randomly selected from the two strains each week for 8 weeks. They were blood sampled 7-9 hours after lights on, immediately before being killed.

All turkeys were blood sampled and killed as described in 2.2. After killing, the total body weight was recorded for each bird. The abdominal cavity was opened and the muscular cord of the ventral ligament was dissected out and weighed as described in 2.4. The oviduct was removed and its length and weight were recorded. The uterus and vagina were removed from the oviduct and weighed. The ovary was removed from the abdominal cavity and weighed. The ovarian follicles were classified (2.4) and weighed. The residual ovary weight was also recorded. The *sphincter ani* muscle

(Harvey *et al.* 1968) was removed from the outside of the vent and the surrounding skin and fat were dissected from it before it was weighed.

Samples were taken for histological investigation in both experiments. From each turkey a sample was taken from the uterus, vagina, muscular cord of the ventral ligament and *sphincter ani* muscle. Samples were also taken from four additional male-line turkeys that had prolapsed 5 weeks after photostimulation. The samples from the uterus and the ventral ligament were pinned to balsa wood to maintain their shape. Samples were placed in individual Tissue Tek Mega-cassettes (Diagnostics Division, Miles Inc. USA) and fixed in buffered neutral formalin. After at least two weeks in buffered neutral formalin the samples were dehydrated using a Shandon Hypercentre XP tissue processor (Shandon Scientific Limited, Astmoor, Runcorn, UK) and embedded in paraffin wax. Sections were cut, mounted on albumin coated microscopy slides and stained with Haematoxylin and eosin or Van Gieson's stains. Haematoxylin and eosin strains nuclear structures purple and cytoplasmic and intercellular structures pink, while Van Gieson's stains muscle yellow and connective tissue red. The procedures for these stains are given in Appendix 2. The vaginal, uterine and *sphincter ani* sections were cut transversely while longitudinal sections were cut from the muscular cord of the ventral ligament.

In the first experiment samples were taken from the uterus and vagina for immuno-histochemical investigation of the presence of collagen type I and III. The sections were taken and treated as described in 2.7. Collagen type I and III antibodies were obtained from Chemicon International, Ltd. (Harrow, UK). Both primary antibodies were rabbit anti-chick collagen and the second antibody was FITC conjugated rabbit IgG raised in a goat (Sigma Chemical Company, Poole, UK). The protocol used for the immuno-staining is given in Appendix 4. Negative controls were carried out by replacing the incubation with the primary antibody with an incubation in the second antibody.

The samples for measurement of oestradiol were initially extracted using antibody-sepharose binding. Oestradiol was measured in the extract by a double antibody

radioimmunoassay as described in 2.6.1. Plasma progesterone was measured by radioimmunoassay (2.6.2).

Analysis of variance was used to test the significance of differences between the two strains at first egg. The statistical model included effects for strain and pen.

Regression analysis was used to compare changes in the mean length and weight of the structures over the seven weeks following photostimulation between the two strains. A range of non-linear models were evaluated and the model with the smallest residual mean square was selected. The models used, their equations and an explanation of their parameters are given on Table 3.1. The R^2 value represents the percentage variance accounted for by the fitted curve.

Values for plasma oestradiol and progesterone concentrations were transformed to natural logs to ensure normal distribution of the residuals. Analysis of variance and regression analysis were used to test for significant strain or week effects.

The graphs of Melnychuk *et al.* (1997) (Figures 8 and 9) were scanned into TIFF files and the original data were reconstructed using NIH Image Analysis software. Non-linear regression analysis was used to compare differences between ovary and oviduct growth in the male- and female-lines.

TABLE 3.1 Models used for regression analysis

Model	Equation	Parameters	
Logistic	$y=C+A/(1+EXP(-R(x-M)))$	A	Upper asymptote
		C	Lower asymptote
		R	Rate parameter
		M	Point of inflection
Exponential	$y=A+BR^x$	A	Upper asymptote
		B	Scaling factor
		R	Rate parameter

RESULTS

Experiment 1

Gross anatomy

There was no significant difference in time to first egg between the traditional- and male-line. Both strains took an average of 23.8 ±1.95 days of photostimulation to come into lay. The total body weight, oviduct length and weights of the oviduct, uterus, vagina, total and residual ovary, *sphincter ani* muscle and muscular cord of the ventral ligament, and number of hierarchical and post ovulatory follicles are shown on Table 3.1.

Table 3.1. Mean and SED of body weight, length and weight of oviduct and weight of uterus, vagina, residual ovary, sphincter-ani muscle, muscular cord of ventral ligament and number of hierarchical follicles and post ovulatory follicles for traditional- and male-line turkeys at first egg.

	Traditional-line	Male-line	SED	Significance
Total body (kg)	5.6	17.5	0.77	***
Oviduct (mm)	73	85	4.0	*
Oviduct (g)	70	123	8.2	***
Uterus (g)	18	27	2.4	*
Vagina (g)	7.3	11.6	1.33	*
Total ovary (g)	78	247	18.7	***
Residual ovary (g)	5.8	25.9	2.67	***
Number follicles	8.5	22.2	1.61	***
Number POFs	2.7	6.2	0.76	**
Sphincter ani (g)	6.3	11.5	0.49	***
Ventral ligament (g)	0.53	0.88	0.140	ns

The male-line had greater body weight at first egg compared to the traditional-line. The oviduct was longer and heavier in the male-line than the traditional-line. The uterus and vagina were also heavier in the male-line strain compared to the traditional-line. The total and residual ovary weighs were greater in the male-line. There were significantly more hierarchical follicles and post ovulatory follicles in the male-line compared to the traditional-line. The *sphincter ani* muscle was also heavier

cord of the ventral ligament between the two strains although it was heavier in the male-line.

Plasma oestradiol concentration

The turkeys came into lay between 17 and 30 days after photostimulation. As the blood samples were taken three times a week from photostimulation until the onset of lay, there were varying numbers of samples for individual turkeys.

Figure 3.1 shows the mean plasma oestradiol concentrations for each strain plotted against the number of days from photostimulation that the sample was taken. Towards the right hand side of the graph the number of birds in each group decreased as more turkeys came into lay.

The same data arranged as the sample number before the onset of lay are presented in Figure 3.2. The right-most points represent the final sample taken for each bird before first egg. In this graph the number of birds in each group decreases towards the left hand side of the graph.

Linear regression analysis showed no significant effect of either days since photostimulation, or sample number before first egg on plasma oestradiol concentration. There was a significant effect of strain, as plasma oestradiol concentration was greater in the traditional-line (256.9pg/ml) than the male-line (194.4pg/ml), $P<0.01$, SED=16.9.

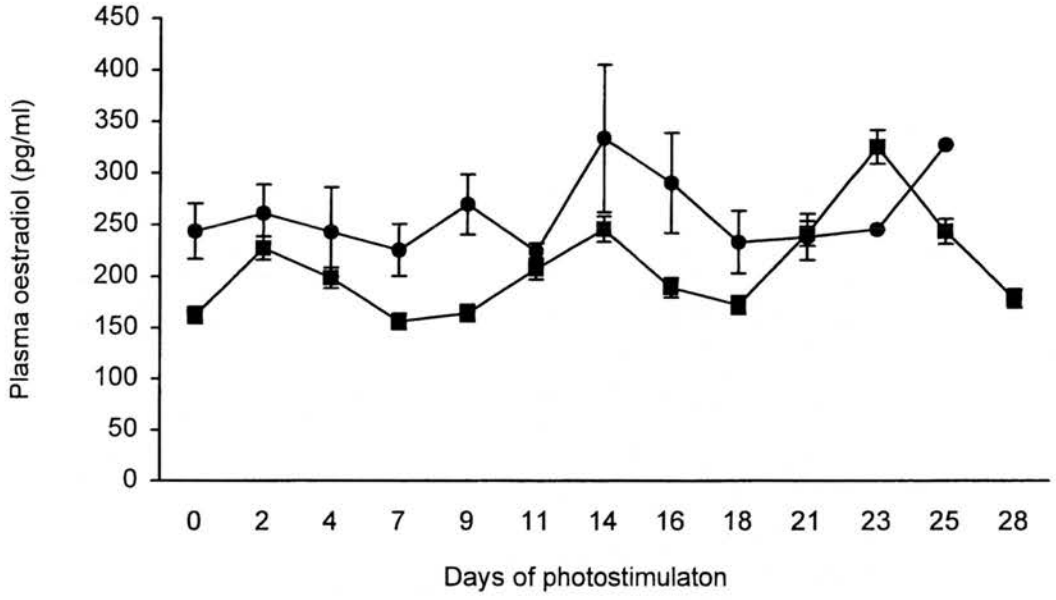


FIGURE 3.1 Mean (±SEM) plasma oestradiol concentration by day of photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

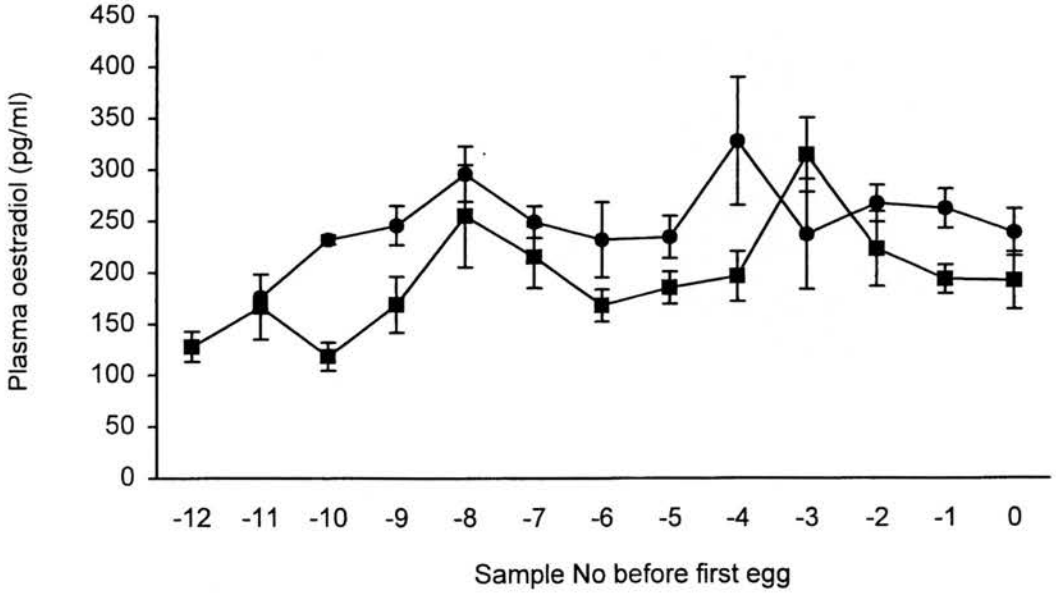


FIGURE 3.2 Mean (±SEM) plasma oestradiol concentration by sample number before first egg in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

Histological investigation

There were no significant differences between the traditional- and male-line turkeys in the histological sections from the uterus, vagina, muscular cord of ventral ligament or *sphincter ani* muscle at the onset of lay. The structures closely resembled the ones taken at 5 weeks post photostimulation presented later in this chapter. No differences were found in either the haematoxylin and eosin or Van Gieson's stained slides.

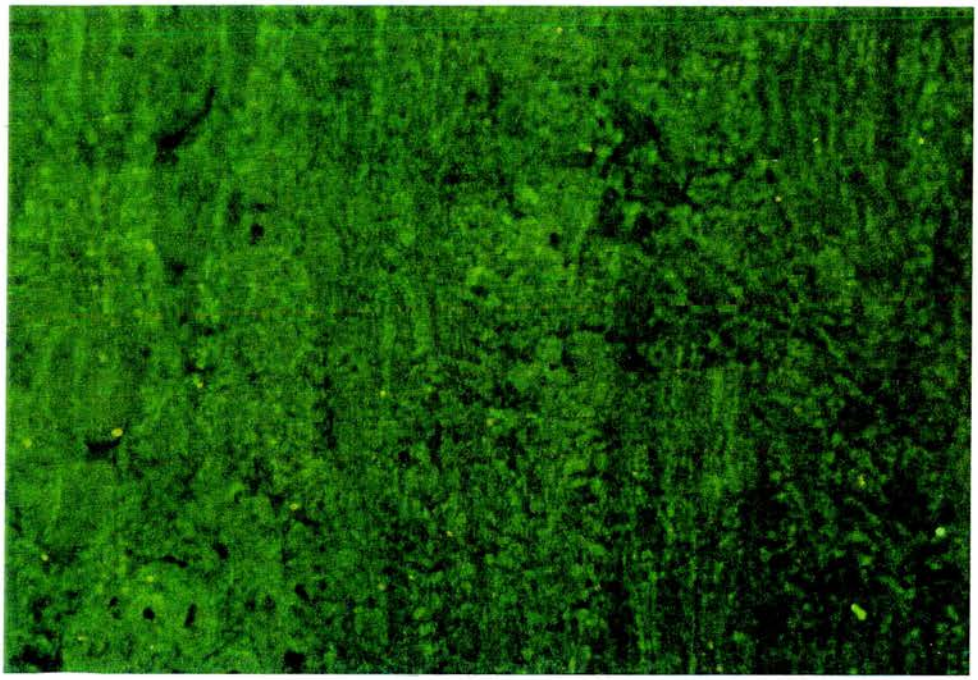
The immunohistological investigation into the distribution of collagen type I and III showed both types of collagen to be widely distributed across the uterus and vagina in both the traditional- and male-line strains while there was no staining on the control sections (Figures 3.3-3.7). There were no obvious differences in the distribution of type I and III collagen between the traditional- and male-line turkeys that was detectable by immunohistochemical techniques.

Experiment 2

Gross Anatomy

The male-line turkeys were significantly heavier than the traditional-line turkeys (16.0±0.31 kg compared to 5.4±0.07 kg, $P<0.001$). Figures 3.8 and 3.9 respectively show the length and weight of the oviduct in the traditional- and male-line from weeks 0-7 post photostimulation. Figures 3.10 and 3.11 respectively show the change in weight of the uterus and vagina from 0-7 weeks after photostimulation and similar data for the residual ovary weight is presented in Figure 3.12. A logistic curve gave the best fitting model for oviduct length and weight, residual ovary weight and weight of the uterine and vaginal sections of the oviduct. The fitted curves are shown on the figures: the parameters of the fitted curves and their standard errors are given on Table 3.3.

(a)



(b)

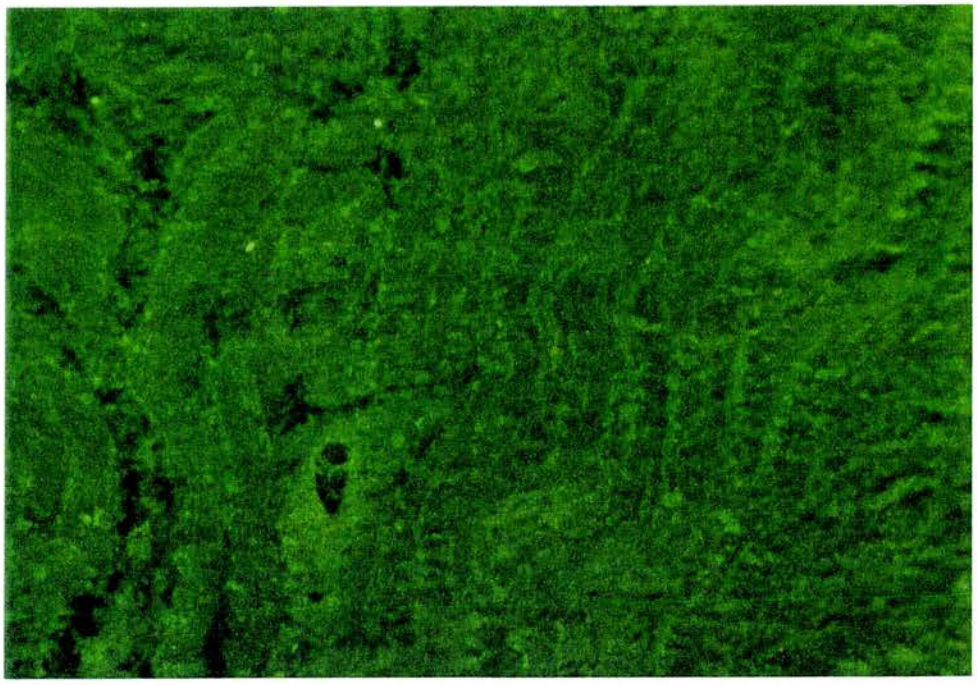
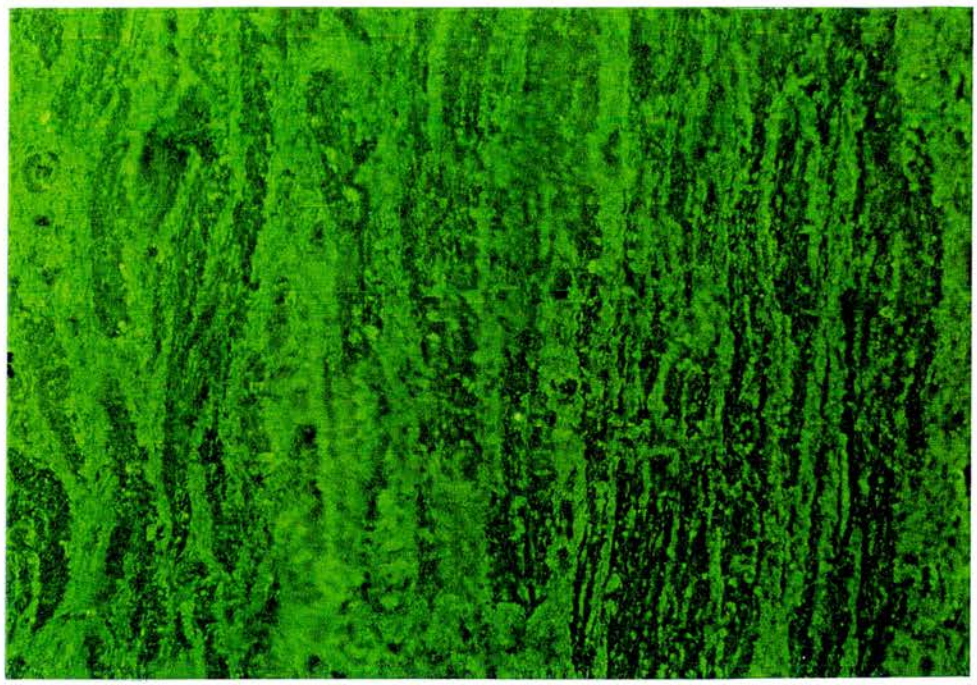


FIGURE 3.3 Vaginal cross sections with immunofluorescent staining representing collagen type I. (a) traditional line; (b) male-line. $_{\times}$144 magnification.

(a)



(b)

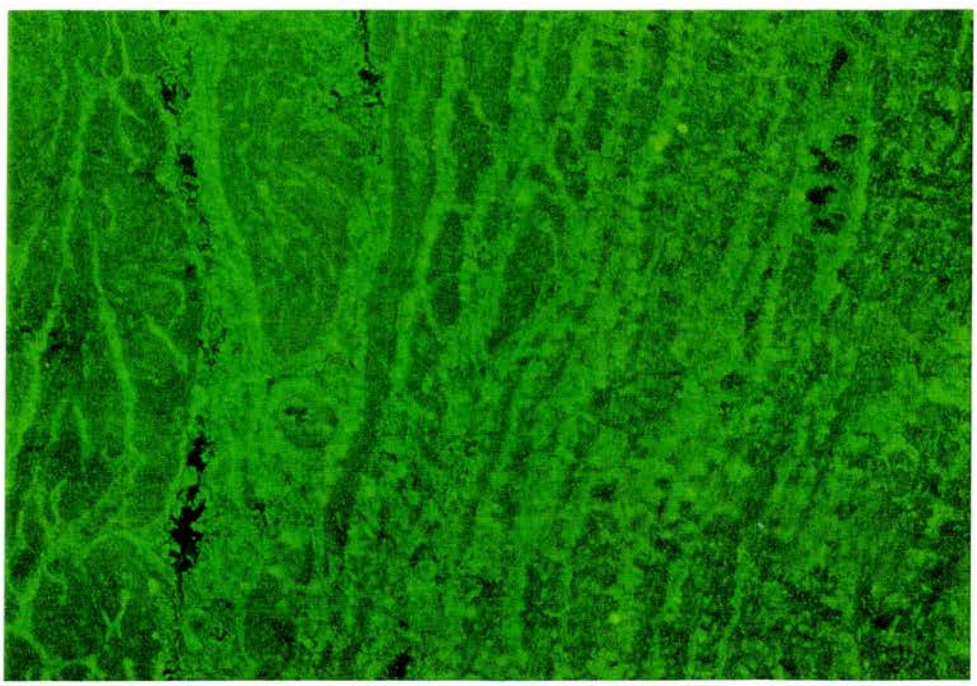
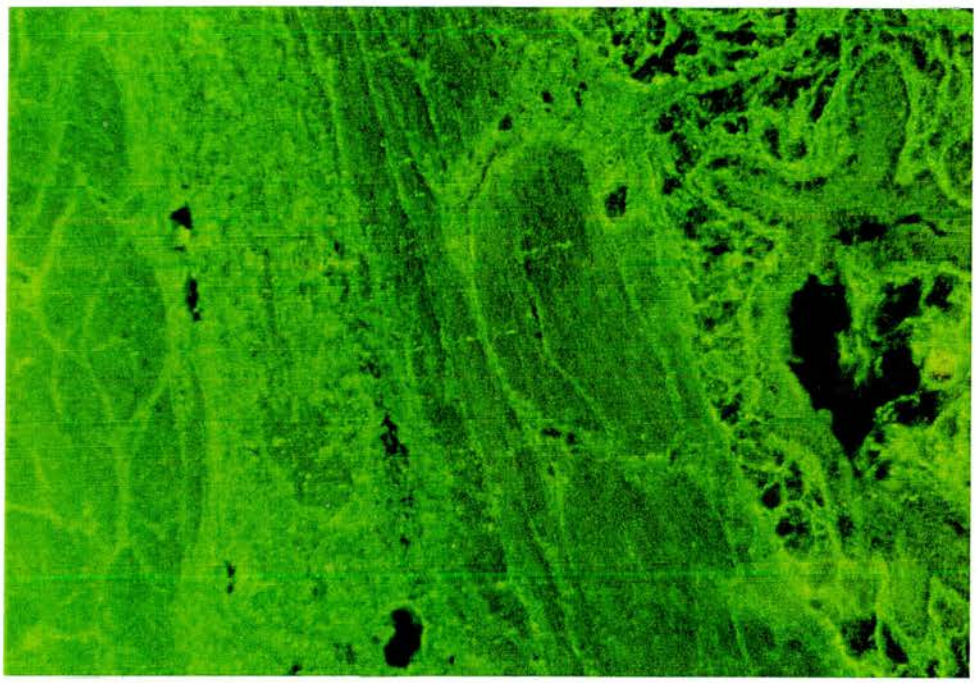


FIGURE 3.4 Vaginal cross sections with immunofluorescent staining representing collagen type III. (a) traditional line; (b) male-line; (c) negative control. $_{\times}$230 magnification.

(a)



(b)

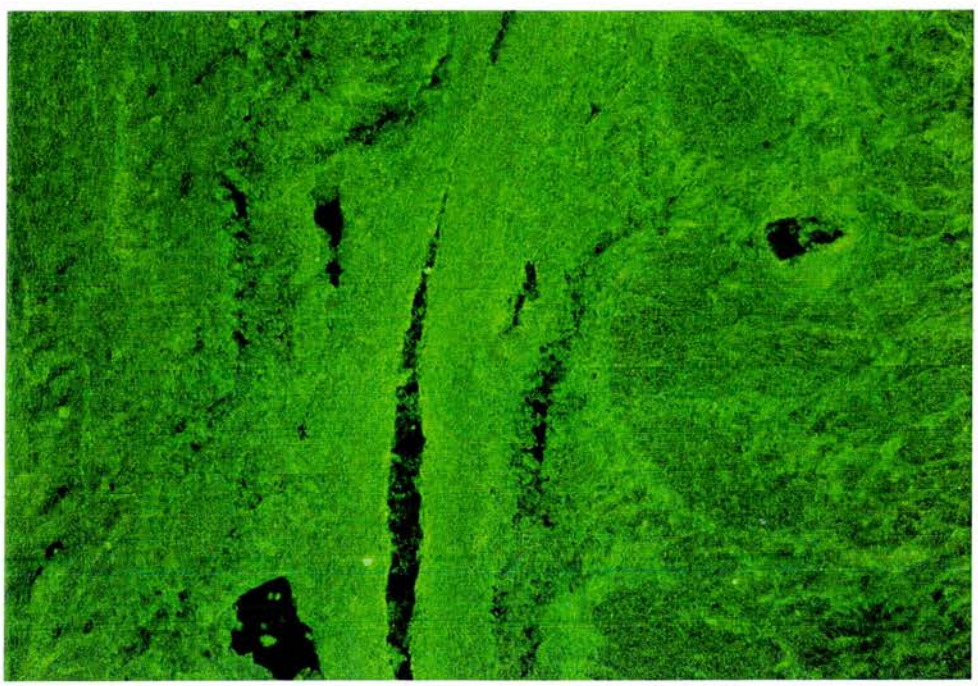
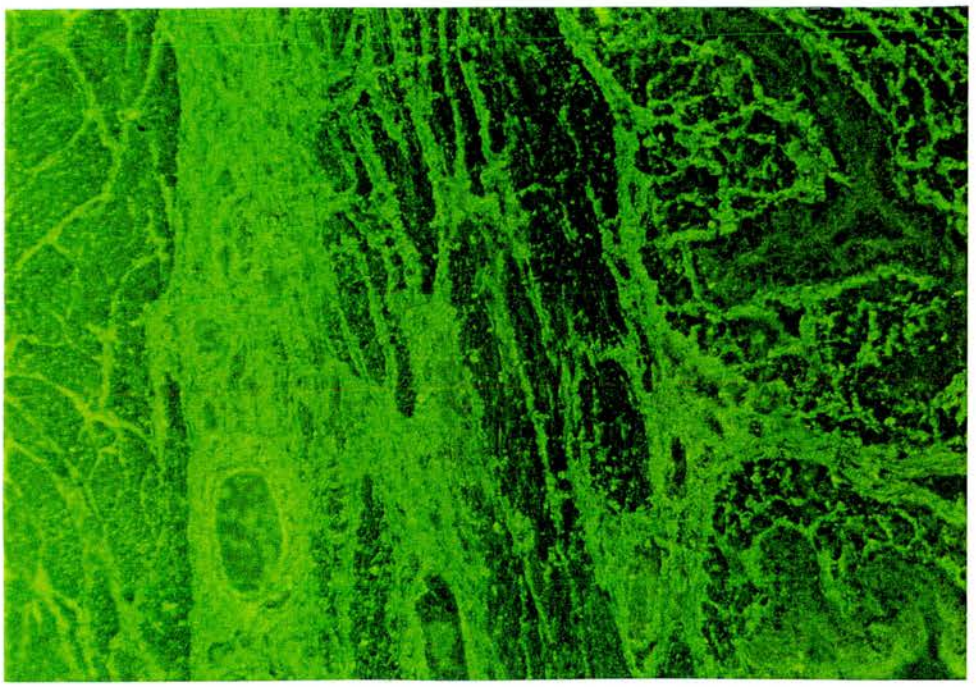


FIGURE 3.5 Uterine cross sections with immunofluorescent staining representing collagen type I. (a) traditional line; (b) male-line; (c) negative control. $_{\times}$230 magnification.

(a)



(b)

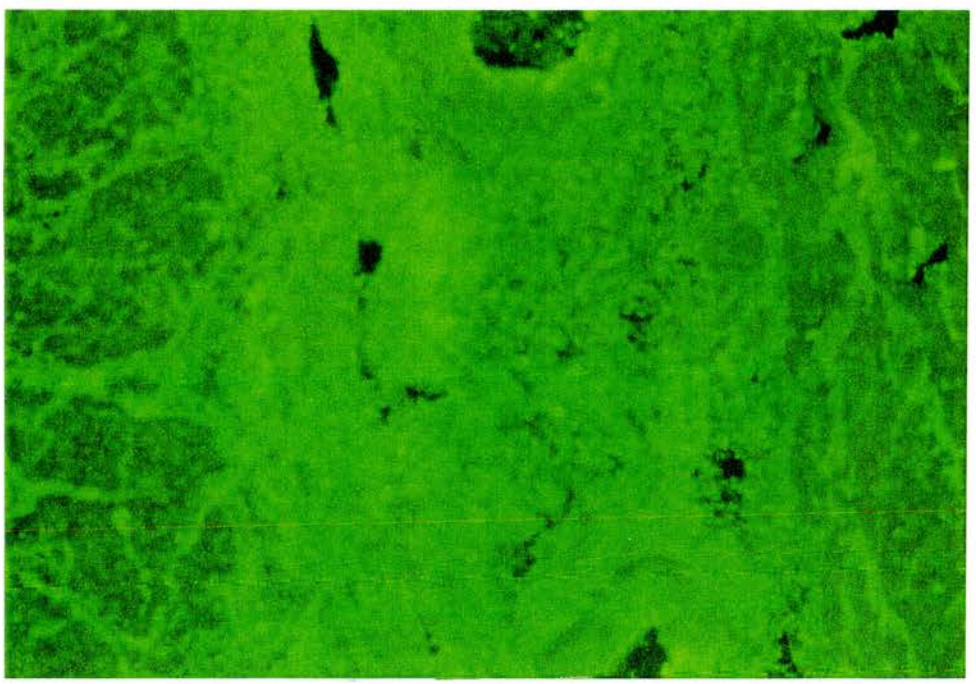
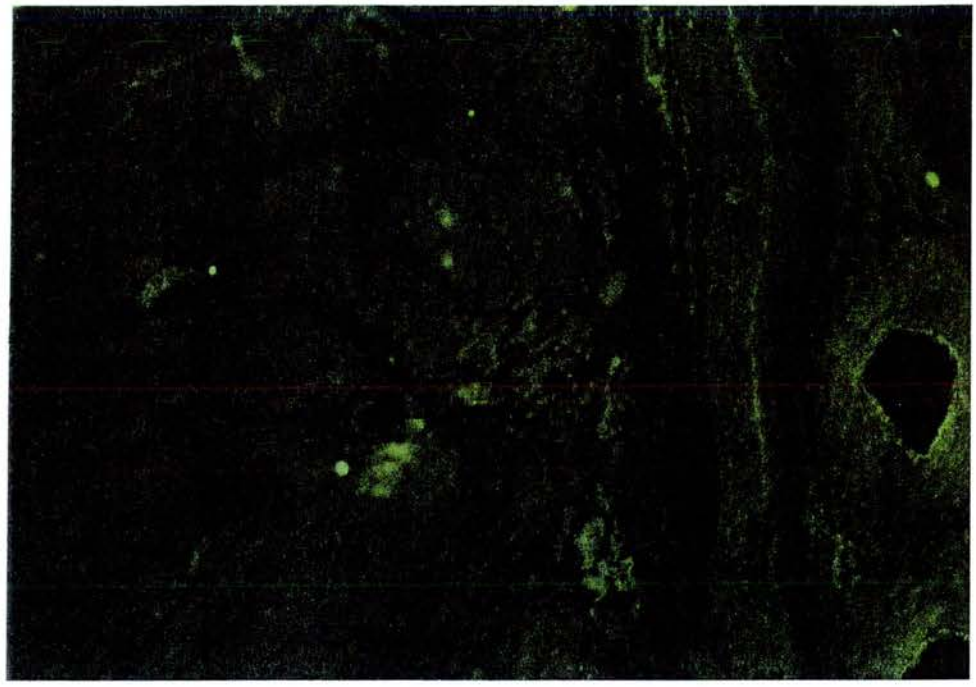


FIGURE 3.6 Uterine cross sections with immunofluorescent staining representing collagen type III. (a) traditional line; (b) male-line; (c) negative control. $_{\times}$230 magnification.

(a)



(b)

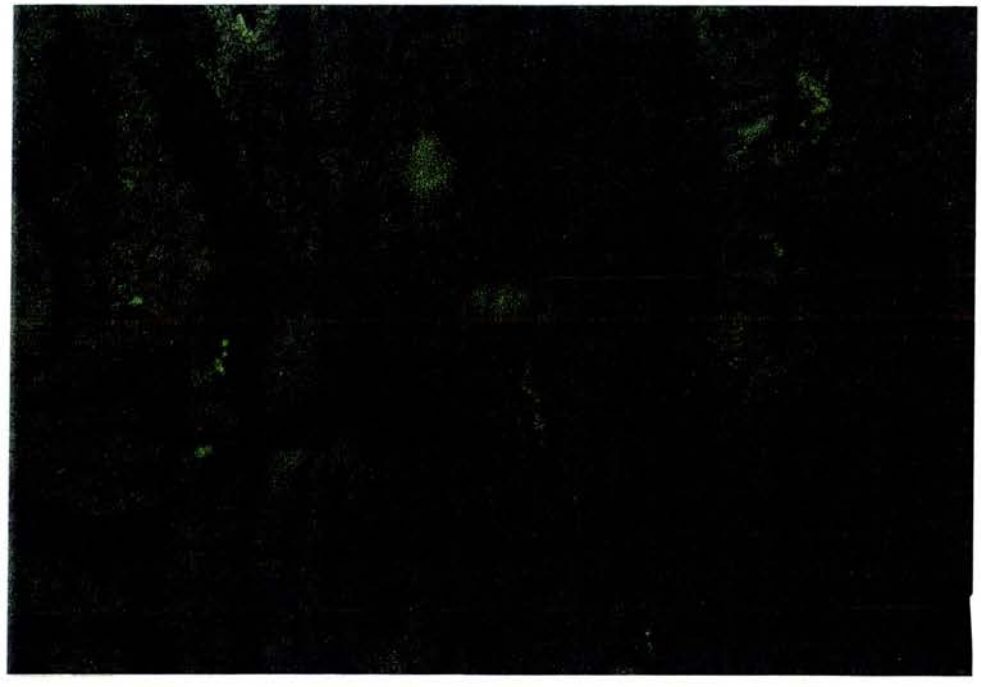


FIGURE 3.7 Negative control sections with the first antibody replaced with goat anti-rabbit IgG. (a) vagina; (b) uterus. $_{\times}$230 magnification.

TABLE 3.3 Estimates ±SE of fitted parameters of logistic curves for oviduct length, weight, residual ovary weight and weight of the uterine and vaginal portions of the oviduct in traditional- and male-line turkeys

	Rate parameter	Point of inflection (weeks)	Upper asymptote (g)	Lower asymptote (g)	R^2
Oviduct length					
Traditional-line	1.1 ± 0.37	1.7 ±0.46	724 ±156	81 ± 139	96.8
Male-line	1.1 ±0.37	1.7 ±0.45	751 ±159	166 ±142	
Oviduct weight					
Traditional-line	1.8 ±0.72	1.8 ±0.26	76 ±11.3	0.02 ±9.91	97.0
Male-line	1.3 ±0.36	1.9 ±0.26	120 ±15.9	-4.2 ±14.0	
Residual ovary					
Traditional-line	0.9 ±1.27	2.3 ±1.60	7.8 ±5.72	0.7 ±4.65	95.6
Male-line	1.4 ±0.57	2.3 ±0.34	19.5 ±3.27	6.2 ±2.67	
Uterus weight					
Traditional-line	2.6 ±1.60	1.9 ±0.20	20.5 ±2.75	0.8 ±2.31	95.6
Male-line	1.6 ±0.56	2.0 ±0.23	29.7 ±3.70	0.9 ±3.17	
Vagina weight					
Traditional-line	1.8 ±1.62	1.0 ±0.78	6.0 ±3.15	-0.8 ±2.95	87.4
Male-line	2.0 ±1.5	0.9 ±0.52	8.0 ±3.06	-0.8 ±2.90	

Each of these five fitted models showed a significant change ($P<0.001$) in the residual mean square when the constant and linear parameters (upper and lower asymptotes) were fitted separately for each strain but there were no significant change when the non-linear parameters (rate parameter and point of inflection) were fitted. The point of inflection of the curve gives an indication of the time when half of the growth is complete. There was no significant strain difference in point of inflection for oviduct length, weight, residual ovary weight, or weight of the uterus or vagina. The point of inflection was numerically greater in both strains for ovary weight compared to oviduct weight, but there was no difference between the traditional- and male-lines.

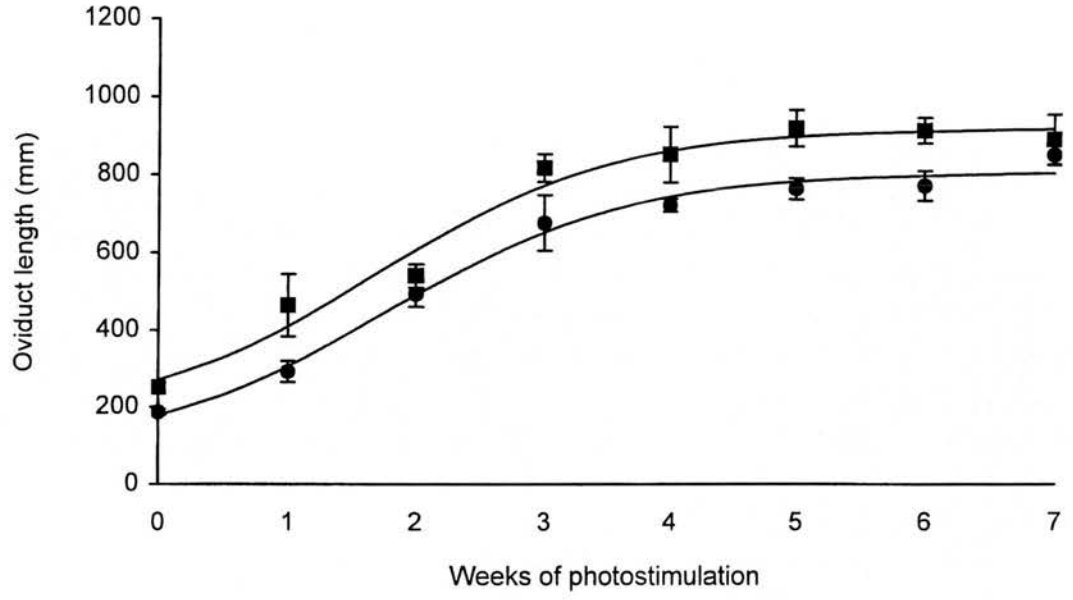


FIGURE 3.8 Mean (±SEM) length of the total oviduct from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

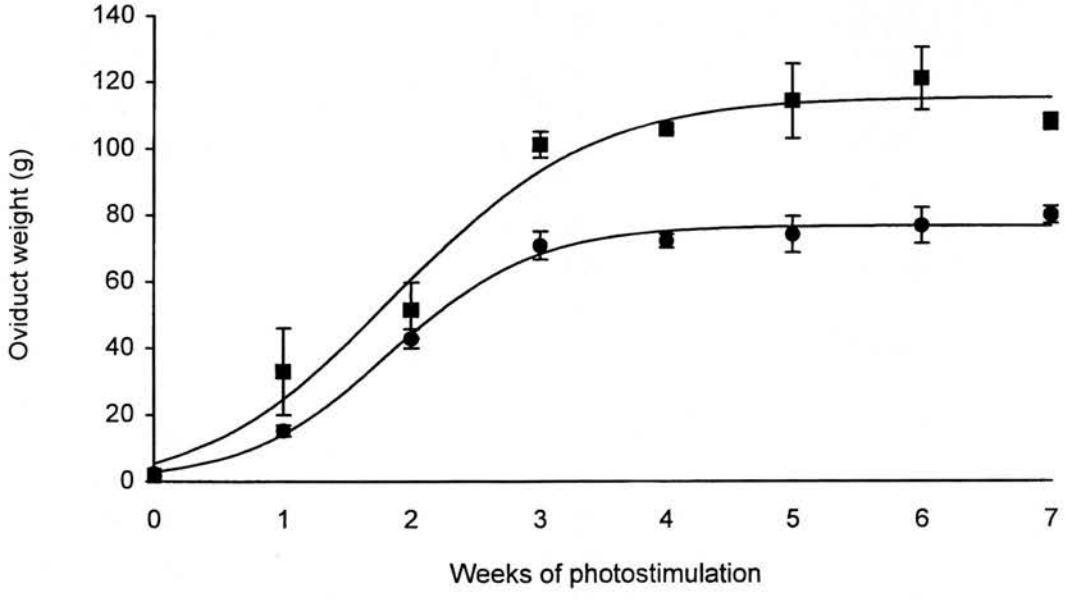


FIGURE 3.9 Mean (±SEM) weight of the total oviduct from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

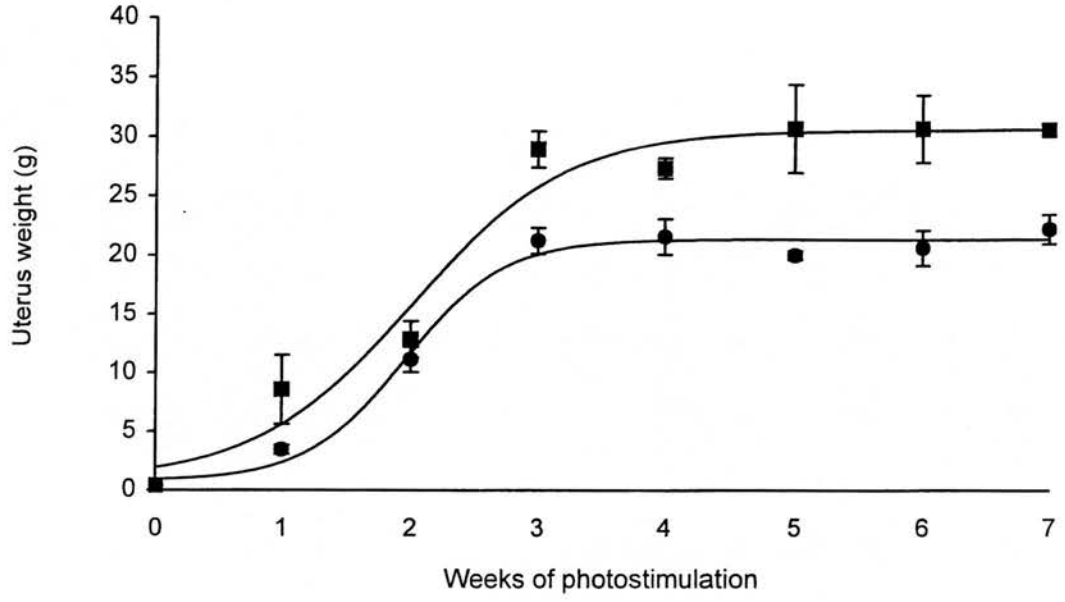


FIGURE 3.10 Mean (±SEM) weight of the uterine section of the oviduct from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

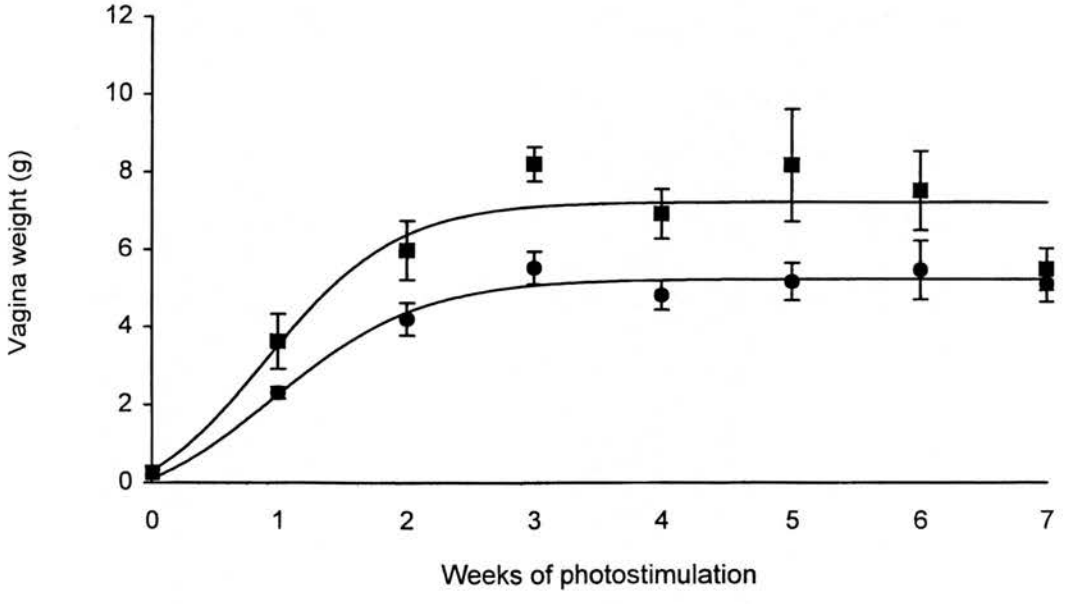


FIGURE 3.11 Mean (±SEM) weight of the vaginal section of the oviduct from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

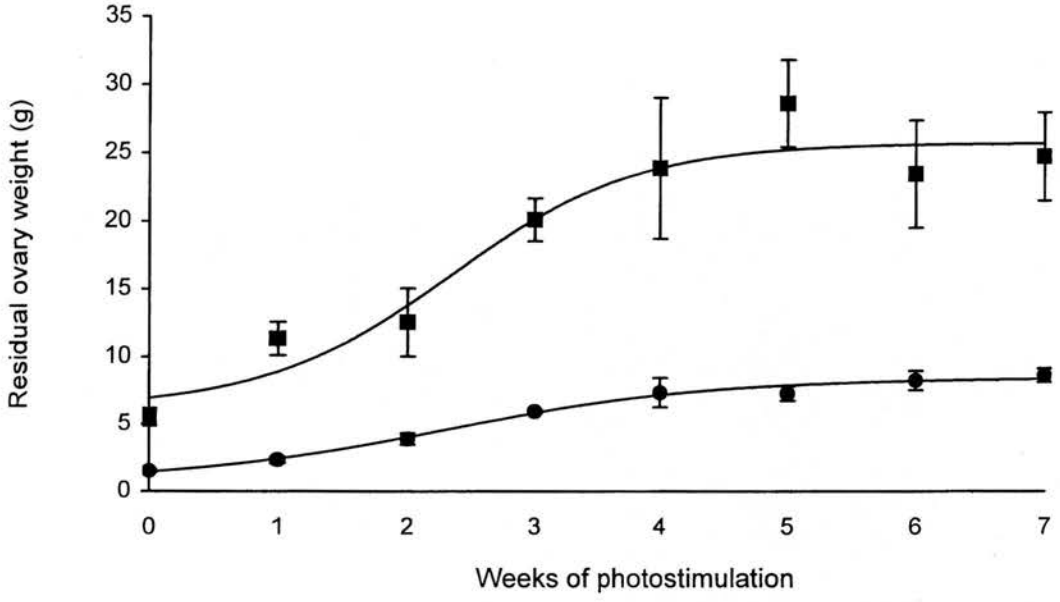


FIGURE 3.12 Mean (±SEM) weight of the residual ovary from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

The mean number of follicles (>8mm) in the hierarchy of traditional- and male-line turkeys for seven weeks after photostimulation and the changes in weight of the muscular cord of the ventral ligament are shown on Figures 3.13 and 3.14 respectively. Exponential models were fitted to the weekly means for the number of hierarchical follicles and ventral ligament weight. The estimates for the fitted parameters are given on Table 3.4. The fitted models for both number of hierarchical follicles and weight of ventral ligament showed a significant change ($P<0.001$) when the linear parameters (upper asymptote and scaling factor) were fitted separately for each strain but no significant change in the residual mean squares was found when the non-linear parameters were fitted.

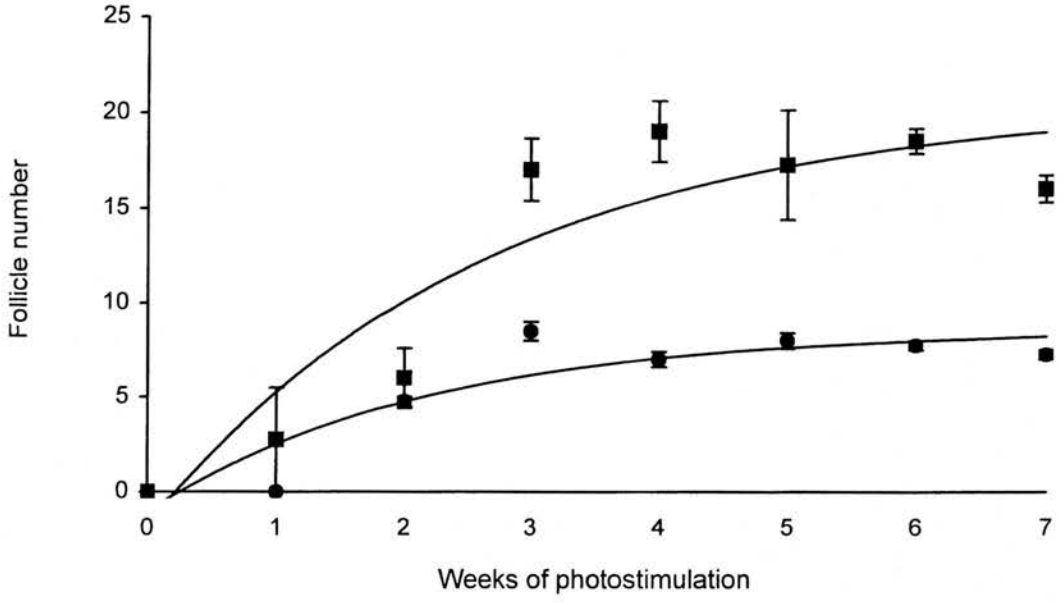


FIGURE 3.13 Mean (±SEM) number of hierarchical follicles (<0.5g) from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

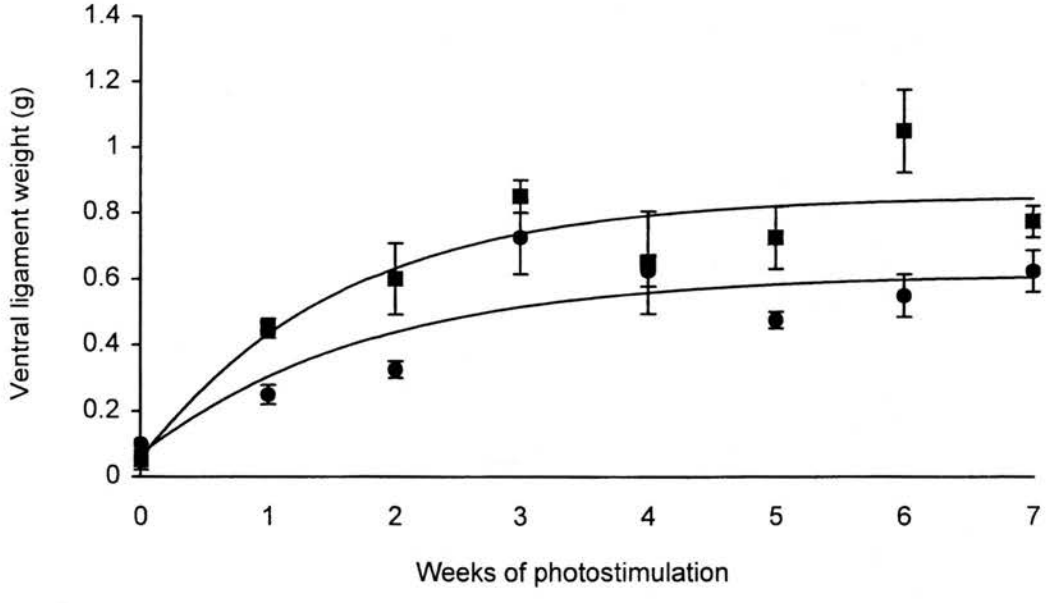


FIGURE 3.14 Mean (±SEM) weight of the muscular cord of the ventral ligament from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

TABLE 3.4 Estimates ±SE of fitted parameters of exponential models for weight of the muscular cord of the ventral ligament, the number of hierarchical follicles (<0.5g) and the total ovary weight in traditional- and male-line turkeys

	Rate	Scaling factor	Asymptote (g)	R^2
Number of Follicles				
Traditional-line	0.63 ±0.267	-9.6 ±3.26	8.6 ±2.80	84.2
Male-line	0.69 ±0.119	-22.3 ±3.77	20.6 ±3.72	
Ventral ligament weight (g)				
Traditional-line	0.57 ±0.228	-0.55 ±0.148	0.62 ±0.107	76.0
Male-line	0.53 ±0.157	-0.80 ±0.145	0.85 ±0.093	
Total ovary weight (g)				
Traditional-line	0.52 ±0.445	-150 ±133	75.7 ±27.8	84.9
Male-line	0.56 ±0.158	-393 ±109	221.1±32.8	

Mean total ovary weights of both strains for seven weeks following photostimulation are presented in Figure 3.15. Regression analysis did not result in any reasonable fitting model for the changes in total ovary weight from weeks 0-7. However an exponential model was fitted to the data for weeks 1-7, as shown on Figure 3.15. The estimated parameters from this model are shown on Table 3.4. There was a significant change ($P<0.001$) in the residual mean squares when the linear parameters (upper asymptote and scaling factor) were fitted separately for each strain but no significant change was found when the non-linear parameters were fitted.

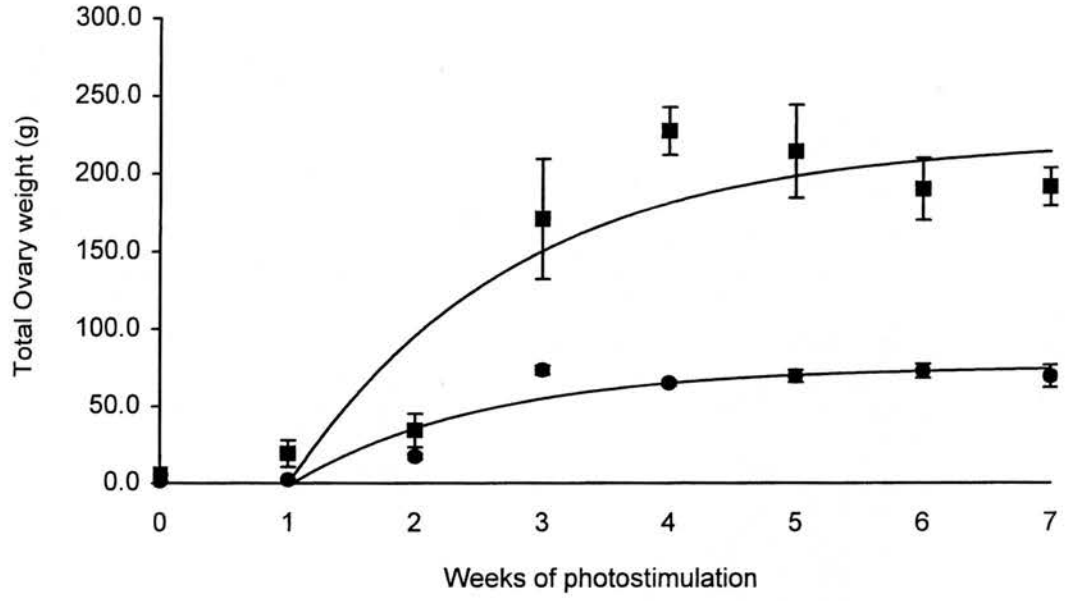


FIGURE 3.15 Mean (±SEM) total ovary weight from 0-7 weeks after photostimulation in traditional- and male-line turkeys
Key: ● Traditional-line; ■ Male-line

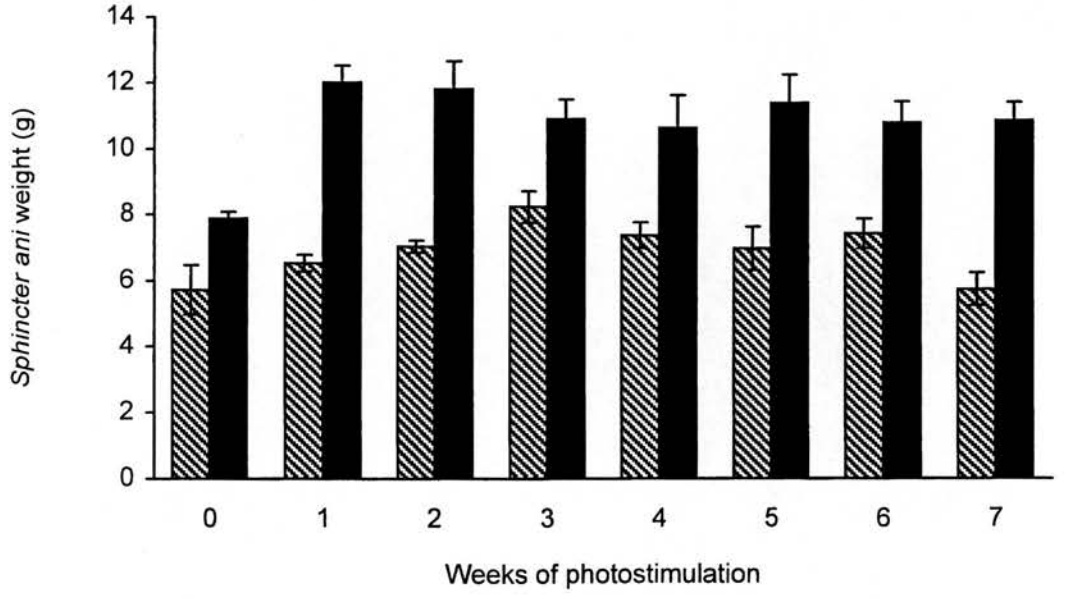


FIGURE 3.16 Mean (±SEM) weight of the *sphincter ani* muscle from 0-7 weeks post photostimulation in traditional- and male-line turkeys
Key: ▨ Traditional-line; ■ Male-line

The mean weight of the *sphincter ani* muscle in the traditional- and male-line turkeys for eight weeks after photostimulation is shown on Figure 3.16. Regression analysis did not result in any reasonable fitted model. Analysis of variance gave a significant effect of strain ($P<0.001$) and week ($P<0.001$) but no significant interaction between strain and week. The *sphincter ani* muscle was heavier in the male-line strain. The weight of the *sphincter ani* muscle appeared to increase initially in both strains after photostimulation and may have decreased slightly during the final weeks of the experiment.

Analysis of Data from Melnychuk et al. (1997)

The reconstructed data of Melnychuk *et al.* (1997) is shown on Figures 3.17 and 3.18. The oviduct and ovary weights were plotted separately, with each curve showing data for both the male- and female-lines, as shown on Figures 3.19 and 3.20. A logistic curve was the best fitting model for both the oviduct weight and ovary weight. There was no significant effect on the fitted model for either oviduct weight or ovary weight when linear parameters for the male-line and female-line were fitted separately. The parameters of the curves are given in Table 3.6.

TABLE 3.6 Estimates ±SE of fitted parameters of the logistic curves for ovary and oviduct as a percentage of mature organ weight in male-line and female-line turkeys. Data extracted from Melnychuk *et al.* (1997)

	Rate	Point of inflection (days)	Upper asymptote (g)	Lower asymptote (g)	R^2
Ovary % mature organ weight					
Male-line	0.28 ±0.071	222 ±0.9	98 ±8.7	5 ±6.5	95.9
Female-line	0.29 ±0.074	221 ±0.9	99 ±8.6	1 ±6.7	
Oviduct % mature organ weight					
Male-line	0.65 ±0.165	226 ±0.5	86 ±4.2	4 ±2.9	97.6
Female-line	0.51 ±0.108	226 ±0.5	96 ±4.5	4 ±3.1	

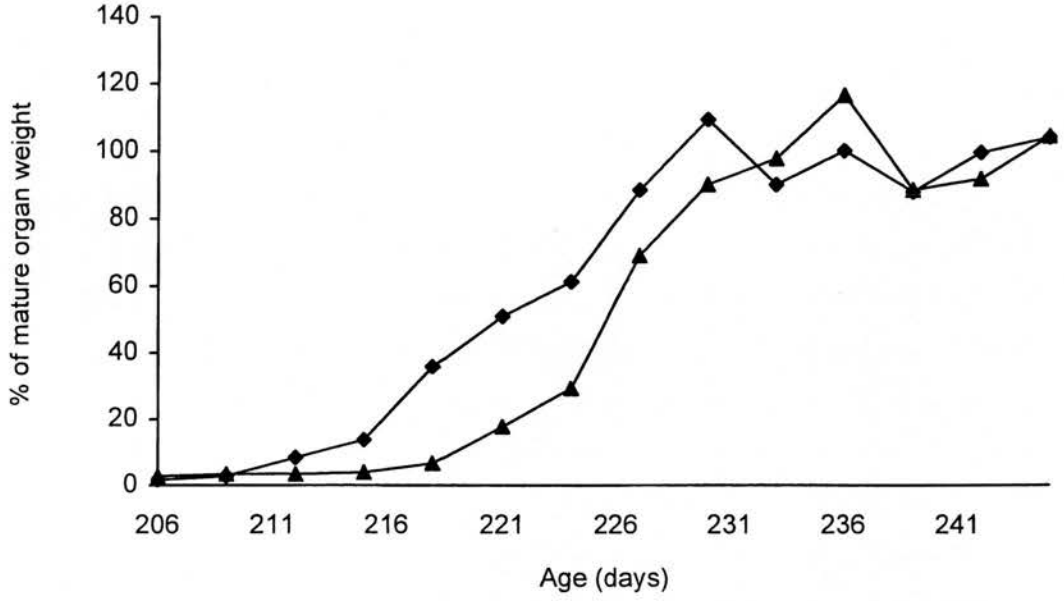


FIGURE 3.17 Weight of the oviduct and ovary in female-line turkeys from 206 to 245 days of age. (Data reconstructed from Melnychuk *et al.* (1997) Figure 9.a)
Key: ◆ Oviduct; ▲ Ovary

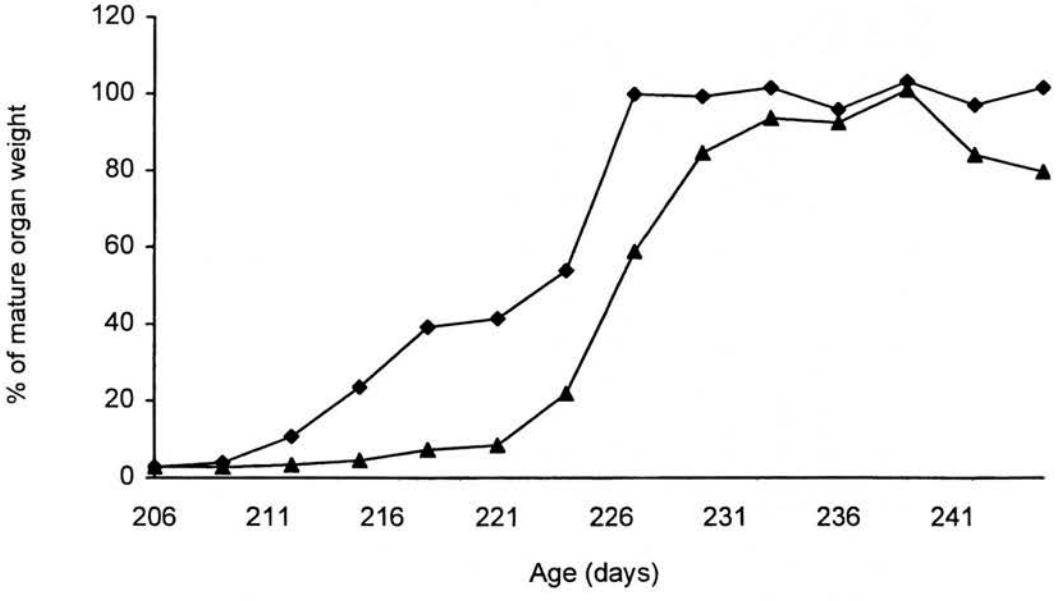


FIGURE 3.18 Weight of the oviduct and ovary in male-line turkeys from 206 to 245 days of age. (Data reconstructed from Melnychuk *et al.* (1997) Figure 8.a)
Key: ◆ Oviduct; ▲ Ovary

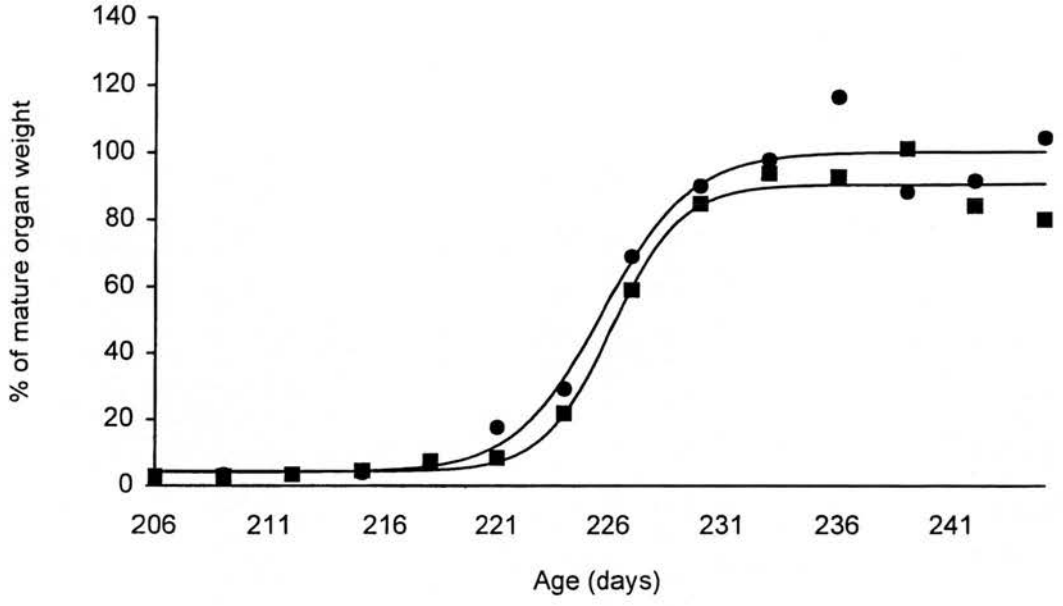


FIGURE 3.19 Ovary weight as a percentage of mature organ weight from 206 to 245 days of age in female- and male-line turkeys. (Data derived from Melnychuk *et al.* (1997))
Key: ● Female-line; ■ Male-line

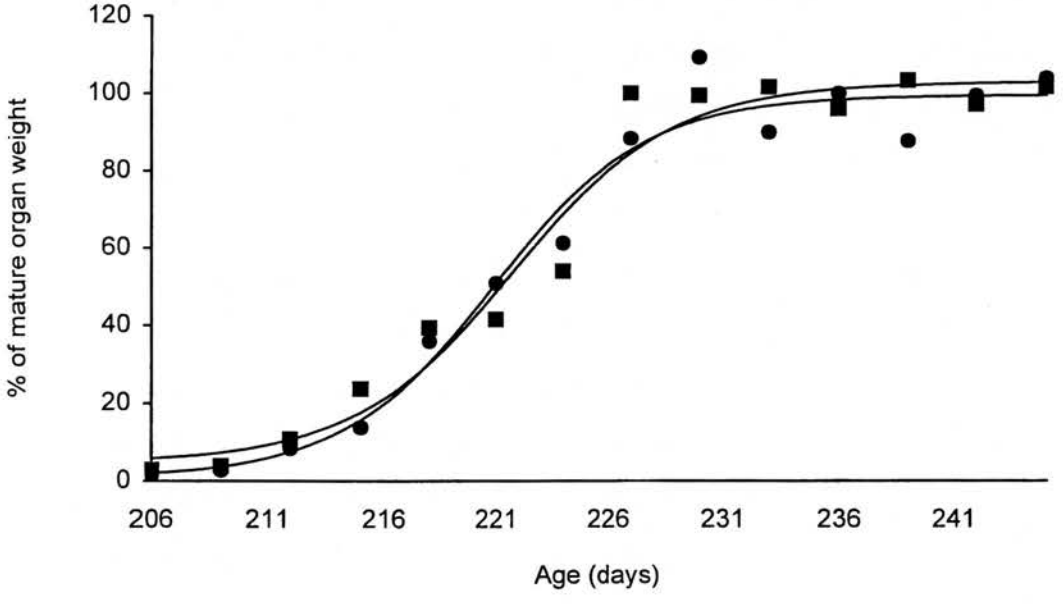


FIGURE 3.20 Oviduct weight as a percentage of mature organ weight from 206 to 245 days of age in female- and male-line turkeys. (Data derived from Melnychuk *et al.* (1997))
Key: ● Female-line; ■ Male-line

Plasma oestradiol and progesterone concentration

Plasma oestradiol from 0-7 weeks post photostimulation is shown in Figure 3.21. There was no statistical evidence to support the fitting of a curvilinear model to the mean plasma oestradiol concentration for either strain. Analysis of variance showed a significant effect of week on plasma oestradiol ($P<0.001$). This effect was due entirely to the low levels of plasma oestradiol at week zero. There was no significant change in plasma oestradiol from weeks one to seven after photostimulation. The male-line turkeys had a lower overall mean plasma oestradiol concentration (189±21) than the traditional-line (202±13), although this difference was not significant.

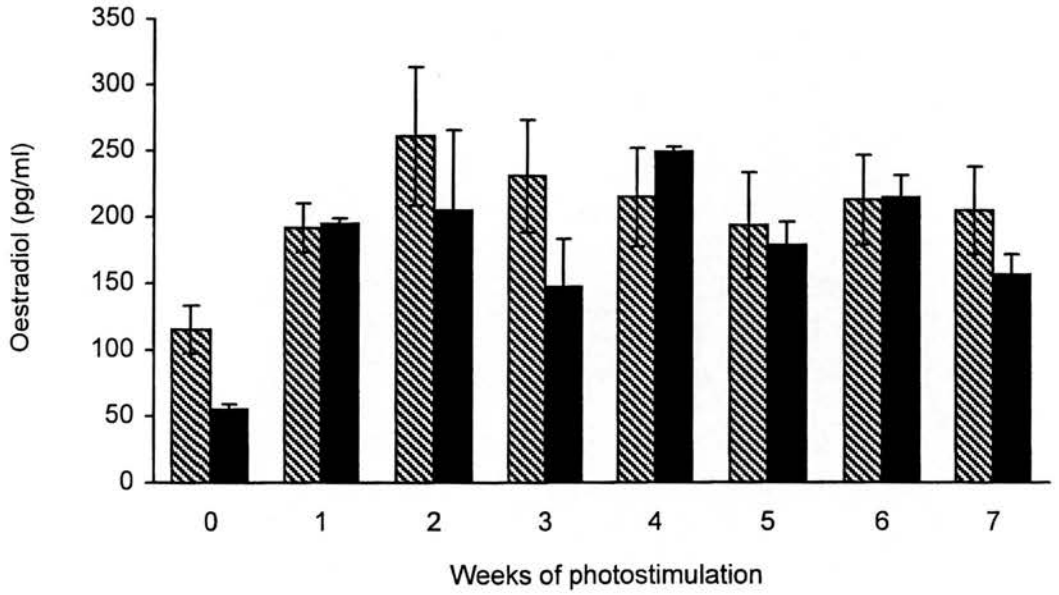


FIGURE 3.21 Mean (±SEM) plasma oestradiol concentration from 0-7 weeks post photostimulation in traditional- and male-line turkeys
Key: ▨ Traditional-line; ■ Male-line

Plasma progesterone concentrations in traditional- and male-line turkeys from 0-7 weeks post photostimulation are presented in Figure 3.22. Analysis of variance of the log transformed values for plasma progesterone concentration showed a significant effect of week on progesterone ($P<0.001$) and a significant interaction between week and strain ($P<0.05$), as progesterone was higher in the traditional-line at weeks 2 and 3 after photostimulation. A quadratic by linear curve was the best fitting model for the change in plasma progesterone from weeks 0 to 8, but this did not give a very good fit ($R^2=38.5$) and therefore is not shown. There was no significant change in the residual mean square when the curve was fitted separately for the two strains. In both traditional- and male-line turkeys plasma progesterone increased from photostimulation, peaked at five weeks post photostimulation and decreased thereafter.

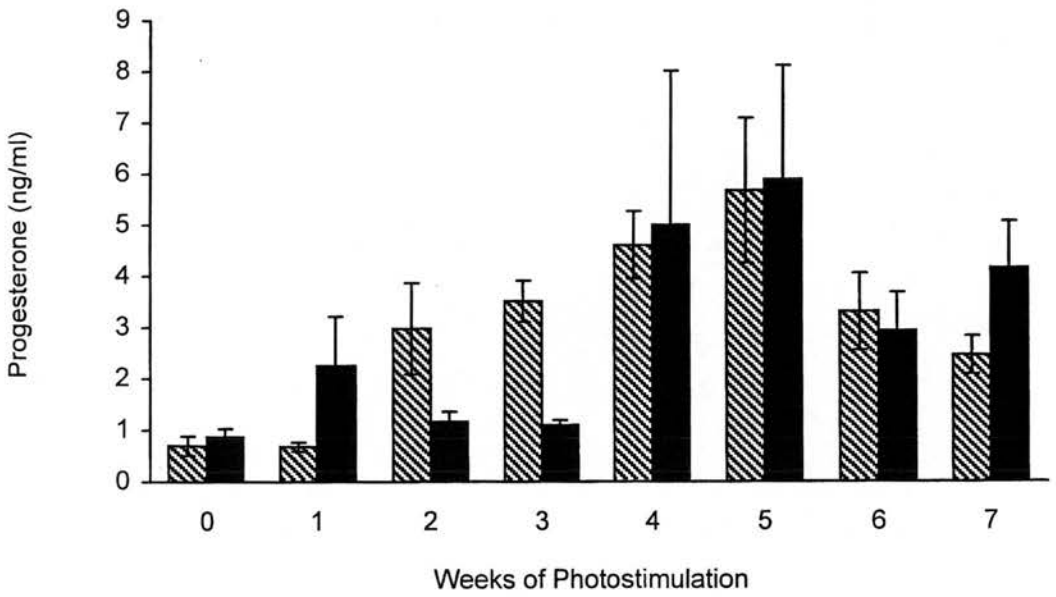


FIGURE 3.22 Mean (±SEM) plasma progesterone concentration from 0-7 weeks post photostimulation in traditional- and male-line turkeys
Key: ▨ Traditional-line;■ Male-line

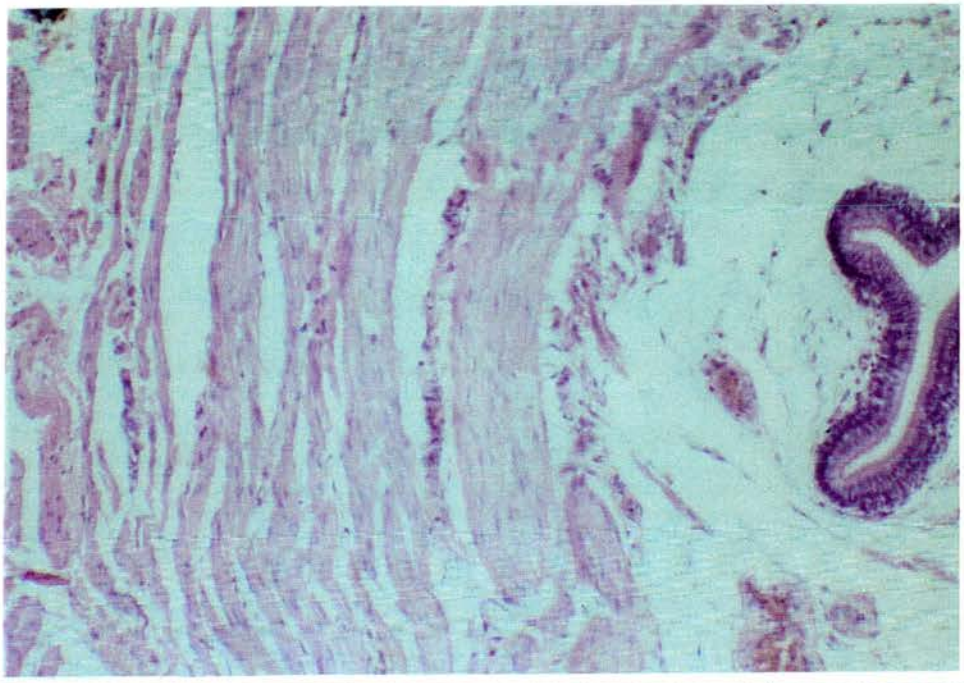
Figure 3.23 and 3.24 show vaginal cross-sections from traditional-, (a), male-line, (b), and prolapsed male-line, (c), turkeys respectively stained with either haematoxylin and eosin or Van Gieson's stains. The two layers of the muscle were clearly visible, with the longitudinal muscle layer outermost and the circular muscle layer lying between the longitudinal layer and the vaginal epithelium. Figures 3.25 and 3.26 show the uterine cross sections from the traditional-line, male-line and prolapsed male-line, with the same stains. The surrounding circular and longitudinal muscle layers were also found in the uterine sections although they were much thinner than in the vagina.

Longitudinal sections of the muscular cord of the ventral ligament from the three groups are shown in Figures 3.27 and 3.28. In traditional-, male-line and prolapsed male-line turkeys the muscular cord of the ventral ligament consisted mainly of muscle fibres and connective tissue. Sections from the *sphincter ani* muscle are shown on Figures 3.29 and 3.30. Sections from all three groups investigated showed a tight band of circular muscle fibres surrounded by connective tissue.

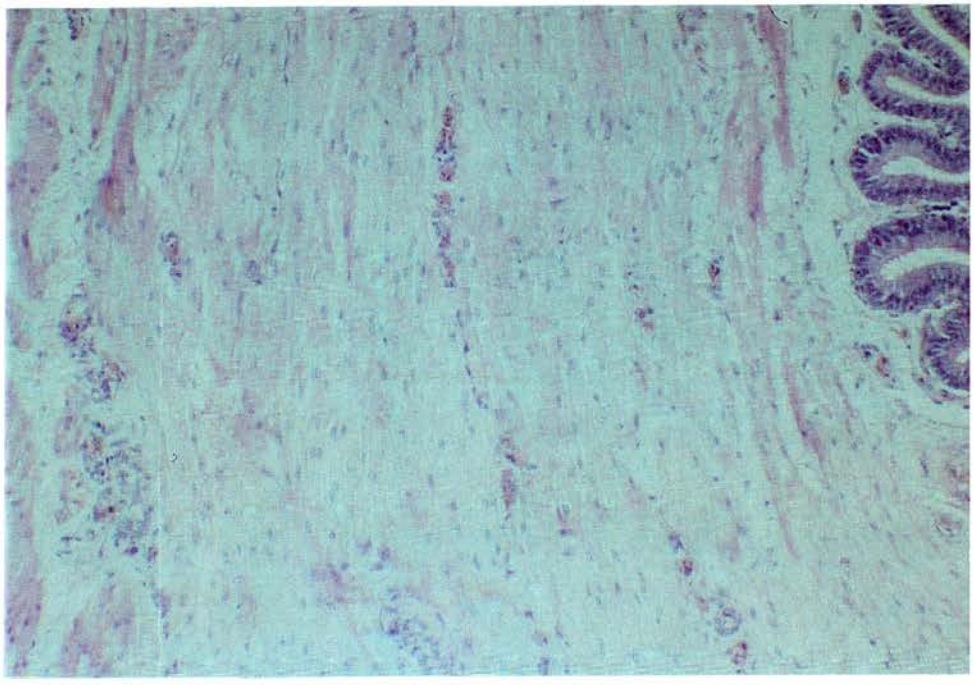
In all four structures investigated no differences were observed in the histological slides prepared with either haematoxylin and eosin or Van Gieson's stains from samples from the male-line, the traditional-line or the prolapsed male-line.

FIGURE 3.23 Vaginal cross sections from traditional- line (a), male-line (b) and prolapsed male-line (c) turkeys with haematoxylin and eosin stain. ×144 magnification.

(a)



(b)



(c)

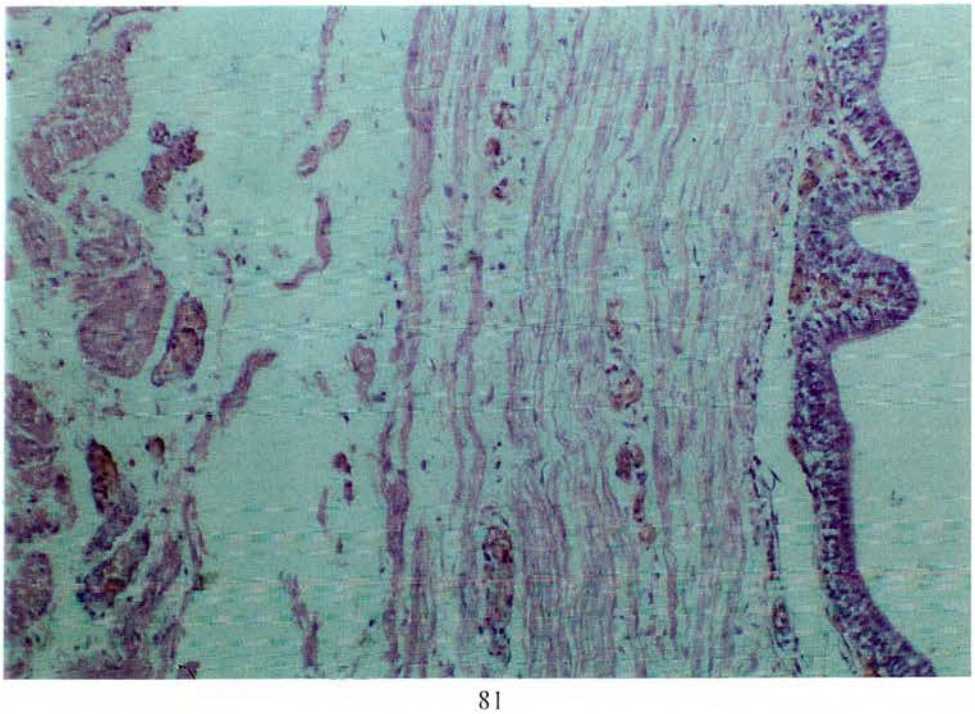
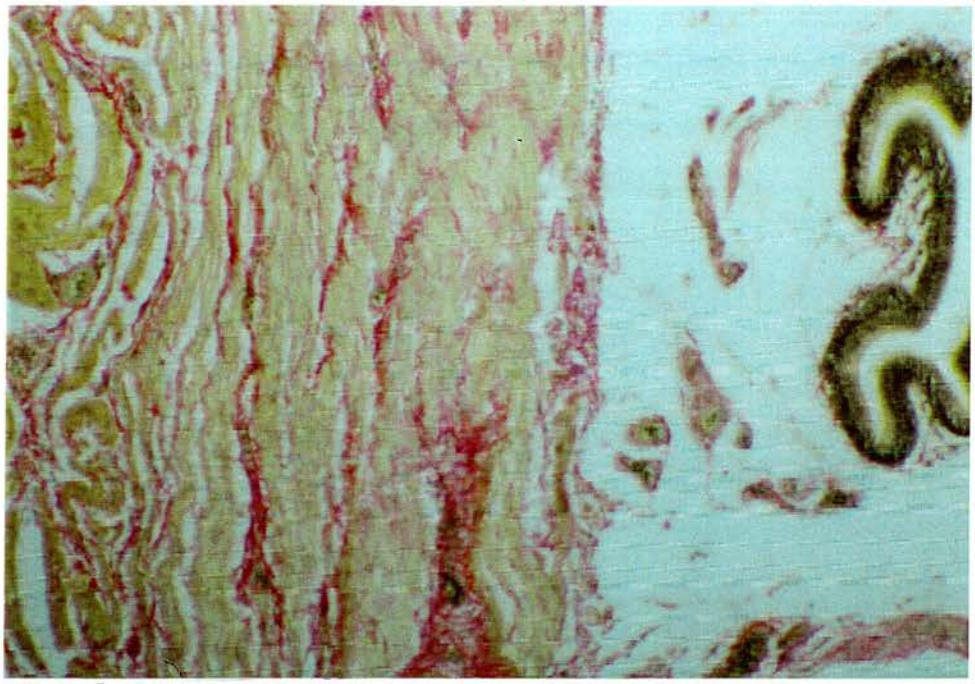
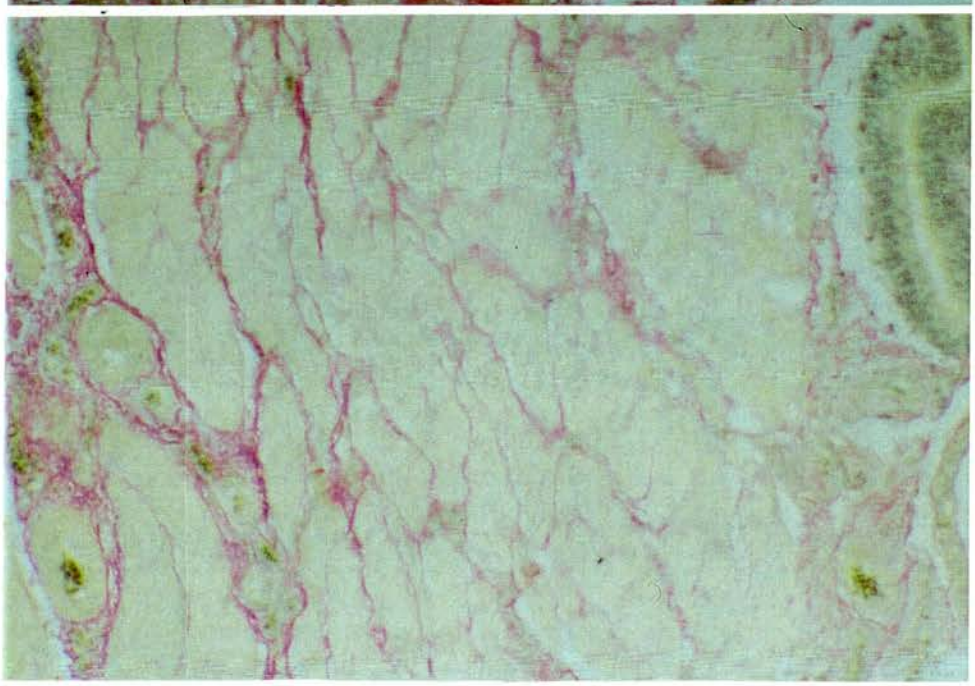


FIGURE 3.24 Vaginal cross sections from traditional-line (a), male-line (b) and prolapsed male-line (c) turkeys with Van Gieson's stain. ×144 magnification.

(a)



(b)



(c)

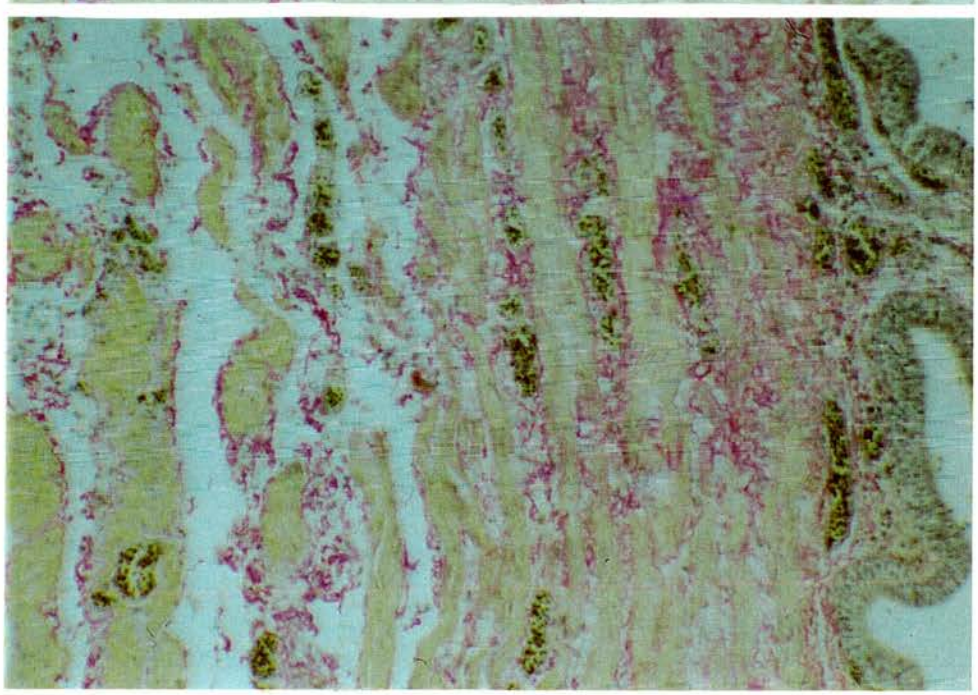
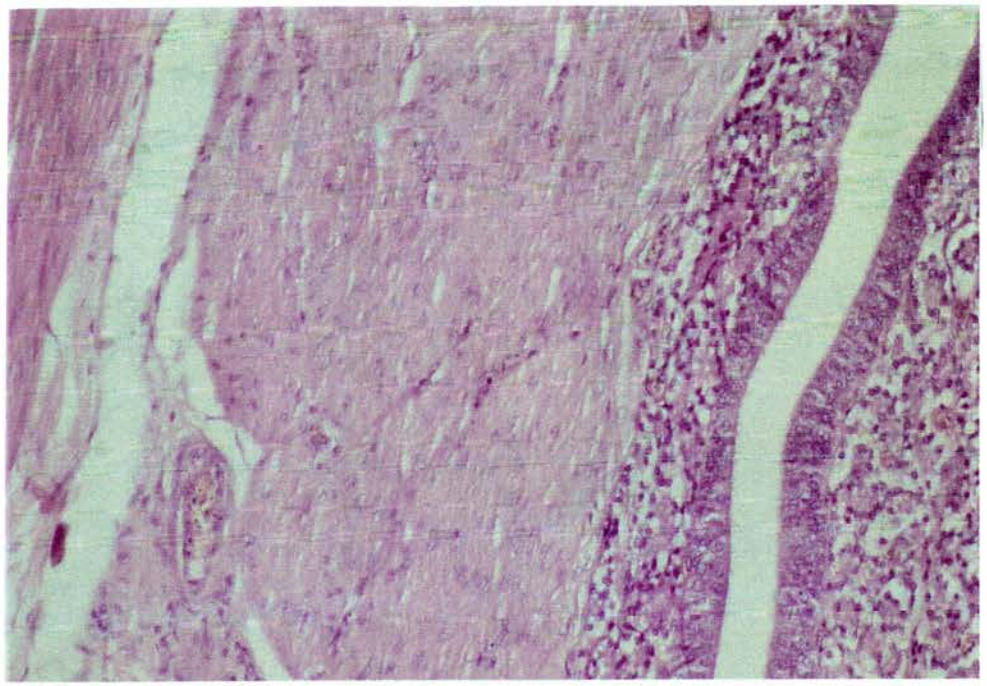
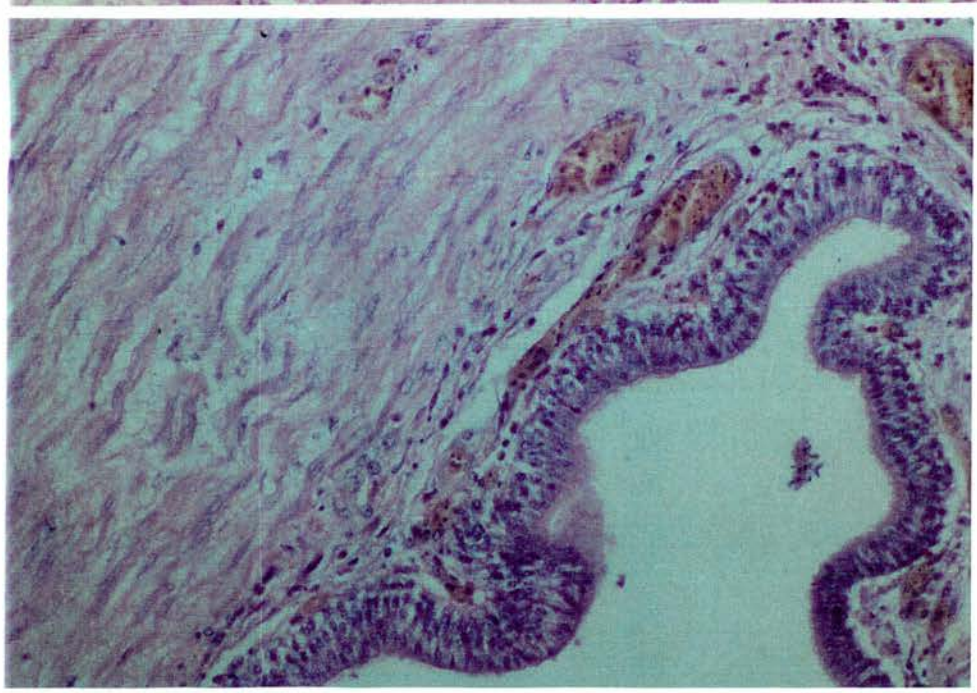


FIGURE 3.25 Uterine cross sections from traditional-line (a), male-line (b) and prolapsed male-line (c) turkeys with haematoxylin and eosin stain. ×230 magnification.

(a)



(b)



(c)

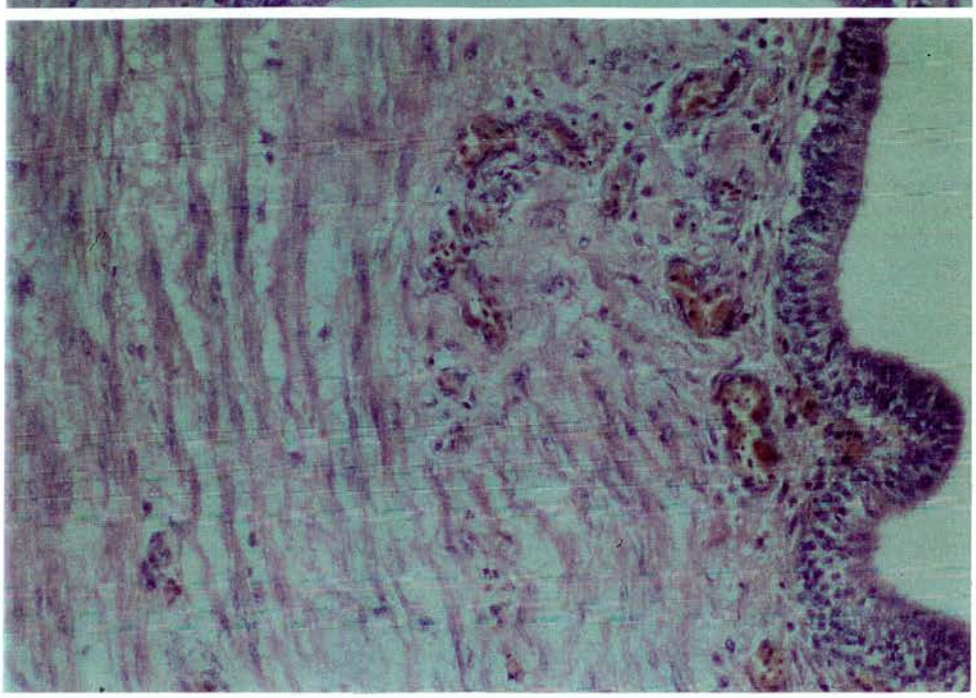
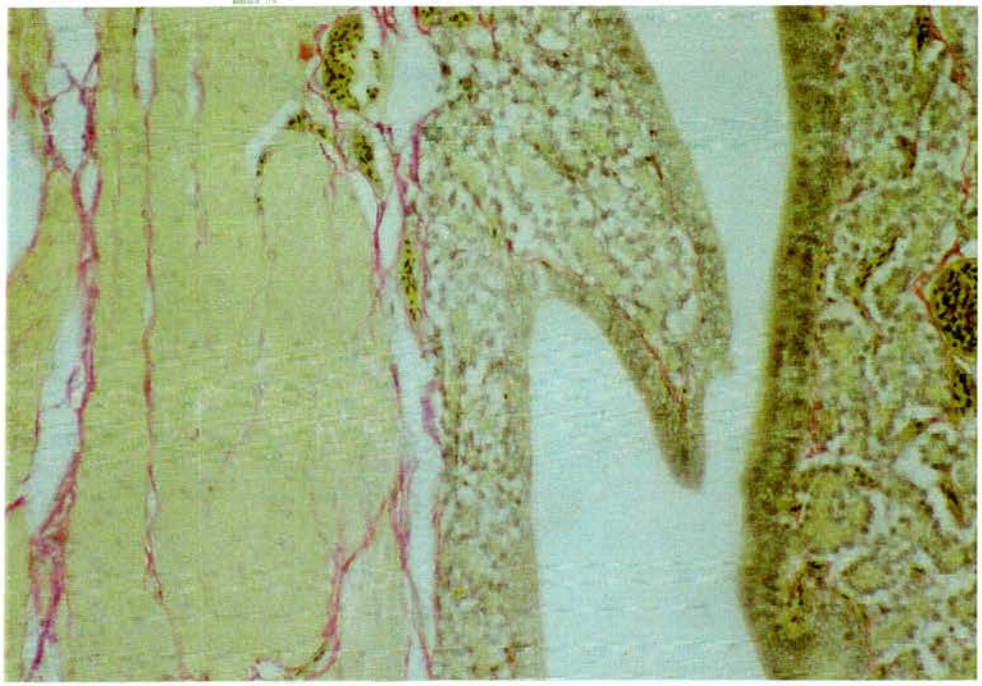
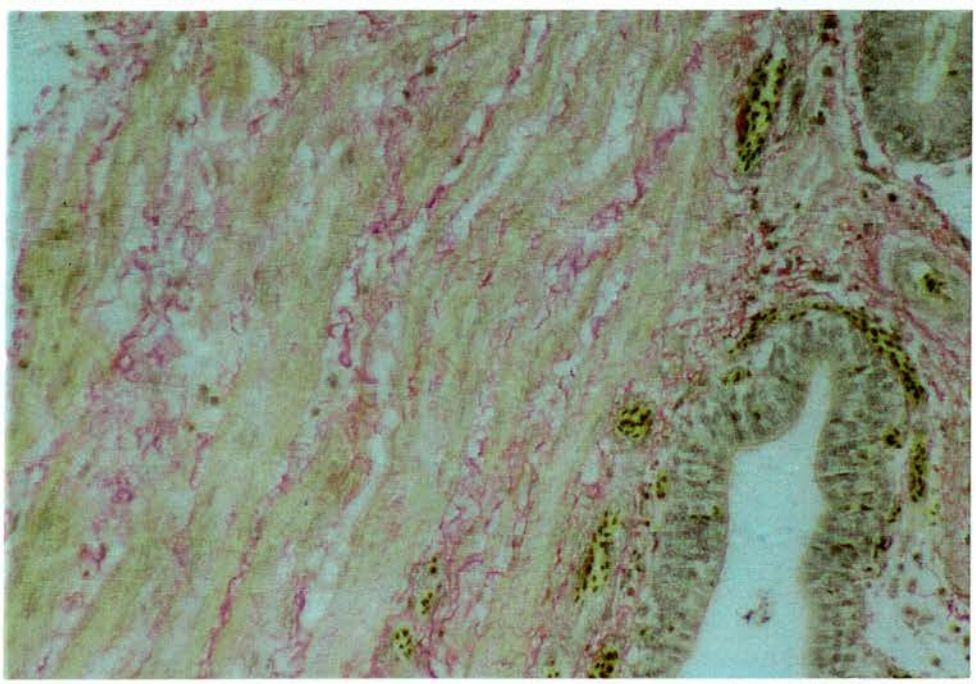


FIGURE 3.26 Uterine cross sections from traditional-line (a), male-line (b) and prolapsed male-line (c) turkeys with Van Gieson's stain. ×230 magnification.

(a)



(b)



(c)

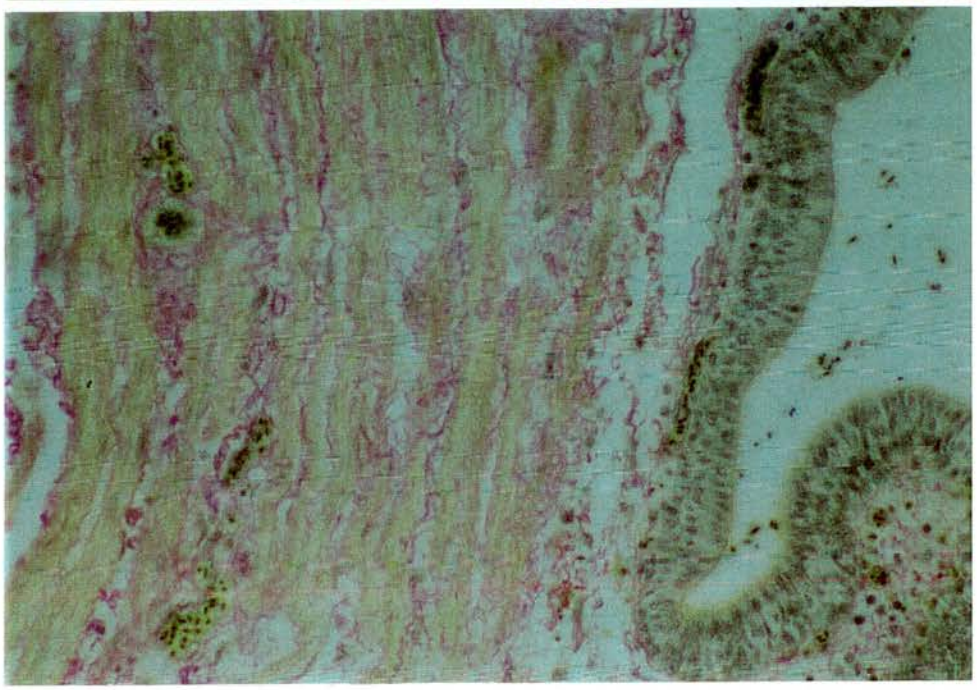
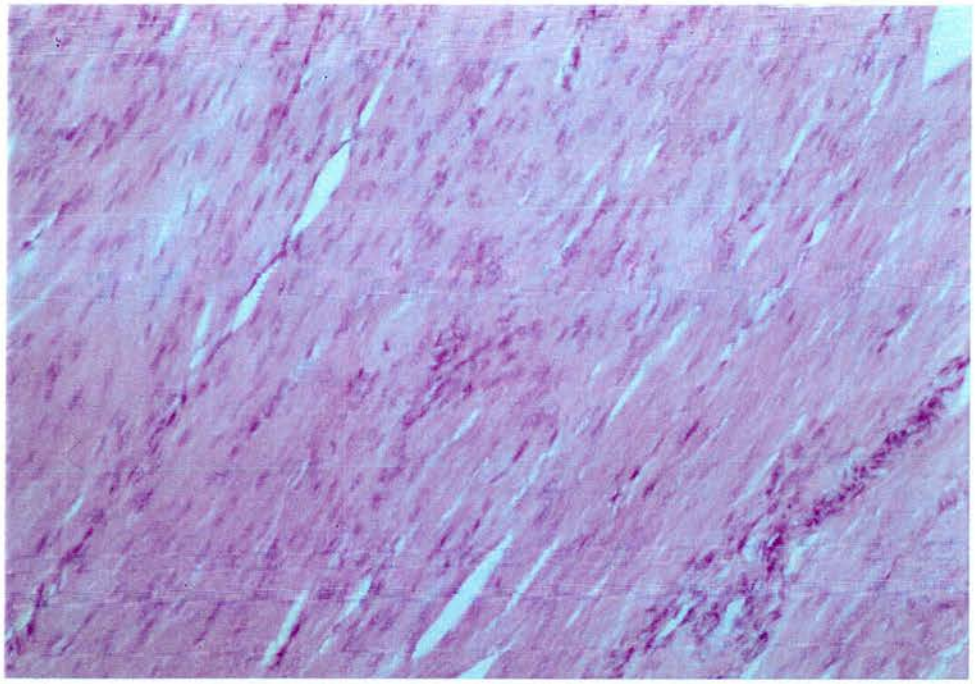
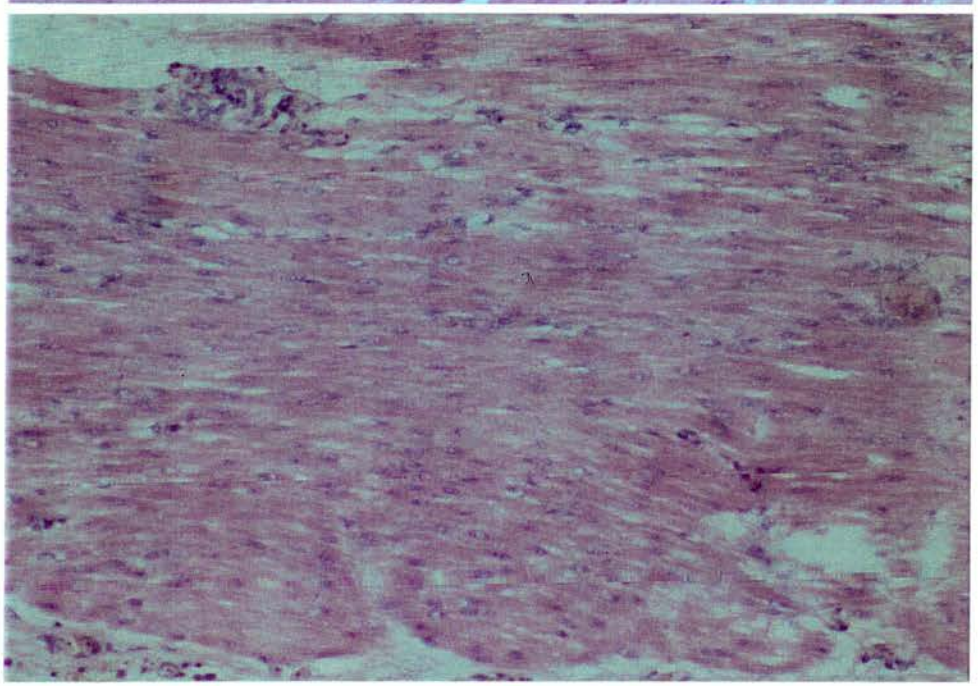


FIGURE 3.27 Longitudinal sections of the muscular cord of the ventral ligament from traditional- (a), male-line (b) and prolapsed male-line (c) turkeys with haematoxylin and eosin stain. ×230 magnification.

(a)



(b)



(c)

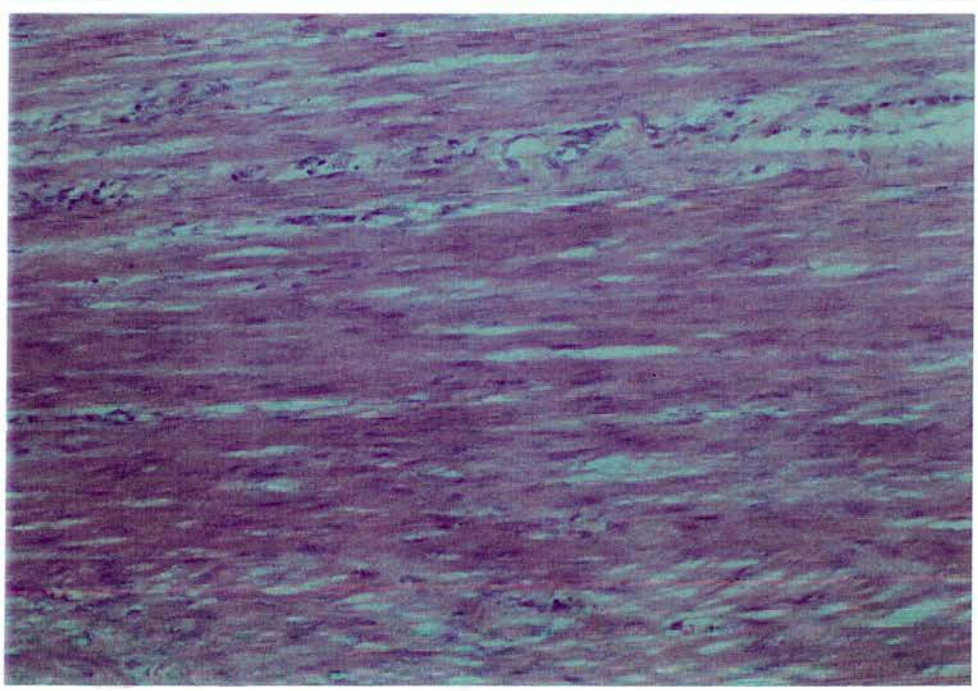
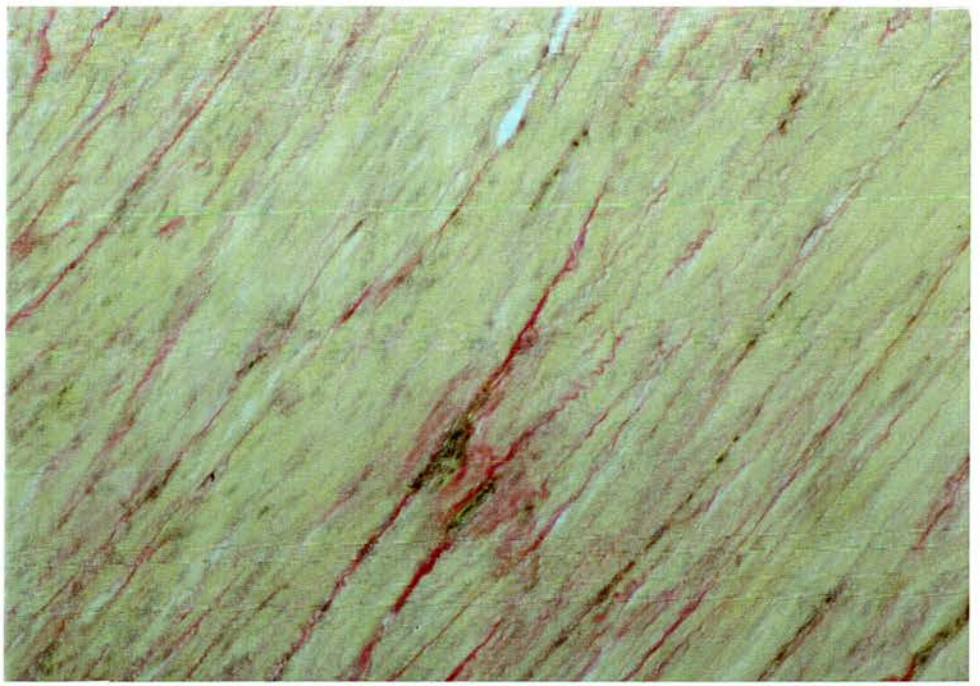
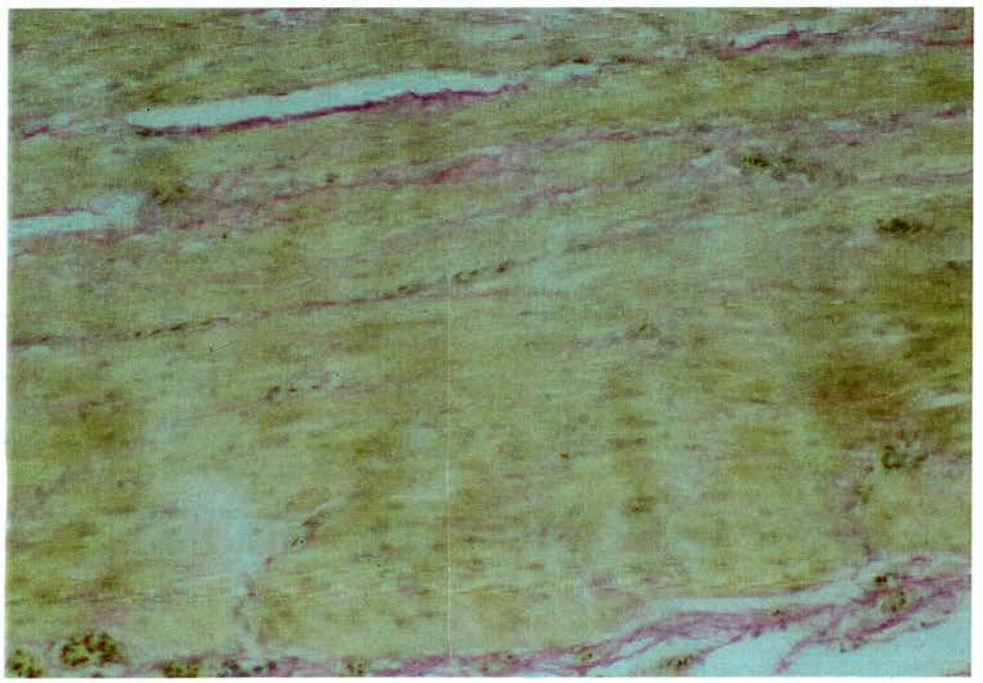


FIGURE 3.28 Longitudinal sections of the muscular cord of the ventral ligament from traditional- (a), male-line (b) and prolapsed male-line (c) turkeys with Van Gieson's stain. ×230 magnification.

(a)



(b)



(c)

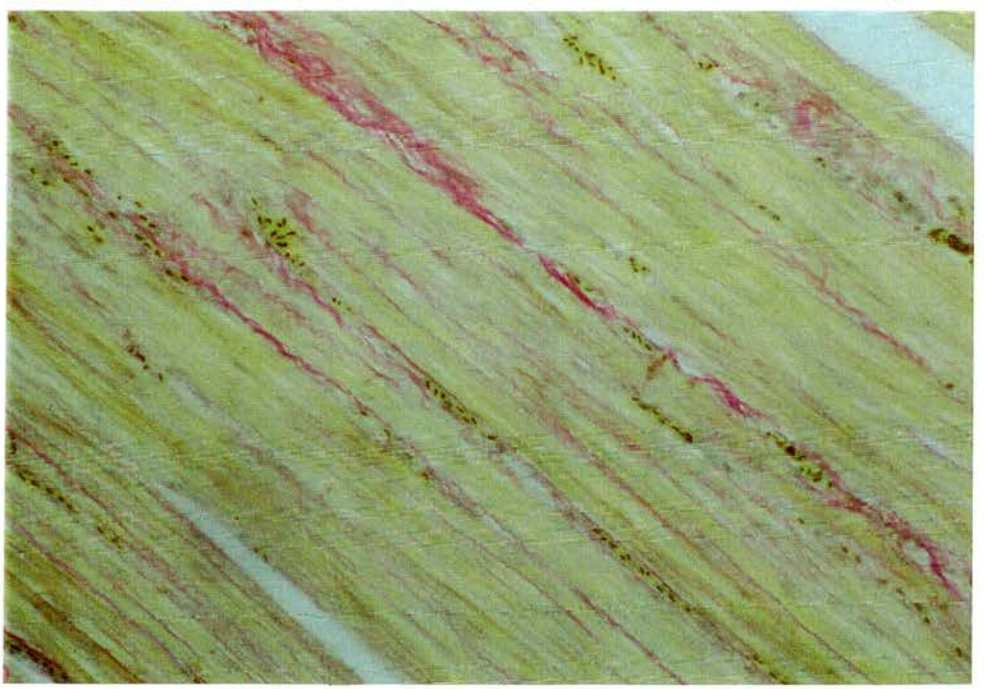
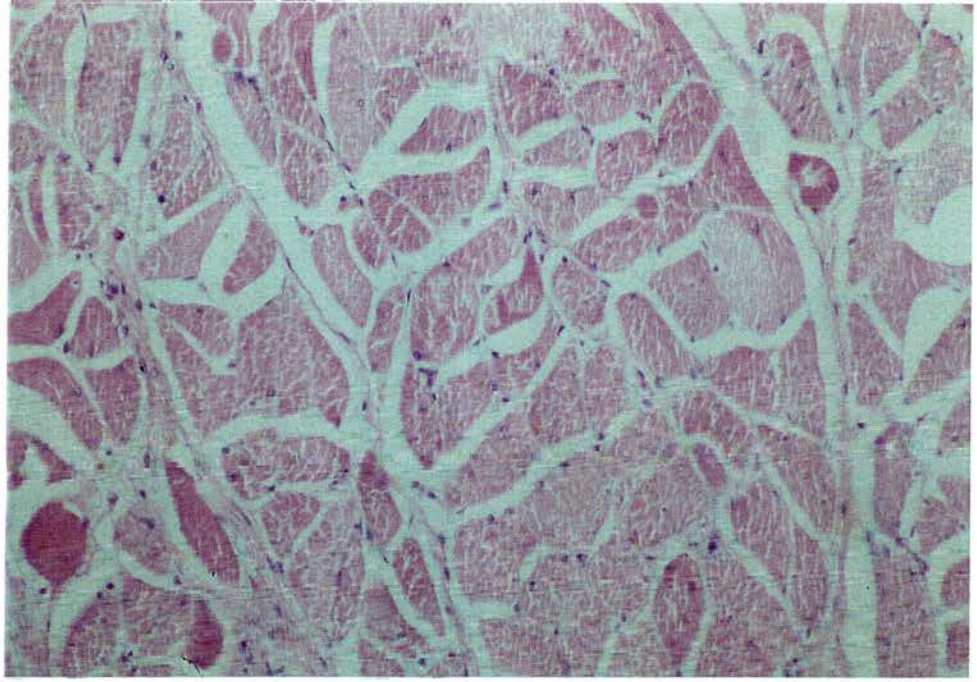
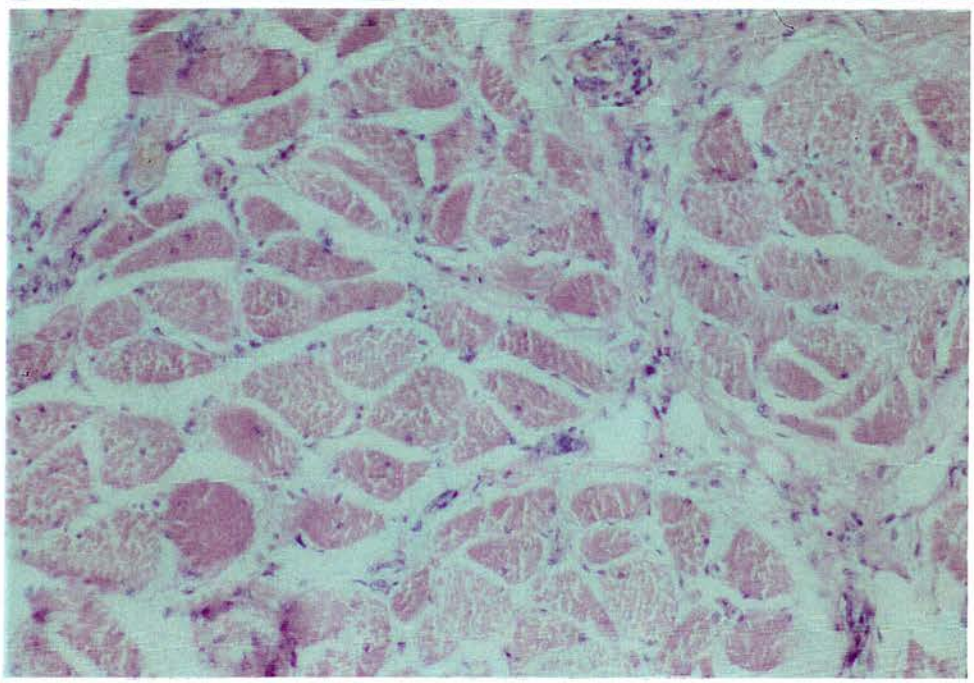


FIGURE 3.29 Sections of the *sphincter ani* muscle from traditional- (a), male-line (b) and prolapsed male-line (c) turkeys with haematoxylin and eosin stain. ×230 magnification.

(a)



(b)



(c)

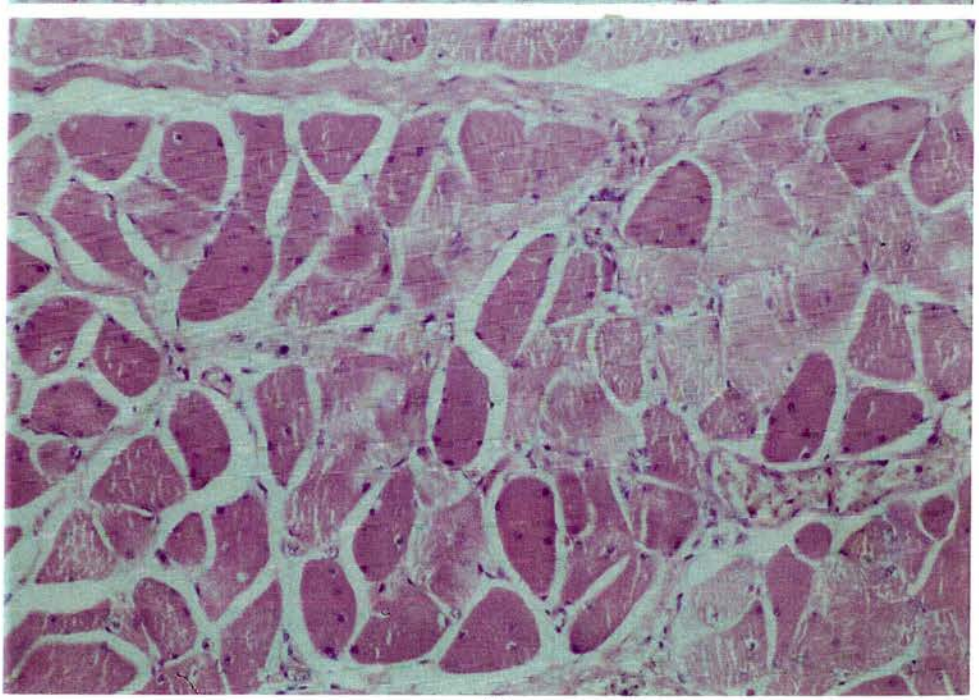
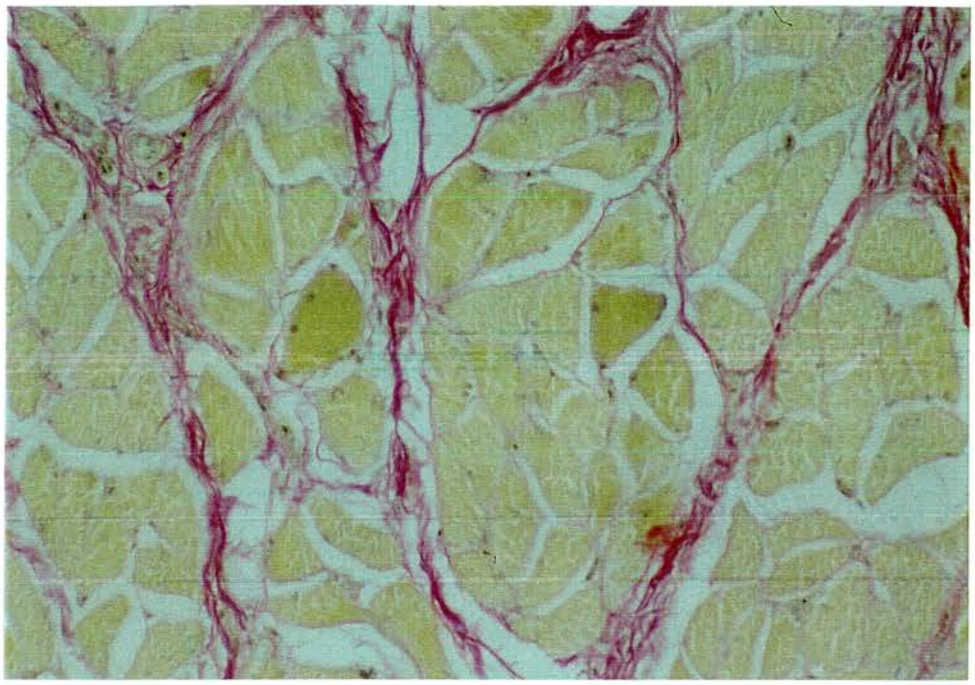
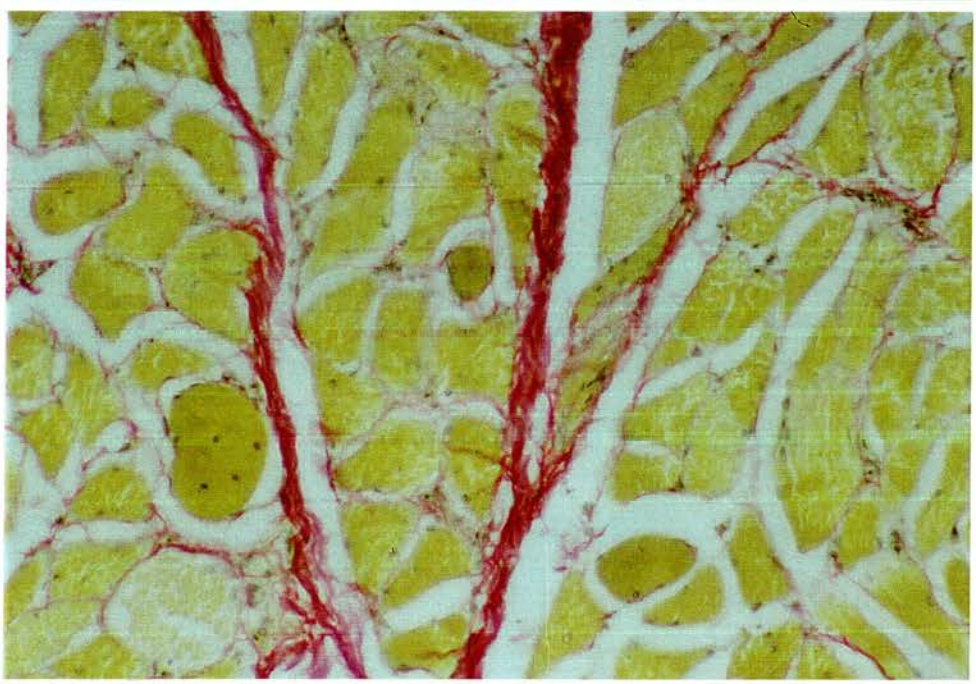


FIGURE 3.30 Sections of the *sphincter ani* muscle from traditional- (a), male-line (b) and prolapsed male-line (c) turkeys with Van Gieson's stain. ×230 magnification.

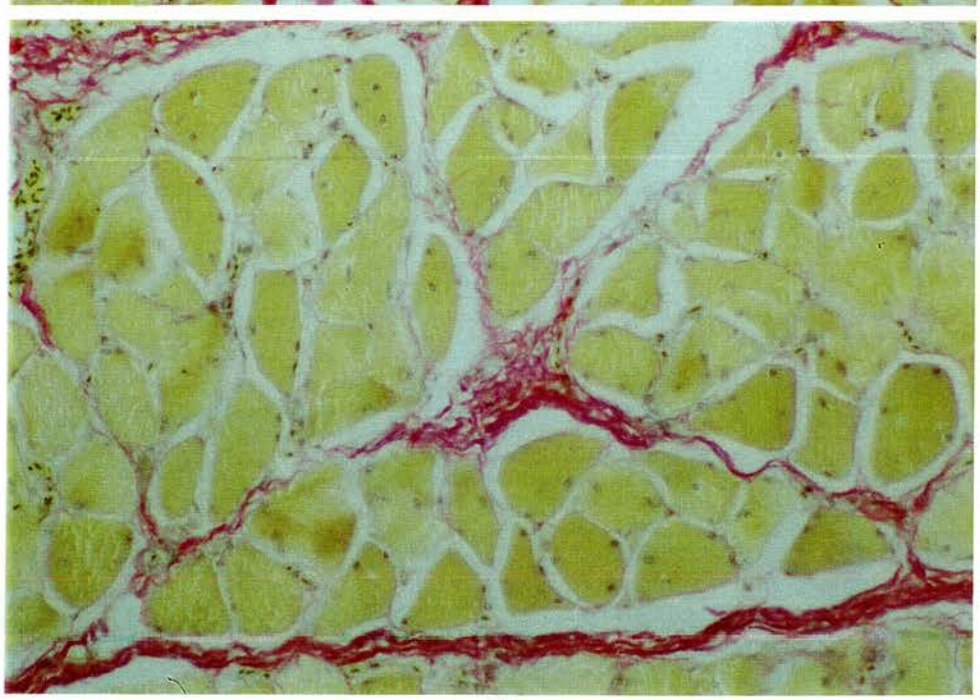
(a)



(b)



(c)



DISCUSSION

The significant effects of strain on the fitted models for oviduct length, weight, total and residual ovary weight and weights of the uterus and vagina from the second experiment reflect the greater size of these structures in the male-line strain compared to the traditional-line. These differences were also observed in the first experiment when the two strains were compared at first egg. These results are unsurprising as the male-line strain has a much greater body weight than the traditional-line.

The muscular cord of the ventral ligament and the *sphincter ani* muscle were also heavier in the male-line, both at first egg and throughout the development of the reproductive system. Again it is thought that this reflects the greater size of the male-line turkey compared to the traditional-line.

In both experiments the male-line was shown to have more follicles in the follicular hierarchy compared to the traditional-line. This agrees with the findings of Hocking (1992b), Hocking and Bernard (1998) and Melnychuk *et al.* (1997) who showed that male-line turkeys have a multiple follicular hierarchy.

Male-line turkeys can have up to 30 follicles arranged in a hierarchy with 2 or 3 follicles at each of the 10 positions corresponding to the daily recruitment of follicles (Hocking 1987). The multiple follicular hierarchy results in two or three ovulations occurring each day, instead of one, which explains the observation of the first experiment that male-line turkeys have an average of six post ovulatory follicles on the ovary the day after the first egg is laid. The traditional turkeys had an average of three post ovulatory follicles, which is consistent with observations that they had all laid at least one egg and the majority had another in the uterus when killed. The results suggest that the male-line turkeys ovulate two follicles for every follicle ovulated by the traditional turkeys.

In the second experiment, the growth of the oviduct and ovary appeared to follow the same time course in the male-line and traditional-line. If there was any difference in their maturation rates this would be reflected by a significant difference in the residual mean squares when the non-linear parameters (point of inflection and rate) were fitted. As fitting these parameters had no significant effect, and there was no significant difference in the values estimated for rate or point of inflection between the two strains, there is no evidence that development occurs at a different rate in the two strains. This suggests that growth of the oviduct in the male-line occurs over the same time-scale as in the traditional-line.

Lilburn and Nester (1993) suggested that growth of the oviduct continued for seven weeks after the onset of lay in their male-line but not in their female-line. The results of the second experiment showed that the growth of the oviduct reached a plateau within the seven weeks of photostimulation. From the first experiment it was shown that the first egg is laid after about three weeks of photostimulation and the results are therefore different from those of Lilburn and Nester (1993).

The proposal of Lilburn and Nester (1993) that oviduct growth in turkeys selected for high meat yield increases through the first 49 days of egg production was based on the observation that the oviduct weight of the male-line strain at 49 days post photostimulation was significantly heavier than at near to the onset of lay. This does not necessarily reflect continuous growth throughout the first 49 days of photostimulation; the increase in oviduct weight could have occurred in the first week after the start of egg production. They also showed a non-significant increase in oviduct weight of their female-line after 49 days compared to the onset of lay. Their results were based on comparing turkeys at 25 days after photostimulation as near to the onset of lay and 49 days after photostimulation with a total of 13 turkeys in each strain. Perhaps the increase in weight of the oviduct for the female-line would have been significant if more turkeys had been sampled and if their timing of the onset of lay sampling had been more accurate.

In the second experiment the time course of growth of the residual ovary was not significantly different for the traditional- and male-line turkeys, as shown by the lack of significant effect on the fitted model when the point of inflection and rate parameters were fitted separately for the two strains. There was also no difference in the time course of increase in follicle number between the two strains, as reflected by the lack of effect of fitting the non-linear parameters separately.

The change in weight of the total ovary throughout the experiment was investigated to allow comparison of the results with those of Melnychuk *et al.* (1997). However the total ovary weight consists of two components, the residual ovary weight and the weight of the follicular hierarchy. The results showed that the residual ovary increased in weight in a logistic fashion while the hierarchical follicle number increased exponentially. The combination of these two patterns may explain why the change in total ovary weight from weeks 0-7 did not fit either a logistic or an exponential model. However an exponential model was fitted to the 1-7 week data, and showed no significant strain effect when the non-linear parameters were fitted. There was no evidence from the results of the second experiment to suggest a difference in the developmental rate of the ovary between the traditional- and male-lines.

The re-analysis of the data extracted and reconstructed from Melnychuk *et al.* (1997) did not provide any evidence that there was a difference in the growth rate of the ovary between the male- and female-lines. Clearly the suggestion that the ovary of the female-line reaches mature weight 3 days after that of the male-line is unsubstantiated.

It was recently suggested by the same group that the ovary of Bronze turkeys also reaches maturity 3 day after the oviduct (Renema *et al.* 1998). This conclusion was based on fitting straight lines to the data for the change in weight of the oviduct and ovary after photostimulation, when a logistic model would have been more appropriate. There was no statistical evidence provided in support of the suggestion that the

maturation of the ovary differed from the oviduct and it is unlikely that proper statistical analysis would substantiate this conclusion.

The second experiment also showed that the growth of the uterus and vagina followed a similar trend with time post photostimulation in the traditional- and male-line and there were no differences in their growth patterns that could influence the stability of the reproductive system. The change in weight of the vagina appeared to reach a plateau before that of the uterus or the total oviduct in both the traditional-line and the male-line. This suggests that the vagina reaches its mature weight before the rest of the oviduct. It has been suggested (Chapter 4) that prolapse of the oviduct in turkeys is associated with structural failure of the vagina. These results do not provide any evidence to suggest that an anatomical immaturity of the vagina of the male-line is involved in predisposing the strain to prolapse.

The change in weight of the structures measured in the second experiment, is a crude measure of the maturity of these tissues. Obviously many factors are involved in the maturation of the tissue that would not necessarily be reflected in the weight of the tissue. However this experiment was carried out to investigate the previous suggestions that there are differences in the growth rate of the reproductive structures in male-line turkeys compared with smaller strains that have not been selected for increased meat yield (Lilburn and Nestor 1993; Melnychuk *et al.* 1994; Melnychuk *et al.* 1997) and has provided no evidence to support these suggestions.

Histological investigation of the uterus, vagina, muscular cord of the ventral ligament and *sphincter ani* muscle did not reveal any differences that could be involved in prolapse of the oviduct. This disagrees with findings of Rao *et al.* (1985) who reported that the muscular cord of the ventral ligament was present only in hens with prolapse. In turkeys there was a distinct muscular cord of the ventral ligament in the traditional-, male-line and prolapsed turkeys. Clearly the hypothesis suggested by Rao *et al.* (1985)

that over development of the ventral ligament caused increased torsion in the oviduct and predisposed affected birds to prolapse is not supported by the present data.

The histological investigations on the uterus and vagina in experiments one and two showed them to consist of an inner epithelial layer surrounded by circular, then longitudinal layers, which were thicker in the vagina than the uterus. These findings are very similar to the observations by Verma and Cherms (1964) on the structure of the vagina in broad breasted bronze turkeys and it would appear that there are no strain differences in the structure of the uterus and vagina that are detectable by standard histological methods.

The immunohistochemical investigation into the distribution of collagen types I and III in the uterus and vagina did not reveal any differences between the traditional- and male-lines. However as both types of collagen were widely distributed throughout the tissue in both strains it was not possible to quantify any differences between them. Further investigation into the ratio of type I and III collagen would be necessary to conclude that there were any differences in their distribution between the two strains.

The results presented here do not provide any evidence for a peak in plasma oestradiol concentration, either just prior to the onset of lay as observed in domestic hens (Senior 1974), or around the onset of lay when prolapse is most common. The results are consistent with previous findings of Bacon *et al.* (1980). There was no evidence for an involvement of a peak in plasma oestradiol associated with prolapse of the oviduct. The lower plasma oestradiol concentration of the male-line turkeys is surprising considering the larger residual ovary weight and the larger number of ovarian follicles in this strain.

The results showed a peak in plasma progesterone concentration five weeks after photostimulation in both the traditional- and male-lines. The lack of difference in plasma progesterone between the two strains does not suggest any involvement of progesterone with prolapse. It was surprising that plasma progesterone was not higher in

the male-line, considering the greater number of mature progesterone producing follicles of this strain.

The plasma concentrations of oestradiol and progesterone in these two experiments were obtained from blood samples taken at one time of day. The plasma hormone concentrations vary throughout the ovulatory cycle, and therefore a more frequent sampling regime would be required to identify if there were any differences in the hormone pattern throughout the cycle between the two strains. This is investigated in Chapter 5. However, the results for both oestradiol and progesterone, from these two experiments suggest that, in relation to the size of the appropriate tissues, the male-line ovary is less steroidogenically active compared to the ovary of the traditional-line. This hypothesis is examined in Chapter 6.

There is no evidence from these results of any underdevelopment of the oviduct or its supporting structures in the male-line compared to the traditional-line. It seems unlikely that any anatomical immaturity of the oviduct is responsible for the high incidence of prolapse in the male-line strain. As no differences were found in the histological investigation of the structures involved in the support of the oviduct, it appears likely that there is a physiological basis to the high incidence of prolapse in the male-line.

4. Plasma oestradiol and progesterone and their relationship with vaginal collagen and prolapse in male-line turkeys

INTRODUCTION

Prolapse of the oviduct in turkeys is most common around the onset of lay, when oestrogen levels are high (Bacon *et al.* 1980). There is a possible connection between prolapse and oestradiol in domestic hens, but the nature of such a relationship is unclear (Wheeler and Hoffman 1948; Shemesh *et al.* 1982; Shemesh *et al.* 1984; Shore *et al.* 1984). In some mammalian species oestradiol has been shown to stimulate collagen degradation while progesterone inhibits such degradation (Pastore *et al.* 1989; Rajabi *et al.* 1991a; Rajabi *et al.* 1991b; Rajabi *et al.* 1991c; Wilcox *et al.* 1992; Bienkiewicz *et al.* 1996). In humans it has been shown that vaginal collagen is lowered in association with prolapse (Jackson *et al.* 1996). The male-lines that have a high incidence of prolapse, have extremely large ovaries with many hierarchical follicles, compared to traditional-lines (Hocking and Bernard 1998). These findings are discussed in more detail in Chapter 1.

It was hypothesised that the larger ovary of the male-line could result in high plasma oestradiol concentrations compared to traditional-line turkeys and that this high circulating oestradiol concentration could stimulate degradation of collagen, thus impairing the structural integrity of the oviduct and predisposing the male-line turkeys to prolapse. Alternatively, collagen degradation could be promoted by low plasma progesterone concentrations in the male-line.

The strength of the collagen is believed to depend predominately on the extent of the cross linking between the collagen fibrils, although the role of the different types of cross links has not yet been defined (Robins 1999). The different types of cross-links are discussed in 1.9.1. It is possible that the proportion of cross-links on the collagen molecule is altered in the oviduct of the male-line, which would influence the strength of the tissue and could also predispose the strain to prolapse.

The aim of these experiments was to compare traditional-, male-line and prolapsed male-line turkeys at the time when prolapse is most common, and to investigate the effect of administration of oestradiol and progesterone on collagen of the oviduct.

METHODS

The strains used in these experiments were the traditional, unselected Nebraska Spot and the Big 6 male-line.

In the first experiment, 19 male-line and 13 Nebraska Spot female turkeys, were reared as described in section 2.1 until five weeks post photostimulation. Prolapsed male-line female turkeys (n=31) were obtained from British United Turkeys' farms at the same time as the experimental turkeys and were killed as soon as possible after discovery of the prolapse, at an average of five weeks post photostimulation.

A second experiment was carried out to test the effect of oestradiol administration on the reproductive system and the vaginal collagen content. Female turkeys, 32 traditional-line and 32 male-line, were reared to five weeks photostimulation as described previously. The turkeys were housed four per pen, in a randomised block design. After five weeks of photostimulation each turkey was given 0, 0.0001, 0.01 or 1.0 mg oestradiol-17β valerate (Sigma Chemical Company, Poole, UK) per kg body weight, daily for 7 days. The oestradiol was dissolved in caster oil, in various concentrations so that the volume given was constant within each strain, and administered by subcutaneous injection over the breast muscle. The injections were administered at the same time each day and the site of injection was varied slightly each day to prevent any build up of damage or bruising. Prior to the start of the experiment and during the week of injections the turkeys were fed fat soluble dye capsules as described in 2.3 to allow egg production per bird to be recorded. The turkeys were killed 24 hours after the last injection.

A third small scale experiment was carried out to assess the effect of progesterone injections on the reproductive system and the vaginal collagen content. Female turkeys, 14 traditional-line and 15 male line, were given progesterone injections, daily for seven days. The experimental design was the same as the previous experiments except that the turkeys were given either 0, 0.0005, 0.05 or 5 mg progesterone (Sigma Chemical Company, Poole, UK) per kg body weight. As before, the turkeys were killed 24 hours after the last injection.

Blood samples were taken from all turkeys by superficial venepuncture of the brachial vein immediately prior to killing with an overdose of sodium pentobarbitone. Treatment of the blood samples was as described in 2.2. Plasma oestradiol concentration was measured after initial extraction using sepharose antibody binding, followed by radioimmunoassay as described in 2.6.1. Plasma progesterone was measured by a similar radioimmunoassay as described in 2.6.2.

The total oviduct was removed and weighed and the vagina and uterus were dissected from it (as described in 2.4) and their respective weights recorded. The uterus and vagina were stored in individual plastic bags at –20°C for future collagen analysis and dry matter determination. The ovary was removed and the follicles classified as described in 2.4. and weighed. The post ovulatory follicles were removed and counted. Due to constraints of time for sampling the total oviduct weight and uterus and vagina weights were only recorded for 11 of the 31 prolapsed turkeys.

In the second and third experiments collagen analysis was carried out only on the vaginal section of the uterus, following the results of the first experiment. Tissue collagen content was determined indirectly by measurement of hydroxyproline content using a colorimetric microassay based on Ehlrich's reaction, as described by Creemers *et al.* (1997). The protocol for measurement of the hydroxyproline content of wet tissue is given in 2.5. The collagen content per gram wet tissue was estimated by multiplying hydroxyproline content by 7.14 (Jackson *et al.* 1996).

The wet weight:dry weight ratio of the uterus and vagina were determined from the uterine and vaginal samples obtained in experiment 1. Each sample was thawed and 2-3g of tissue was weighed in a 5ml uncoated plastic plasma tube. The samples were refrozen, and placed in a freeze dryer for 3 days, until there was no day to day change in weight. They were re-weighed and the final weight/initial weight ×100 gave the % dry weight.

The proportion of intermediate and mature cross links in the vaginal collagen was measured for each of the turkeys from experiment 1. Samples (about 5g) were taken from the vagina and the weight was recorded. Samples were placed in disposable polystyrene containers and washed with 5 changes of distilled water. The samples were equilibrated for one hour in two changes of 0.01 M phosphate buffered saline (PBS, Sigma Chemical Company, Poole, UK). The tissue was minced using scissors and suspended in 3mls PBS. 200 µl of 20 mM sodium hydroxide containing 25 mg/ml potassium borohydrate (Merck Ltd, Leicestershire, UK) was added and samples were incubated at room temperature for 30 minutes with occasional shaking. Tissue was rinsed with 3 changes of distilled water and blotted dry on filter paper before being transferred to glass hydrolysis tubes containing 3 mls 6M hydrochloric acid. After incubation for 18 hours at 107°C samples were filtered and dried using a rotary evaporator. Each sample was resuspended in 1 ml distilled water for measurement of cross-links and collagen content.

In order to relate the amount of cross-links to the collagen present the hydoxyproline content of each sample was measured using Erhlich's colour reaction. Samples were diluted 1/100 and duplicate 100 µl samples were made up to 500 µl with distilled water. Standards ranging from 5-25 nmol/500 µl were also assayed. 500 µl of 2-methoxyethanol (Merck Ltd, Leicestershire, UK) and 200 µl chloramine T solution (0.1 g chloramine T (Merck Ltd, Leicestershire, UK), 1 ml distilled water and 4 ml stock buffer) were added to each sample or standard and they were incubated at room temperature for 5 minutes. 300 µl Ehrlich's reagent (1 g p-dimethylamino-benzaldehyde (Sigma Chemical Company, Poole, UK), 7 ml 2-methoxyethanol, 1 ml

concentrated hydrochloric acid) was added and the samples were incubated for 3 minutes in a boiling water bath. Once cooled to room temperature the optical densities of the standards and samples were read at 553nm using a UV Spectrophotometer (CE 6000, Cecil Instruments Ltd, Cambridge, England).

The amount of the mature cross-links, pyridinoline and deoxypyridinoline, in the hydrolysed tissue samples was measured using an automated sample preparation with extraction columns (ASPEC) system with solid-phase extraction of the cross links followed by reversed phase high pressure liquid chromatography (HPLC) as described by Pratt *et al.* (1992). The amount of intermediate cross-links was measured as the amount of dihydroxy-lysinonorleucine (DHLNL) present in the samples following the borohydride reduction (Robins 1976). This analysis was carried out by Dr Simon Robins and Alexander Duncan at the Rowett Research Institute, using a novel system of ASPEC followed by HPLC, which has not yet been published. The amount of intermediate and mature cross-links present in each sample was expressed per mole of collagen.

In a supplementary experiment the collagen content of the gut was assessed in 12 traditional- and 12 male-line turkeys at five weeks after photostimulation, to investigate whether decreased collagen was a feature of epithelial tissues of male-line turkeys. Samples were taken from the colon section of the gut and the collagen content was measured using the same method as for the uterine and vaginal sections.

Student's t-tests were used to compare the traditional-line and male-line, and prolapsed and non-prolapsed turkeys. Analysis of variance and regression analysis were used to evaluate the effects of the hormone injections. The plasma oestradiol concentrations at the different doses of oestradiol-17β, and plasma progesterone concentrations at the different progesterone doses, were transformed using natural logarithms to ensure normal distributions of the residuals. Regression analysis was used to test the effect of the hormone injection on the mean log plasma concentration of that hormone.

RESULTS

Experiment 1

Field observations on turkeys in various stages of prolapse found that, initially, the vagina was everted, swollen and exposed exterior to the cloaca. As the prolapse developed the uterine section of the oviduct protruded through the vent but remained within the everted vagina. The utero-vaginal sphincter remained intact, preventing the uterus from everting with the vagina. Some turkeys were found to prolapse having never laid while others laid several eggs prior to the prolapse. The prolapsed turkeys sampled in this experiment were mainly in the initial stages of prolapse.

TABLE 4.1 Mean±SEM weights of total body, oviduct, uterus, vagina and residual ovary, number of hierarchical atretic and post ovulatory follicles for traditional-, male-line and prolapsed male-line turkeys at five weeks post photostimulation

	Traditional- line	Male-line	Prolapsed male-line
Total body (kg)	5.06±0.14	19.81±1.2	19.1±0.3
Oviduct (g)	75.0±2.5	138.1±7.1	131.4±8.5
Uterus (g)	22.4±1.2	36.6±2.5	39.7±6.1
Vagina (g)	6.6±0.3	14.5±0.9	14.5±1.8
Residual ovary (g)	5.2±0.58	22.8±1.56	29.4±0.01
No. follicles	7.0±0.43	18.9±0.82	20.5±1.0
No. atretics	0.1±0.09	0.6±0.30	4.00±0.7
No POFs	3.2±0.34	8.7±0.92	3.5±0.5

The mean body weights, weights of the various parts of the reproductive system, and numbers of hierarchical and atretic follicles in traditional-, male-line and prolapsed male-line turkeys are presented on Table 4.1. The male-line turkeys were significantly heavier ($P<0.001$) than the traditional-line, while there was no significant difference between the body weights of the prolapsed and non-prolapsed male-line turkeys. The oviduct, uterus and vagina were all significantly heavier ($P<0.001$) in the male-line compared to the traditional-line, but no significant differences were found in either total oviduct weight, uterus weight or vagina weight between prolapsed and non-prolapsed turkeys.

The residual ovary was nearly five times heavier in the male-line than the traditional-line ($P<0.001$), while it was heavier still in the prolapsed male-line, ($P<0.01$). When considered as a percentage of total body weight, the residual ovary weight remained significantly greater ($P<0.05$) in the male-line (0.096 ± 0.005) than the traditional-line (0.081 ± 0.005). The residual ovary as a percentage of body weight was significantly greater in the prolapsed male-line (0.154 ± 0.013) compared to the non-prolapsed male-line, ($P<0.01$).

There were significantly more hierarchical follicles in the male-line than the traditional-line ($P<0.001$) while there was no significant difference in follicle number between prolapsed and non-prolapsed male-line turkeys. There was no significant difference in the number of hierarchical follicles classed as atretic between the traditional- and male-lines, but the prolapsed male-line had significantly more atretic follicles than the non-prolapsed male-line ($P<0.05$). The male-line had significantly more post ovulatory follicles than the traditional-line ($P<0.05$). The prolapsed male-line had significantly fewer post ovulatory follicles than the non-prolapsed male-line ($P<0.01$).

Mean uterine and vaginal collagen concentrations in the traditional-line, male-line and prolapsed male-line turkeys are shown in Figure 4.1. The vagina had significantly more collagen than the uterus in all three groups investigated ($P<0.001$). The collagen content of the vagina was significantly lower in the male-line than the traditional-line ($P<0.01$) while the vaginal collagen content of those with prolapse was even lower than the non-prolapsed male-line turkeys ($P<0.001$). No significant differences were seen in the uterine collagen content between the traditional-line and male-line the prolapsed and non-prolapsed male-line.

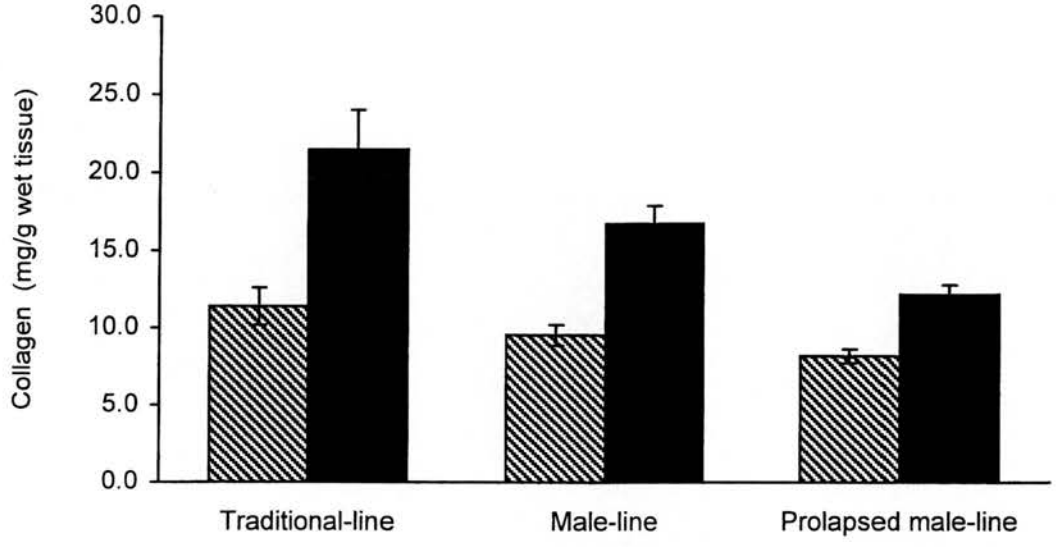


FIGURE 4.1 Mean (±SEM) uterine and vaginal collagen content in traditional-, male-line and prolapsed male-line female turkeys
Key: ▨ Uterus; ■ Vagina

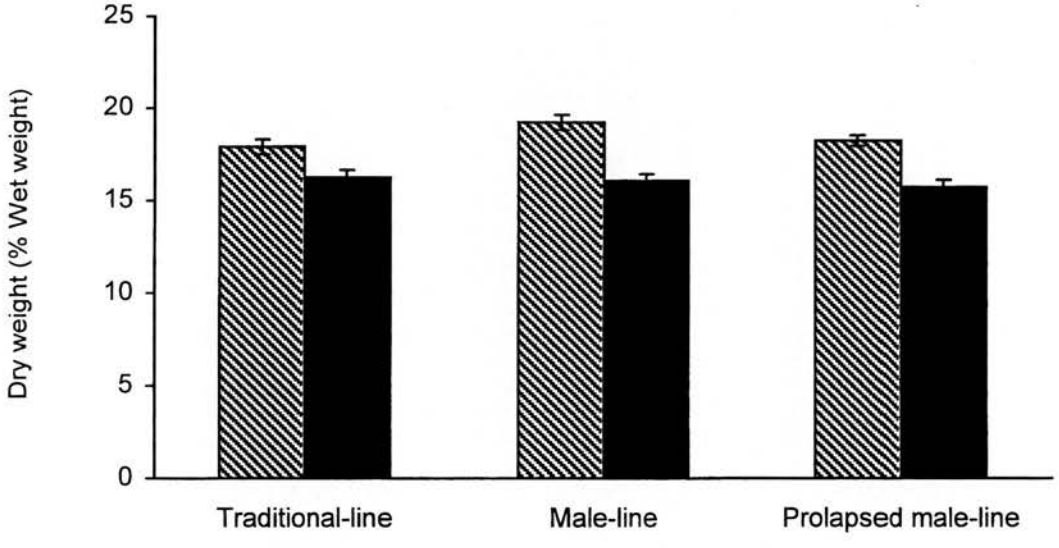


FIGURE 4.2 Mean (±SEM) % dry weight for the uterus and vagina in traditional-, male-line and prolapsed male-line female turkeys
Key: ▨ Uterus; ■ Vagina

The dry weights, expressed as a percentage of the wet weight, of the uterus and vagina for the traditional-, male-line and prolapsed male-line strains are presented in Figure 4.2. There was no significant difference in the percentage dry weight of the vagina between either the traditional-line and the male-line, or the prolapsed and normal male-lines. There was no significant difference in uterine percentage dry weight between the male-line and prolapsed male-line. The uterus of the male-line had a significantly greater percentage dry weight than the traditional-line turkeys ($P<0.05$).

Figure 4.3 and 4.4 respectively show the number of mature cross-link residues, pyridinoline (Pyd) and deoxypyridinoline (Dpd), per vaginal collagen molecule in traditional-, male-line and prolapsed male-line turkeys. There was no significant difference in pyridinoline cross-links between the traditional- and male-line turkeys, but there were significantly fewer ($P<0.05$) in the vaginal collagen of the prolapsed male-line compared to non-prolapsed. The vaginal collagen of the turkeys in all three groups contained very small numbers of deoxypyridinoline residues. There was no significant difference in the number of deoxypyridinoline residues in the vaginal collagen of the male-line compared to the traditional-line, while there were also significantly fewer ($P<0.01$) deoxypyridinoline residues in the vaginal collagen of the prolapsed turkeys compared to non-prolapsed.

Figure 4.5 shows the number of DHLNL residues per collagen molecule, a measure of the number of intermediate cross-links, for the vaginal collagen of the traditional-, male-line and prolapsed male-line turkeys. There was no significant difference in the number of DHLNL residues in the collagen of the traditional-line turkeys compared to the male-line. There were more DHLNL residues in the vaginal collagen of the prolapsed turkeys compared to the non-prolapsed male-line, although this difference was not statistically significant ($P=0.06$).

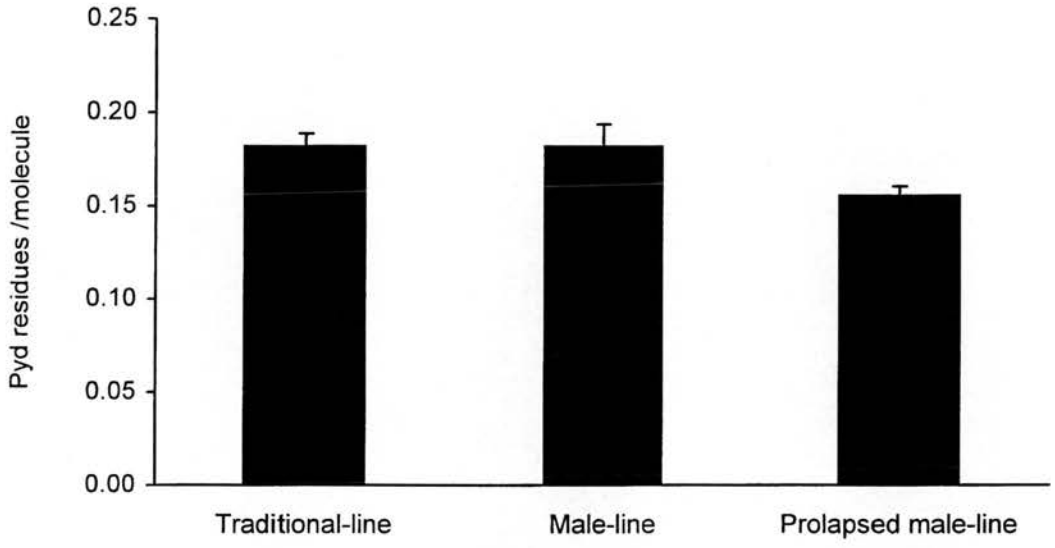


FIGURE 4.3 Mean (±SEM) number of pyridinoline (Pyd) residues per collagen molecule in traditional-, male-line and prolapsed male-line turkeys

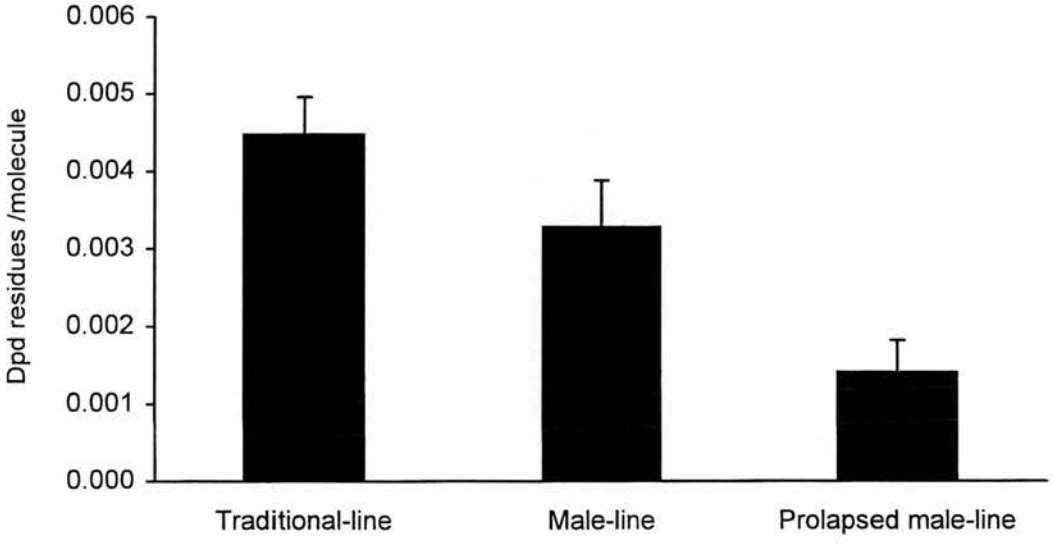


FIGURE 4.4 Mean (±SEM) number of deoxypyridinoline (Dpd) residues per collagen molecule in traditional-, male-line and prolapsed male-line turkeys

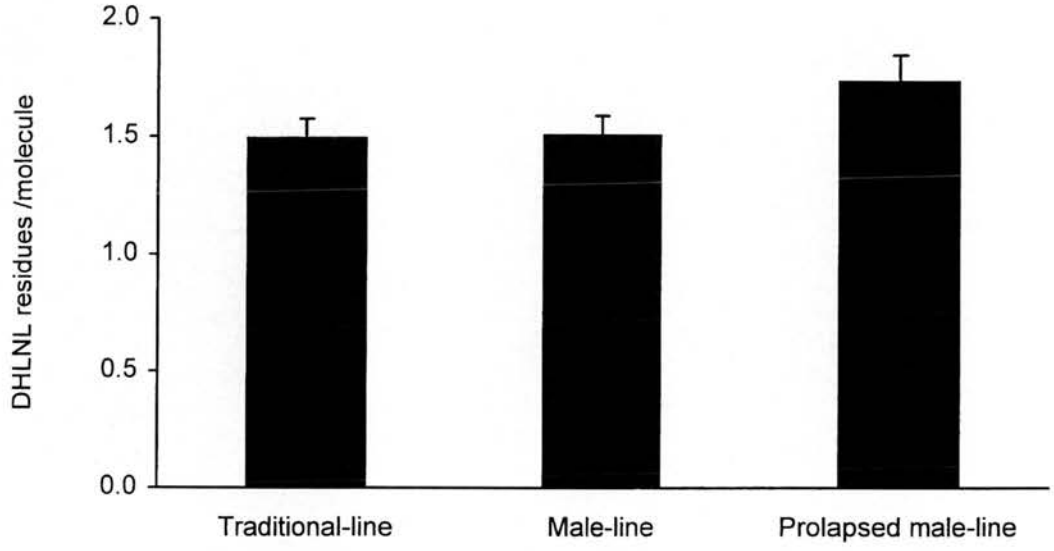


FIGURE 4.5 Mean (±SEM) number of dihydroxy-lysinorleucine (DHLNL) residues per collagen molecule in traditional-, male-line and prolapsed male-line turkeys

Plasma oestradiol concentrations of the traditional, male-line and prolapsed male-line are presented in Figure 4.6. Plasma oestradiol was significantly higher in the traditional-line turkeys compared to the male-line turkeys ($P<0.001$) but there was no significant difference between the normal male-line turkeys and those with prolapse.

Figure 4.7 represents the plasma progesterone concentration for the traditional-line and the prolapsed and non-prolapsed male-line. There were no significant differences in plasma progesterone concentration between any of the groups.

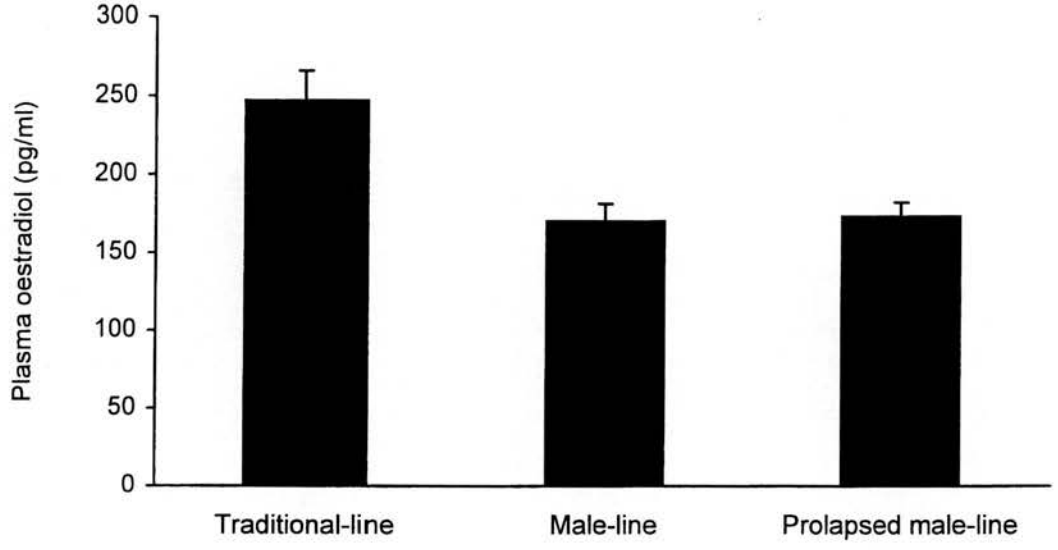


FIGURE 4.6 Mean (±SEM) plasma oestradiol concentration in traditional-, male-line and prolapsed male-line female turkeys

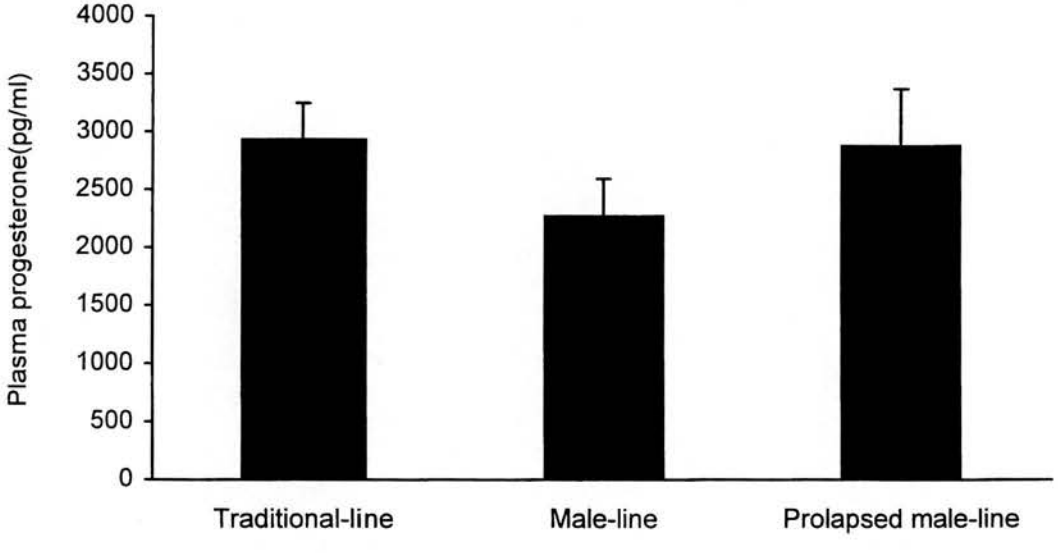


FIGURE 4.7 Mean (±SEM) plasma progesterone concentration in traditional-, male-line and prolapsed male-line female turkeys

Experiment 2

Four of the traditional-line turkeys used in the second experiment had partially developed right oviducts. These individuals had abnormally low plasma oestradiol concentrations and vaginal collagen contents and the data from these birds were excluded.

TABLE 4.2 Total body weight, weigh of oviduct, uterus, vagina, residual ovary, number of hierarchical and post ovulatory follicles and number of eggs laid for traditional and male-line turkeys at the different doses of oestradiol administered

	Dose of oestradiol administered (mg/kg/day)				
	0	0.0001	0.01	1.0	SED
Total body(kg)					
Traditional-line	5104	4839	4966	5026	398.3
Male-line	18205	18225	18561	17746	
Oviduct (g)					
Traditional-line	74.2	74.9	75.5	80.6	5.72
Male-line	133.5	147.8	138.0	150.5	
Uterus (g)					
Traditional-line	20.0	20.3	20.7	22.5	3.75
Male-line	34.4	39.0	33.5	36.6	
Vagina (g)					
Traditional-line	7.0	6.8	6.8	7.6	1.05
Male-line	11.8	14.8	13.8	14.3	
Residual ovary (g)					
Traditional-line	4.1	4.2	3.7	5.9	2.27
Male-line	22.9	25.4	24.6	24.2	
No. follicles					
Traditional-line	7.2	7.6	7.1	7.3	1.13
Male-line	16.6	17.4	19.3	16.9	
No. atretics					
Traditional-line	0.22	0.07	0.04	0.36	0.49
Male-line	0.13	1.12	0.72	0.93	
No. POFs					
Traditional-line	3.8	3.7	3.7	4.4	0.93
Male-line	10.4	8.9	10.0	10.2	
No. Eggs/7days					
Traditional-line	4.3	4.0	4.2	4.8	0.64
Male-line	2.4	3.0	3.0	2.8	

The mean body weights, weights of the various parts of the oviduct and ovary, and numbers of hierarchical and atretic follicles at the different doses of oestradiol are presented on Table 4.2. The oestradiol injections had no significant effect on total body weight in either strain. There was a significant effect of oestradiol dose on the total oviduct weight ($P<0.05$) which reflects slightly higher oviduct weights at the highest oestradiol dose in both strains. There was no significant effect of oestradiol dose on the weights of the uterine or vaginal sections of the oviduct. The oestradiol injections also had no significant effect on the residual ovary weight, hierarchical follicle number or number of atretic follicles in either strain.

The vaginal collagen content of the traditional- and male-line turkeys that were injected with oestradiol is presented on Figure 4.8. Administration of 0.0001, 0.01 or 1 mg oestradiol per kg body weight for 7 days had no effect on vaginal collagen content in either the traditional- or the male-line.

Mean log transformed plasma oestradiol concentrations for the traditional-line and male-line turkeys at each of the oestradiol doses administered are shown on Figure 4.9. The response of plasma oestradiol concentration to oestradiol administration was described by fitting an exponential model to the mean log transformed values of plasma oestradiol for each strain at the four oestradiol-17β doses administrated. The oestradiol injections had a significant effect on plasma oestradiol ($P<0.001$), with plasma oestradiol concentration increasing exponentially with the dose of oestradiol. Plasma oestradiol was significantly greater in the male-line strain compared to the traditional-line strain for the same dose of oestradiol administered ($P<0.001$).

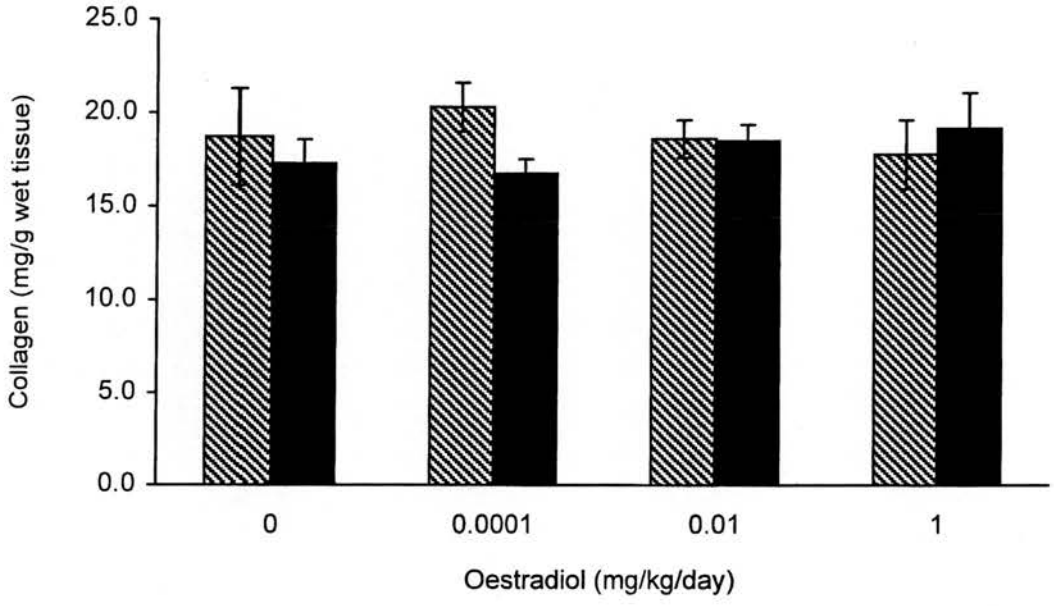


FIGURE 4.8 Mean (±SEM) vaginal collagen content in traditional-line and male-line female turkeys injected with 0-1 mg oestradiol valerate per kg body weight daily for 7 days

Key: ▨ Traditional-line; ■ Male-line

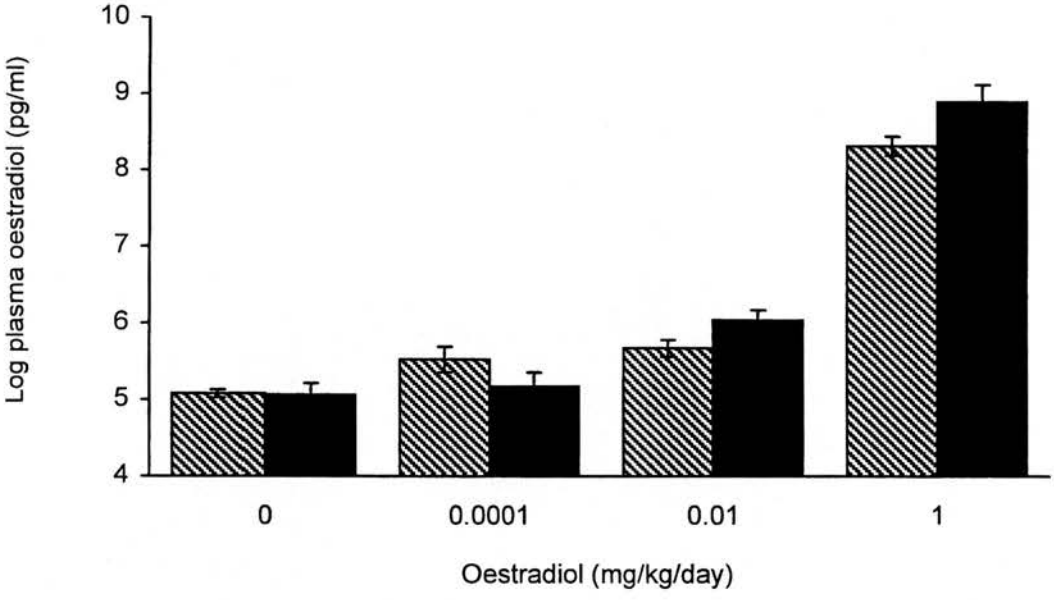


FIGURE 4.9 Mean (±SEM) $\log_e$ plasma oestradiol concentration in traditional- and male-line female turkeys injected with 0 - 1 mg oestradiol valerate per kg body weight daily for 7 days

Key: ▨ Traditional-line; ■ Male-line

Experiment 3

Only one of four traditional-, and three of four male-line turkeys that were receiving 5mg/kg/day progesterone survived to the eighth day of the experiment. Post mortems carried out on these turkeys suggested congestive heart failure as a probable cause of death.

Table 4.3 presents mean total body weight, oviduct, uterus, vagina and residual ovary weight, number of follicles in the hierarchy, number of atretic follicles and number of eggs laid for the two strains at the different doses of progesterone.

TABLE 4.3 Total body weight, weigh of oviduct, uterus, vagina, residual ovary, number of hierarchical, atretic and post ovulatory follicles and number of eggs laid for traditional- and male-line turkeys at the different doses of progesterone administered

	Dose of progesterone administered (mg/kg/day)				
	0	0.0005	0.05	5	SED
Total bird (kg)					
Traditional-line	5338	5228	4990	4976	975
Male-line	17955	18619	17788	18141	
Oviduct (g)					
Traditional-line	76.0	70.9	75.5	85.4	10.3
Male-line	133.9	137.7	127.0	133.8	
Uterus (g)					
Traditional-line	20.9	19.4	20.4	21.9	3.4
Male-line	35.1	37.6	33.2	31.1	
Vagina (g)					
Traditional-line	6.5	6.6	7.0	4.3	1.9
Male-line	13.3	14.4	11.8	11.6	
Residual ovary (g)					
Traditional-line	4.2	4.5	5.9	5.4	3.7
Male-line	18.6	26.2	19.9	28.7	
No. follicles					
Traditional-line	8.0	6.0	6.3	2.5	1.8
Male-line	16.2	17.3	13.5	1.3	
No. atretics					
Traditional-line	0	0	1.7	3.5	2.2
Male-line	0	0	0.25	19.3	
No. eggs/7days					
Traditional-line	5.0	3.3	2.0	1.5	1.1
Male-line	2.0	3.3	3.0	0.33	

The progesterone injections had no significant effect on body weight, oviduct weight or weight of the uterine and vaginal sections of the oviduct. The residual ovary weight was not affected by the progesterone injections. There was a significant interaction between dose and strain for hierarchical follicle number ($P<0.001$) as the number of follicles in the hierarchy decreased as the progesterone dose increased and the decrease was greater in the male-line. There was also a significant interaction effect ($P<0.001$) on the number of follicles classed as atretic, as there were more atretic follicle at the higher doses of progesterone and this increase was greatest in the male-line. The number of post ovulatory follicles was also significantly different ($P<0.05$) with the interaction between progesterone dose and strain. Post ovulatory follicle number decreased as progesterone dose increased and this effect was greater in the male-line compared to the traditional-line.

There was a significant interaction between progesterone dose and strain on egg production ($P<0.05$). The number of eggs laid decreased as the progesterone dose amd egg production in the control group was greater in the traditional-line compared to the male-line. When the daily egg production was investigated, it was observed that in both strains the turkeys receiving the highest dose of progesterone had failed to lay any eggs after the second day of injections and it is this cessation of lay that gave rise to their significantly lower egg production.

The vaginal collagen content of the two strains of turkey at the various doses of progesterone administered is shown on Figure 4.10. The different doses of progesterone had no significant effect on vaginal collagen. Figure 4.11 shows the mean log transformed plasma progesterone concentrations for the traditional- and male-line turkeys at each of the doses of progesterone administered. The response of plasma progesterone to progesterone administration was best described by fitting an exponential model to the mean log transformed values of plasma progesterone for each strain. Progesterone administration had a significant effect on plasma progesterone ($P<0.001$), with plasma progesterone concentration rising exponentially with progesterone dose.

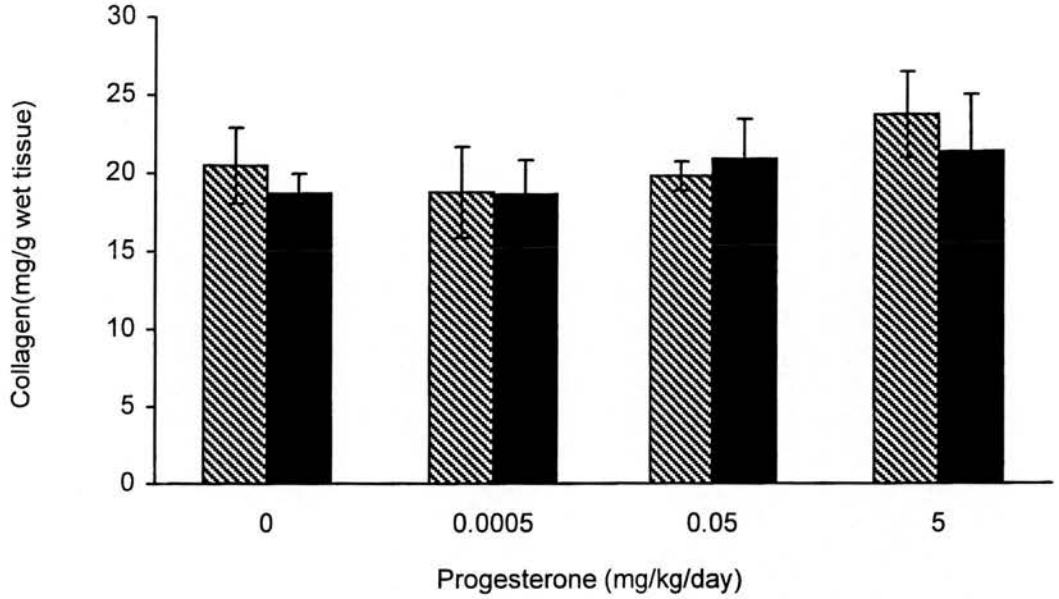


FIGURE 4.10 Mean (±SEM) vaginal collagen content in traditional- and male-line female turkeys injected with 0-5 mg progesterone per kg body weight daily for 7 days

Key: ▨ Traditional-line; ■ Male-line

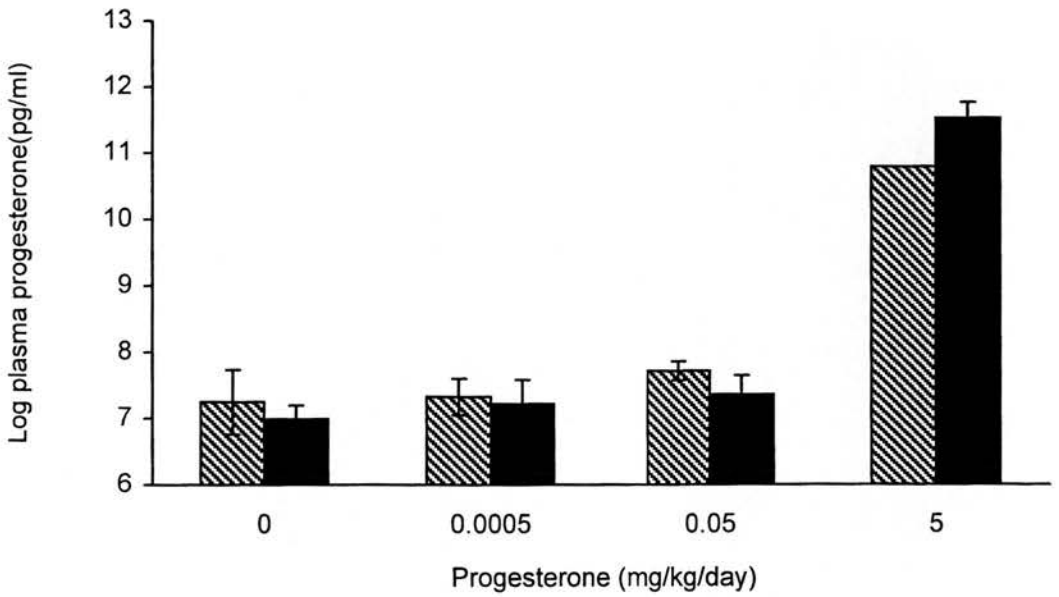


FIGURE 4.11 Mean (±SEM) $\log_e$ plasma progesterone concentration in traditional- and male-line female turkeys injected with 0 - 5 mg progesterone per kg body weight daily for 7 days. The plasma progesterone value for the traditional-line at the highest dose is based on the one turkey in that group which survived until the eighth day of the experiment, therefore no SEM is available for this value

Experiment 4

There was no significant difference in the collagen content of the colonic section of the gut between the traditional- (28.0±1.01mg/g wet tissue) and the male-line (29.1±0.93) turkeys.

DISCUSSION

The results of the first experiment suggest that the low vaginal collagen content of the prolapsed male-line, compared to the control male-line turkeys, could be the underlying cause of prolapse. These results support the findings of Jackson *et al.* (1996) who demonstrated that vaginal collagen was decreased in association with prolapse in humans. The hypothesis was further supported by the low collagen content of male-line turkeys, that are susceptible to prolapse, compared to the traditional-line. As there were no significant differences in vaginal percentage dry weights between the different strains the observed differences between the collagen contents cannot be due to differences in water content of the tissue. The higher dry weight of the male-line uterus was a small difference and unlikely to be biologically important.

It was also found that the vaginal collagen of the prolapsed turkeys had fewer mature cross links per collagen molecule, as shown by the decreased numbers of pyridinoline and deoxypyridinoline residues, compared to the non-prolapsed turkeys. The decreased number of mature cross links would have reduced the structural strength of the collagen and therefore is likely to have contributed to the impairment of the vaginal structure that led to the prolapse.

The prolapsed turkeys had an increased proportion of intermediate cross links, as shown by the increased number of DHLNL residues per molecule of collagen, although this difference did not reach statistical significance. Taken together the results suggest that the collagen of the prolapsed vagina was less mature than the

non-prolapsed vagina and may reflect an increase in collagen turnover. An increase in the proportion of intermediate cross links was also associated with prolapse in women (Jackson *et al.* 1996).

The lower plasma oestradiol concentration of the male-line compared to the traditional-line did not support the hypothesis that high plasma oestradiol concentrations stimulated increased collagen degradation and predisposed the turkeys to prolapse. However the results were based on a single sample from each bird after five weeks of photostimulation. It is possible that the male-line turkeys could have higher cycling levels of oestradiol during the ovulatory cycle compared with the traditional-line. Alternatively, it is possible that there is a difference in the pre-lay pattern of plasma oestradiol concentration between the two strains that could be involved in predisposing the male-line to prolapse. These aspects are investigated in Chapters 5 and 3 respectively.

The field observations of prolapsed male-line turkeys suggested that a structural failure of the vagina was likely to be the immediate cause of prolapse. The finding of a collagen deficiency associated with prolapse in the vaginal part of the oviduct is consistent with these observations. A defect in the vaginal tissue that hindered the ability of the tissue to support the oviduct would predispose the male-line to prolapse. The reduced collagen of the male-line was observed only in the vagina and was not a feature of the uterus or the gut, which supports the field observation that failure of the uterus does not appear to be involved in the initiation of prolapse. The greater collagen content of the vagina in comparison to the uterus in all 3 groups supported the hypothesis that the vagina has a supportive role in the oviduct.

In the second experiment, the changes in plasma oestradiol concentration in response to oestradiol administration showed that the injections were effective in increasing plasma oestradiol concentrations. Administration of oestradiol had no significant effect on the collagen content of the vagina in either strain. The results suggests that

oestradiol alone is not involved in stimulating the reduction in vaginal collagen associated with prolapse of the oviduct.

The highest dose of oestradiol caused a 40 fold increase in plasma oestradiol. This had no significant effect on the reproductive system, except on the total oviduct weight. This probably reflects the involvement of oestrogens in oviduct growth and development (Etches 1996a). The increase in oviduct weight was probably due to an increase in weight of the upper section of the oviduct as there was no significant effect of oestradiol on the uterine or vaginal sections of the oviduct. The lack of significant effects of increased oestradiol on the reproductive structures was surprising and raises questions about the involvement of oestradiol in the maintenance of reproduction in turkeys.

The third experiment showed that the injections of progesterone increased plasma progesterone, which showed that the injections were a successful method of administrating progesterone. Increasing plasma progesterone did not increase vaginal collagen content, and it seems unlikely that progesterone inhibits vaginal collagen degradation in turkeys.

The highest dose of progesterone prevented any ovulations occurring after the second day of injections. It is thought that progesterone production by the maturing follicle is responsible for initiating ovulation, by stimulating release of luteinizing hormone from the hypothalamus (1.7). It is possible that the continuous extremely high plasma progesterone concentration has a direct negative effect on the hypothalamus or anterior pituitary blocking the pre-ovulatory surge in luteinizing hormone concentration and prevented ovulation from taking place. The highest dose of progesterone caused deaths in several turkeys (50% mortality compared to zero mortalities in the other progesterone groups). Heart failure was considered the likely cause of death on post mortem examination. Progesterone injections may be associated with fluid retention (Martindale 1989) and it is possible that, at the highest

dose, progesterone increased fluid retention in the body placed an increased strain on the heart resulting in congestive heart failure.

As there were no effects of progesterone on vaginal collagen content and several turkeys had died due to the progesterone administration it was decided that there were no justification for repeating the progesterone trial as a full scale experiment.

The results of these experiments suggest that low vaginal collagen content predisposes the male-line to prolapse. The structural strength of the vagina in the prolapsed male-line turkeys is likely to be further impaired by a reduction in the proportion of mature cross links on the collagen. Plasma oestradiol concentrations were higher in the traditional-line compared with the male-line and there was no difference in plasma oestradiol between turkeys with or without prolapse. It is therefore unlikely that high plasma oestradiol concentrations are involved in stimulating the decreased vaginal collagen associated with prolapse. This conclusion is further supported by the absence of an effect of oestradiol administration on vaginal collagen content. There was no evidence of a role for progesterone in prolapse of the oviduct.

5. Changes in plasma progesterone, oestradiol and luteinizing hormone concentrations during the ovulatory cycle of turkeys

INTRODUCTION

Male-line turkeys have large ovaries compared to traditional-lines (Hocking and Bernard 1998). These large ovaries contain a multiple follicular hierarchy, with two or more follicles at each position on the hierarchy, that results in the ovulation of two or more follicles each day. This decreases the production of eggs suitable for incubation because of the increased number of double-yolked, soft-shelled and mis-shapen eggs, and through internal ovulations. It is not known whether these multiple ovulations occur separately throughout the day or at the same time.

Medium body weight modern turkeys have a pre-ovulatory peak in luteinizing hormone 4-8 hours before ovulation, which is accompanied by an increase in plasma progesterone (Hammond *et al.* 1981b; Sharp *et al.* 1981). Ovulatory changes in progesterone and luteinizing hormone in female-line turkeys were investigated by Yang *et al.* (1997) who also demonstrated pre-ovulatory peaks in progesterone and luteinizing hormone. Opel and Arcos (1978) measured plasma oestradiol throughout the ovulatory cycle in turkeys, but found no consistent pattern of oestradiol 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) compared the changes in plasma luteinizing hormone during the ovulatory cycle in a multiple ovulating strain of White Leghorns and a commercial laying strain. There was no increase in the number of luteinizing hormone peaks during the ovulatory cycle and they concluded that there were no abnormalities in the secretion of luteinizing hormone in the multiple ovulating hens. They proposed that the cause of multiple ovulation was a defect in the development of the follicular hierarchy.

It was originally hypothesised (1.14) that prolapse of the oviduct in male-line turkeys could be due to high plasma oestradiol concentrations stimulating degradation of vaginal collagen. It has been shown that plasma oestradiol was significantly lower in male-line turkeys compared to the traditional-line (Chapter 4). However this result was based on samples taken at one time of day. If plasma oestradiol concentrations changed during the ovulatory cycle it is possible that plasma oestradiol peaked at a higher concentration in the male-line than the traditional-line and that this could be involved in predisposing the strain to prolapse.

A serial sampling experiment was carried out to compare plasma oestradiol, progesterone and luteinizing hormone concentrations throughout the ovulatory cycle in a single ovulating traditional-line and a multiple ovulating male-line of turkeys. The aim of this experiment was to detect any differences in the plasma hormone profiles between the two strains and any changes in the oestradiol concentrations of the two strains during the ovulatory cycle that could be involved in predisposing the male-line to prolapse of the oviduct.

The experiment would also show whether multiple ovulations are associated with multiple pre-ovulatory luteinizing hormone peaks throughout the ovulatory cycle or a prolonged increase in luteinizing hormone. Both of these situations would suggest that the multiple ovulations were occurring at different times throughout the day. Alternatively if the pre-ovulatory luteinizing hormone peak of the male-line resembled that of the single ovulating traditional-line, it would suggest that several follicles were ovulated together in response to a single peak of luteinizing hormone in the multiple ovulating strain.

METHODS

The two strains used in this experiment were the Big 6 male-line and the unselected traditional-line, Nebraska Spot. Female turkeys (24 from each strain) were reared as described in 2.1, with four birds in each pen, in a randomised block design. Individual egg production was recorded by feeding capsules containing fat soluble dyes three times a week (2.4). Within each pen, the turkeys with the most regular egg production were selected for the experiment, as they were more likely to ovulate during the sampling period. As the laying pattern of the male-line was more irregular than the traditional-line, 12 male-line and 7 traditional-line turkeys were used for the experiment.

The sampling period started six weeks after the onset of photostimulation. The turkeys were handled daily in the week prior to 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. The lighting schedule was 14L:10D, with the lights coming on at 02.00 and going off at 16.00 hours.

Each turkey was blood sampled by superficial venepuncture of the brachial vein (2.2) every 3 hours for 36 hours starting at 10.00 hours. The sampling took about 1 hour but the turkeys were always sampled in the same order to ensure a 3 hour gap between each sample. During the hours when the lights were off, spectacle type head torches (RS Components Ltd, Corby, Northamptonshire) were used for the sampling to minimise disruption in the lighting schedule. Egg production was recorded hourly for the final 24 hours of the sampling.

Turkeys were killed 12 hours after the final sample by overdose of sodium pentobarbitone (2.2), the abdominal cavity was opened and the ovary was removed. Ovarian follicles were classed and recorded as described in 2.4. Post ovulatory follicles were removed and counted. The sequence of previous ovulations was estimated by arranging the post ovulatory follicles according to size, with ones of similar size grouped together as likely multiple ovulations. This data supplemented the egg

production data and allowed comparison of the ovulation pattern to the hormone profile for each bird. The residual ovary weight was also recorded.

Radioimmunoassays were carried out to measure plasma progesterone, oestradiol and luteinizing hormone as in 2.6.

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 base-line value for each bird. The duration of each peak was estimated as the difference in sample time from the start of the steep rise in concentration to the time when the concentration had returned to base-line levels. The height and duration of the peaks in progesterone and luteinizing hormone were compared between the two strains 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 luteinizing hormone was significantly different from zero, and a two sample t-test was used to compare this time difference between the two strains.

RESULTS

Eggs were obtained from five of the traditional-line and four of the male-line turkeys during the sampling time. From the eggs laid after the sampling period and the presence of ovulated follicles in the oviduct when the birds were killed, it appeared that all of the traditional-line and 9 of the male-line turkeys had ovulated during the sampling period.

The male-line had significantly more post ovulatory follicles than the traditional-line (Table 5.1.). The largest post ovulatory follicles were from the most recently ovulated follicles. The male-line strain also had a significantly greater incidence of two or more post-ovulatory follicles at the largest position. No more than one egg was collected from each turkey during the 36 hour period, therefore these additional post ovulatory follicles

must represent follicles lost through internal ovulation or soft shelled eggs that were destroyed before they could be collected.

TABLE 5.1 Mean±SEM number of post ovulatory follicles (POFs) and number of post ovulatory follicles of the largest size in traditional-line and male-line turkeys

	Traditional-line	Male-line	Significance
Number of POFs	3.1±0.34	7.9±1.15	**
Number of largest POF's of similar size	1.0±0.00	2.0±0.19	***

Figures 5.1 and 5.2 respectively show typical traces of the changes in plasma oestradiol, progesterone and luteinizing hormone throughout a 36 hour period in a traditional- and a male-line turkey which had ovulated during the sampling period. Plasma luteinizing hormone peaks were clearly visible in ten of the male-line and all of the traditional-line turkeys. The two male-line turkeys that showed no luteinizing hormone peak were two of the three that were not thought to have ovulated during the sampling period. Figure 5.3 shows the changes in plasma hormone concentrations in one of the male-line turkeys that did not ovulate during the sampling period. The third turkey had a distinct peak in luteinizing hormone although no ovulated follicles were found when the turkey was killed (Figure 5.4). The observed peak in luteinizing hormone was presumably associated with internal ovulations that had been absorbed by the time the turkey was killed.

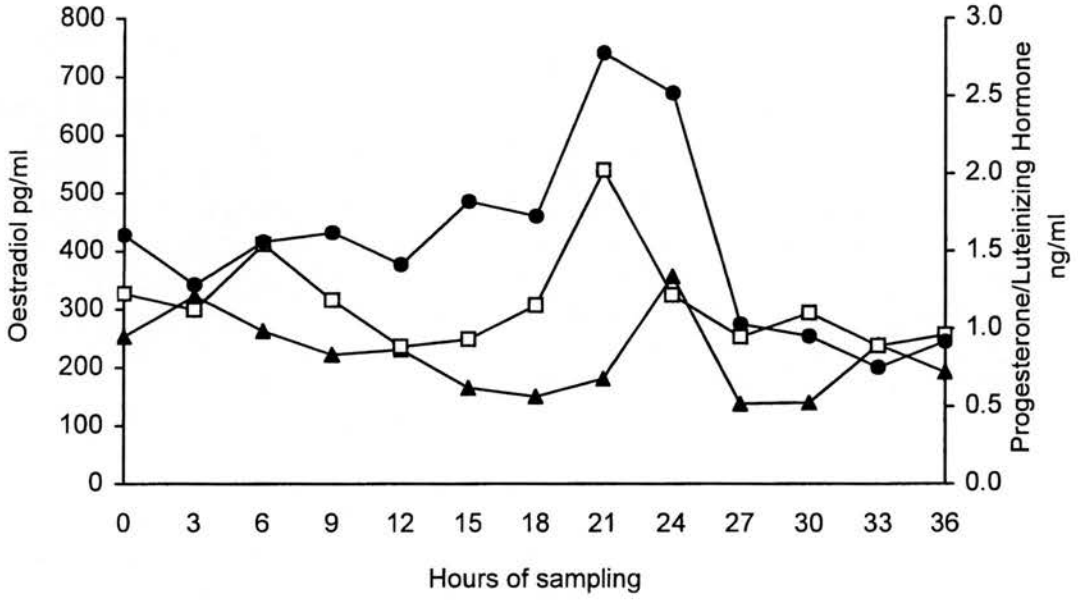


FIGURE 5.1 Changes in plasma concentration of oestradiol, progesterone and luteinizing hormone in a traditional-line turkey during a 36 hour sampling period
Key: ● Progesterone; ▲ Oestradiol; □ Luteinizing hormone

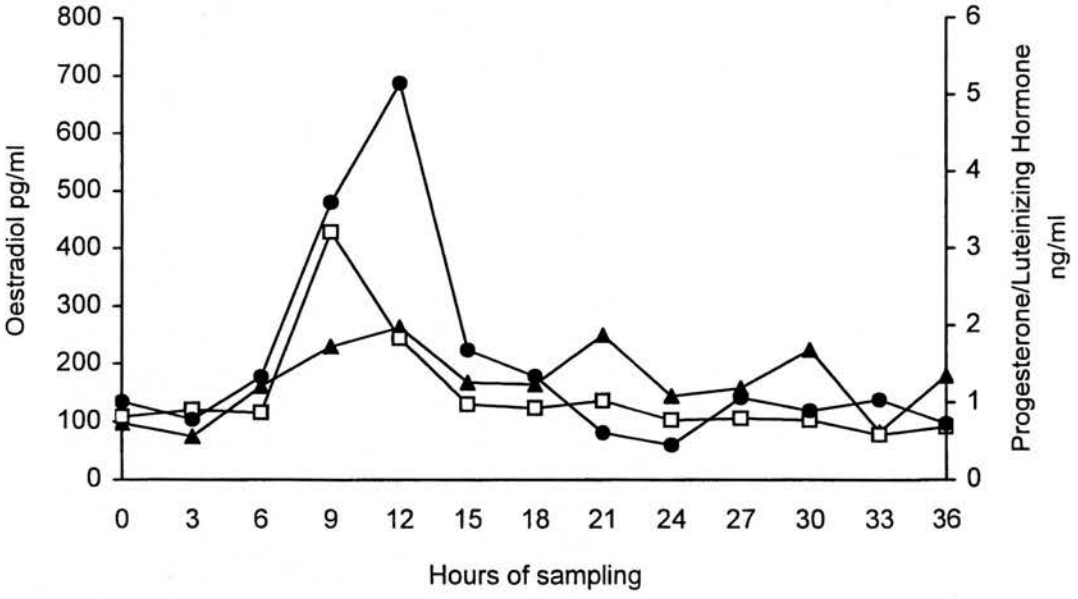


FIGURE 5.2 Changes in plasma concentration of oestradiol, progesterone and luteinizing hormone in a male-line turkey during a 36 hour sampling period
Key: ● Progesterone; ▲ Oestradiol; □ Luteinizing hormone

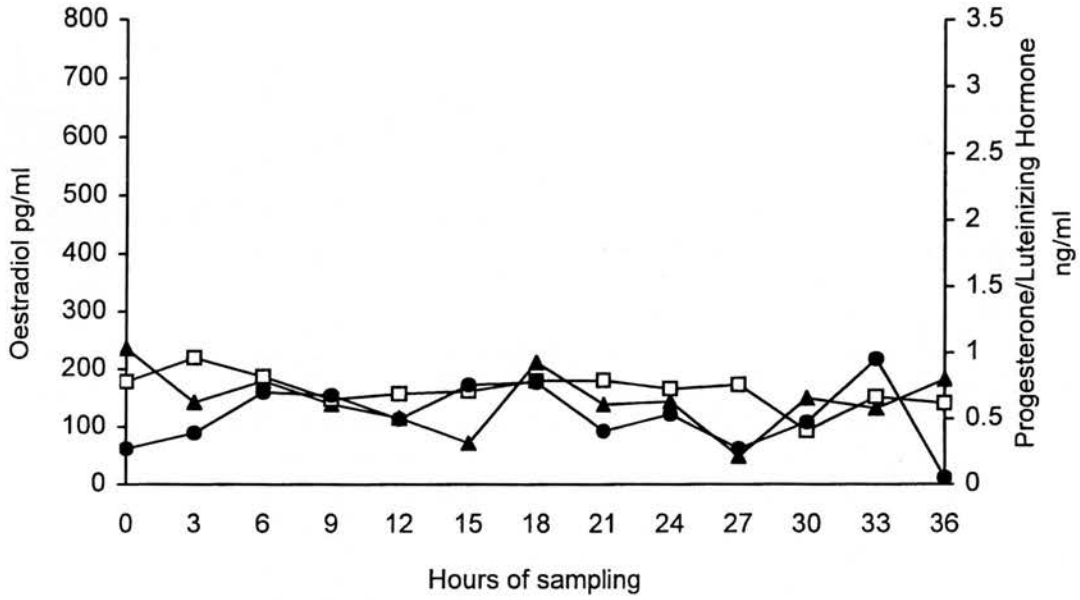


FIGURE 5.3 Changes in plasma concentration of oestradiol, progesterone and luteinizing hormone in a male-line turkey during a 36 hour sampling period during which no ovulation occurred

Key: ● Progesterone; ▲ Oestradiol; □ Luteinizing hormone

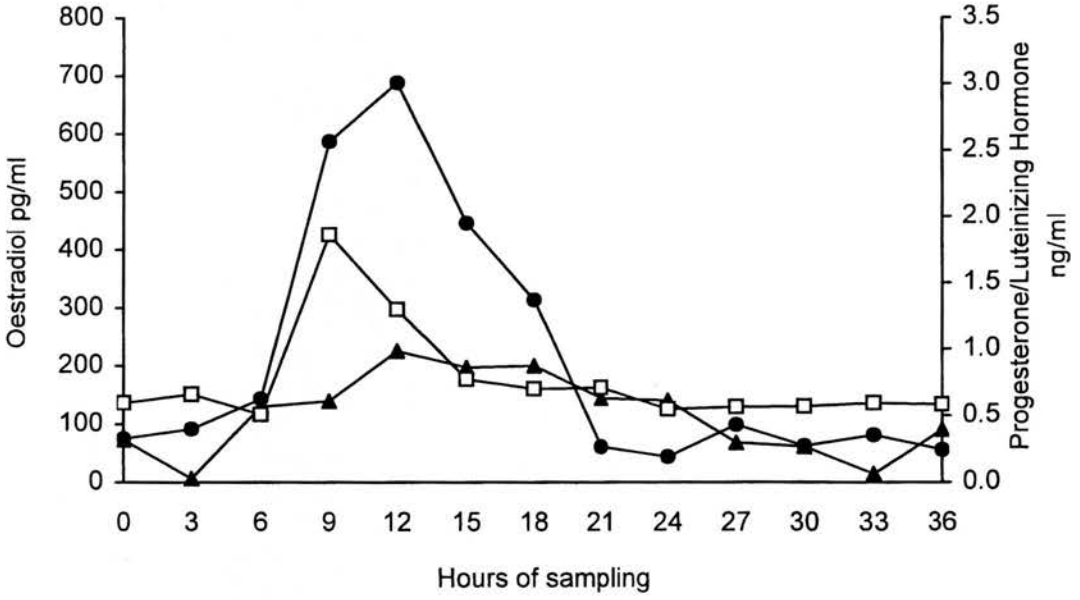


FIGURE 5.4 Changes in plasma concentration of oestradiol, progesterone and luteinizing hormone in a male-line turkey during a 36 hour sampling period during which an internal ovulation is assumed to have occurred

Key: ● Progesterone; ▲ Oestradiol; □ Luteinizing hormone

The luteinizing hormone peaks were no more frequent in the male-line turkeys compared to the traditional-line. No turkey showed more than one complete peak during the sampling period. Mean duration of the luteinizing hormone peak for the two strains is given in Table 5.2. There was no difference in the duration of the peak in luteinizing hormone concentration between the two strains.

The mean concentration of plasma luteinizing hormone and height of the peak for the two strains are shown on Figure 5.5. The mean plasma luteinizing hormone concentration was significantly greater in the traditional-line than the male-line ($P<0.001$). However when the height of the luteinizing hormone peaks was compared there was no significant difference between the two strains.

TABLE 5.2 Mean±SEM duration of pre-ovulatory peak in plasma concentration of progesterone and luteinizing hormone concentration in traditional-line and male-line turkeys

	Traditional-line (hours)	Male-line (hours)	Significance
Progesterone	11.2±1.28	11.6±1.19	NS
Luteinizing hormone	10.5±0.67	10.5±0.80	NS

Plasma progesterone concentration showed a pre-ovulatory increase that coincided with the increase in plasma luteinizing hormone in both the traditional- and male-lines (Figures 5.1 and 5.2) No such increase was visible in either of the male-line turkeys that did not ovulate during the sampling period (Figure 5.3). Progesterone reached peak concentration significantly later than the peak in luteinizing hormone (2±0.5hours, $P<0.001$) but there was no difference in this time period between the two strains. The duration of the peak in plasma progesterone concentration is given on Table 5.2. There was no significant difference in duration of progesterone peak between the two strains. The progesterone peak lasted slightly longer than the luteinizing hormone peak in both strains, although this difference was not statistically significant.

Figure 5.6 shows the mean plasma progesterone concentration and mean height of the progesterone peak in the traditional- and male-line. The mean concentration of progesterone was not significantly different between the traditional-line and the male-line. There was also no significant difference in the height of the plasma progesterone peak between the two strains.

There was no consistent pattern of changes in plasma oestradiol concentration in either the traditional-line or male-line. Some individuals showed peaks in plasma oestradiol at the same time as the luteinizing hormone peak (Figures 5.1 and 5.2), while others had peaks before or after the luteinizing hormone peak and some turkeys had no distinct oestradiol peak at all. The mean plasma oestradiol concentration for the two strains is shown on Figure 5.7. Plasma oestradiol was significantly lower in the male-line compared to the traditional-line ($P<0.001$).

To test the hypothesis that plasma oestradiol reaches a higher peak concentration in the male-line that could be involved in predisposing the strain to prolapse of the oviduct, the highest plasma oestradiol concentrations for each bird throughout the 36 hour sampling period were compared between the two strains. The mean highest oestradiol concentration reached was significantly higher ($P<0.05$) in the traditional-line (446 ± 52.5pg/ml) than the male-line (306 ± 26.0pg/ml).

Figure 5.8 shows the number of follicles in the hierarchy and the residual ovary weight for the traditional- and male-lines. The male-line had significantly more hierarchical follicles ($P<0.001$) and a significantly greater residual ovary weight ($P<0.001$) compared to the traditional-line.

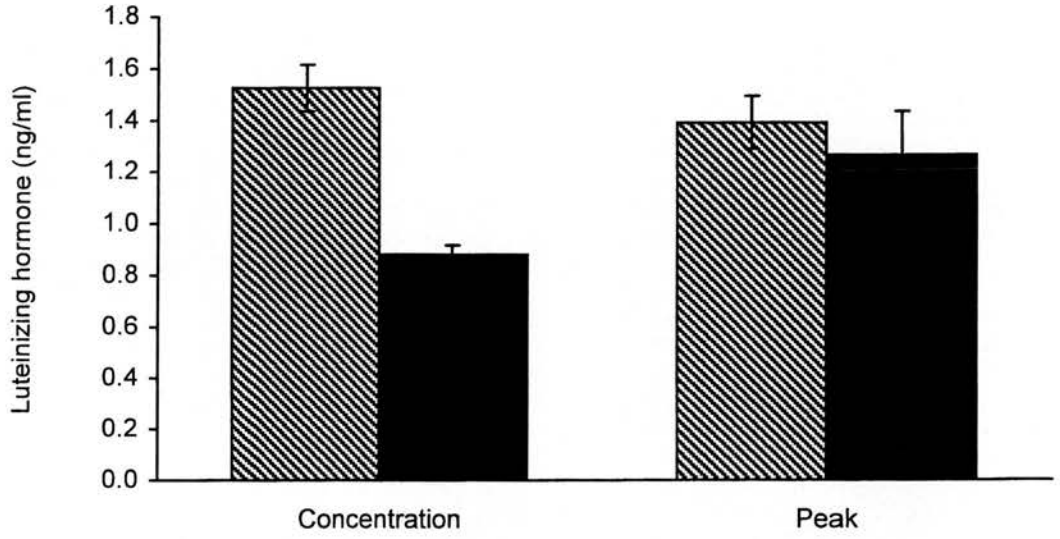


FIGURE 5.5 Mean (±SEM) concentration and height of pre-ovulatory peak of luteinizing hormone in traditional-line and male-line turkeys sampled every 3 hours for 36 hours
Key: ▨ Traditional-line; ■ Male-line

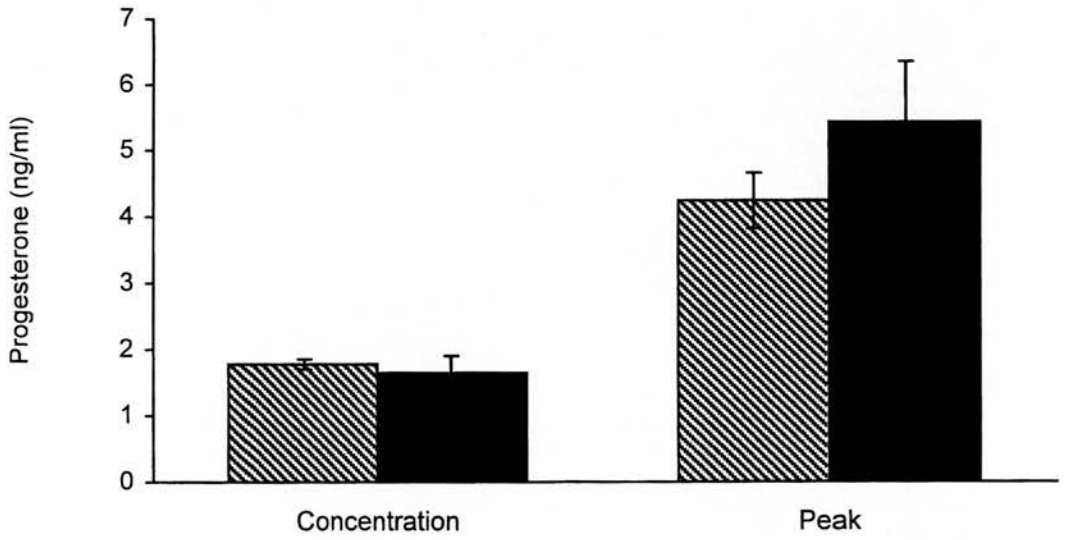


FIGURE 5.6 Mean (±SEM) concentration and height of pre-ovulatory peak of progesterone in traditional-line and male-line turkeys sampled every 3 hours for 36 hours
Key: ▨ Traditional-line; ■ Male-line

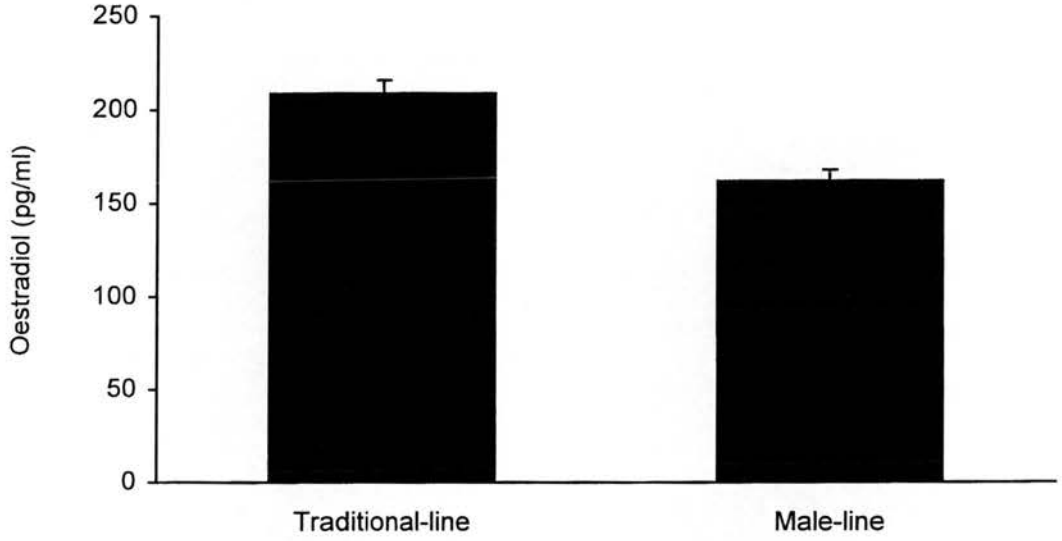


FIGURE 5.7 Mean (±SEM) concentration of oestradiol in traditional-line and male-line turkeys sampled every 3 hours for 36 hours

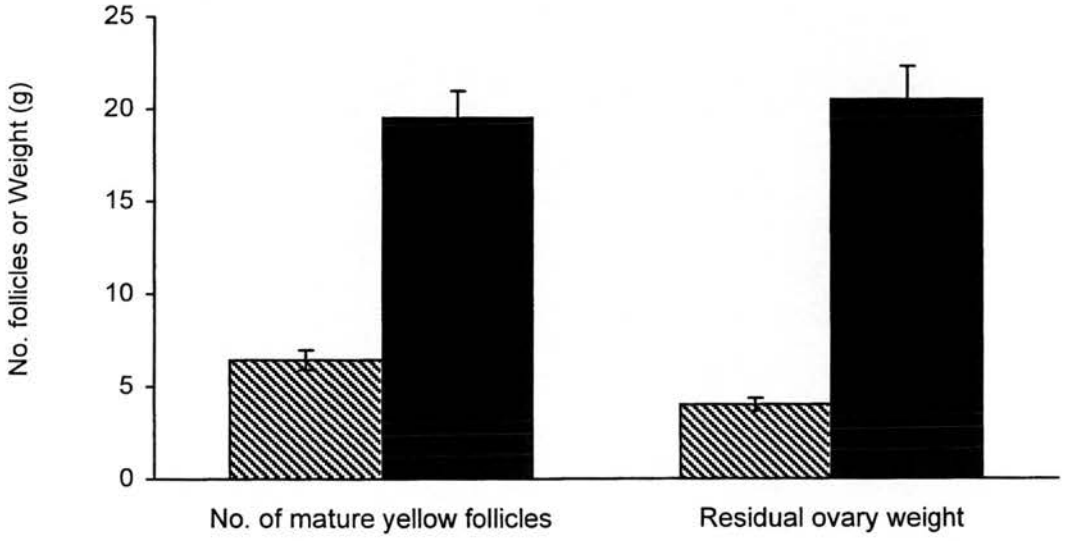


FIGURE 5.8 Mean (±SEM) number of hierarchical follicles and residual ovary weight in traditional- and male-line turkeys killed 12 hours after the end of the sampling period
Key: ▨ Traditional-line; ■ Male-line

DISCUSSION

The data from the 36 hour sampling period showed peaks in plasma progesterone and luteinizing hormone associated with ovulation in both traditional and male-line turkeys. This is in agreement with previous findings in turkeys (Hammond *et al.* 1981b; Sharp *et al.* 1981) and domestic hens (Etches 1996d). The lack of consistent patterns of oestradiol changes during the ovulatory cycle in either strain of turkey also agrees with previous findings (Opel and Arcos 1978).

It was not possible to calculate the time between ovulation and subsequent oviposition, as most of the eggs laid during the sampling period would have been ovulated prior to the start of the sampling period. The results did not show a clear relationship between oviposition and ovulation, such as exists in hens (Etches 1996d) where ovulation tends to occur 30-45 minutes after oviposition of the previous egg. In order to determine the time interval between ovulation and oviposition a longer period of recording egg production following the sampling period would have been necessary. However in this experiment it was necessary to record the presence of fresh post ovulatory follicles in order to establish if ovulation had occurred, therefore a long egg collection period was not possible.

The luteinizing hormone peaks were no more frequent in the male-line than the traditional-line which suggests that multiple follicles are ovulated in response to a single pre-ovulatory peak of luteinizing hormone and progesterone. This is similar to the findings of Sharp *et al.* (1976) who found that multiple ovulations in hens occurred following single luteinizing hormone peaks. However they found no significant difference in the mean concentration of luteinizing hormone between the single and multiple ovulating groups, while these results showed that plasma luteinizing hormone was much lower in the multiple ovulating male-line turkeys. Williams *et al.* (1986) showed a significant difference in plasma luteinizing hormone concentration between fat-line and lean-line chickens, with a greater luteinizing hormone concentration in the lean-line. It is unclear why selection for different body traits such as increased abdominal fat or increased meat yield should alter plasma luteinizing hormone

concentration. It is possible that the hypothalamus of the male-line turkeys has not increased in hormone production capacity sufficiently to keep up with the increased body size of the strain.

Multiple ovulations must have occurred in the male-line during the sampling period as most of the male line turkeys had two or more fresh post ovulatory follicles without associated yolks or eggs. It seems likely that most of these ova were lost as internal ovulations. The abdominal cavity of male-line breeding turkeys frequently contained intact ovulated follicles or yolky fluid which was assumed to have come from burst ova. The results suggest that a high proportion of ovulated follicles are lost as internal ovulations into the abdominal cavity in male-line turkeys.

The peaks in plasma progesterone and luteinizing hormone were no more prolonged in the male-line compared to the traditional-line which further supports the hypothesis that several follicles are released in response to a single luteinizing hormone 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 luteinizing hormone concentration. If the follicles were ovulated several hours apart several peaks or an extended increase in luteinizing hormone would probably have been seen in the male-line.

The release of several follicles from the ovary around the same time 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 at the same time it is likely that this would result in a double yolked egg or two mis-shapen eggs as the ovulated follicles would be too close together in the oviduct to both become fully formed hard shelled eggs.

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 compared to the traditional-line. When the mean plasma oestradiol concentration over the 36 hour sampling period was compared between the two strains, plasma oestradiol was lower in the male-line, which confirmed the results reported in Chapters 3 and 4. Clearly male-line turkeys do not have a higher

plasma oestradiol concentration that could be involved in their predisposition to prolapse of the oviduct.

No difference was found in the mean progesterone concentration over the 36 hour sampling period between the two strains which again confirms the results obtained from the single sample experiment described in Chapter 3. In both strains progesterone increased to a concentration 2-4 times its basal level in a pre-ovulatory peak that occurred slightly later than the pre-ovulatory luteinizing hormone peak. This is concurrent with the finding of Sharp *et al.* (1981) who showed that progesterone peaked about 4 hours after luteinizing hormone. A more frequent sampling regime would be required to determine whether the duration of elevated progesterone levels is significantly longer than the duration of elevated plasma luteinizing hormone.

The preovulatory surge in progesterone is though to be the trigger for ovulation in domestic hens (1.7). It was hypothesised that an initial small increase in plasma progesterone concentration due to progesterone production by the largest preovularoy follicles stimulates luteinizing hormone release from the anterior pituitary. The resulting increase in luteinizing hormone stimulates increased progesterone production by the largest ovarian follicles and plasma progesterone concentration further increases, resulting in ovulation. The high concentration of progesterone stimulates negative feedback on the hypothalamus, decreasing the release of luteinizing hormone and therefore plasma luteinizing hormone concentration falls. This reduces the stimulatory effect of luteinizing hormone on ovarian progesterone production and plasma progesterone also decreases.

In this experiment the preovulatory increase in plasma luteinizing hormone concentration did not precede the increase in progesterone. The results also showed that plasma progesterone reached its peak concentration later than luteinizing hormone in both the traditional- and male-line turkeys. The progesterone peak outlasted the luteinizing hormone peak although this difference was not significant. The results support the hypothesis that progesterone initially stimulates luteinizing hormone release which in turn stimulates further progesterone release resulting in ovulation.

The ovary is a major source of progesterone and oestradiol, with the largest follicles predominately producing progesterone while the smaller follicles and residual ovary predominately produce oestrogens (Armstrong 1984; Porter *et al.* 1991a). It was therefore surprising that the male-line turkeys, which have many more mature follicles and a much bigger residual ovary, do not have higher concentrations of progesterone and oestradiol compared to the traditional-line. The results suggest that the steroidogenic capacity of the ovarian cells may be reduced in the large ovary of the male-line. A reduction in the steroidogenic capacity of the ovarian cells may result in a loss of negative feedback to the hypothalamus and anterior pituitary that could cause over-stimulation of ovarian growth and follicular recruitment resulting in the multiple follicular ovary.

As previously discussed progesterone production by preovulatory follicles is believed to initiate the events leading to ovulation. A reduction in the progesterone output from the largest follicles may reduce the strength of this signal and delay ovulation. Perhaps several follicles of the largest size are required to produce sufficient progesterone to trigger ovulation in the male-line strain. If this were the case then selection may have inadvertently resulted in the development of the multiple follicular hierarchy.

Decreased steroidogenesis may therefore have resulted in the multiple ovulating condition by at least two different routes, via over stimulation of ovarian growth due to a lack of control by negative feedback, and through steroid output from several follicles being required for ovulation. If these hypotheses are substantiated then future research into methods of controlling multiple ovulations should focus on the interaction between selection for high growth rate and the steroidogenic capacity of ovarian tissue. Ovarian steroidogenesis in traditional-line and male-line turkeys is investigated in Chapter 6.

6. Comparison of ovarian steroid hormone production in multiple ovulating male-line and single ovulating traditional-line turkeys

INTRODUCTION

Male line turkeys, that have been selected for high meat yield have large ovaries with many maturing ovarian follicles, compared to traditional, unselected turkeys and female lines that have been selected for egg production (Hocking 1992b; Melnychuk *et al.* 1994; Hocking and Bernard 1998; Melnychuk *et al.* 1997).

The small ovarian follicles and ovarian stroma are the main source of oestradiol in hens (Senior and Furr 1975) and they contain about 50% of the total ovarian aromatase activity (Armstrong 1984). Oestradiol production by small white follicles has also been demonstrated in turkeys (Porter *et al.* 1989b; Porter *et al.* 1991b; Porter *et al.* 1991c).

The hierarchical follicles of hens have been shown to produce progesterone (Yu *et al.* 1992b). The granulosa cells are the source of progesterone (Huang and Nalbandov 1979) and production is greatest in cells from the largest pre-ovulatory follicle (Huang *et al.* 1979). Theca cells, collected from the second and third largest pre-ovulatory follicles in hens were found to produce oestrogen and testosterone which lead to the hypothesis of a 2 cell model for ovarian steroidogenesis, where the granulosa cells produce progesterone, which is then converted to testesterone and oestrogens by the theca cells (Huang *et al.* 1979; Marrone and Hertelendy 1983a).

Porter et al (1989a) investigated steroidogenesis in the granulosa, theca externa and theca interna cell layers from turkey ovarian follicles and proposed the 3 cell model of steroidogenesis, where progesterone produced by the granulosa cells was converted to testosterone by theca interna cells, and subsequently to oestradiol by theca externa cells. The same characteristics of the different cell types have since been demonstrated in domestic hens (Rodriguez-Maldonado *et al.* 1996) which supported the 3 cell theory of ovarian steroidogenesis.

As follicles mature their steroid hormone output changes from predominantly producing oestradiol to mainly producing progesterone (Marrone and Hertelendy 1985; Robinson and Etches 1986; Porter *et al.* 1991a). It is generally accepted that the largest preovulatory follicle produces the greatest amount of progesterone, and that oestradiol production declines as the follicles mature. However the male-line ovary has many maturing follicles, which appear to be arranged as double and triple follicles at each position in the hierarchy according to their individual weights. The steroid output from single and multiple follicular hierarchies has not previously been investigated.

In Chapter 5 it was shown that although the male-line turkeys have many more maturing ovarian follicles and a much larger residual ovary, they do not have higher plasma concentrations of oestradiol or progesterone compared to traditional-line turkeys. In fact plasma oestradiol concentration was significantly lower in the male-line compared with the traditional-line (Chapters 3, 4 & 5). This led to the suggestion that ovarian steroidogenesis may be reduced in male-line turkeys.

An experiment was carried out to compare the output of progesterone and oestradiol from the follicular hierarchy and small ovarian follicles in traditional- and male-line turkeys. The aim of the experiment was to examine the hypothesis that steroidogenesis was reduced in the male-line and to evaluate the effect of the multiple follicular hierarchy on the hormone output from the different follicles of the ovary.

Plasma luteinizing hormone stimulates ovarian hormone production in turkeys and domestic hens (Huang *et al.* 1979; Hammond *et al.* 1981a; Asem *et al.* 1983; Calvo and Bahr 1983). The responsiveness of the follicles to luteinizing hormone would therefore influence their steroid hormone production, and it is possible that the sensitivity of the ovarian follicles to luteinizing hormone varies between the two strains. Luteinizing hormone stimulates oestradiol production by small ovarian follicles in both hens (Wells *et al.* 1985; Robinson and Etches 1986) and turkeys

(Porter *et al.* 1991c). Asem *et al.* (1983) showed that turkeys were less responsive to luteinizing hormone stimulation than chickens and hypothesised that there may be a connection between reduced sensitivity to luteinizing hormone stimulation and low egg production. A second experiment was carried out to measure the increase in oestradiol output from the small follicles in response to luteinizing hormone in traditional- and male-line turkeys to compare their sensitivity to luteinizing hormone stimulation.

Bacon *et al.* (1980) showed that turkey plasma contained oestrone and another unidentified oestrogen and Lien *et al.* (1989) measured oestrone production from turkey ovarian theca cells and small white follicles. It is possible that oestradiol is not the main active oestrogen in male-line turkey plasma. The ovary of the male-line could be producing large quantities of oestradiol that is subsequently metabolised to another oestrogen, which is not detected by the oestradiol radioimmunoassay used in Chapters 3, 4 and 5. If this is occurring then high plasma total oestrogen concentrations could be involved in predisposing the male-line to prolapse of the oviduct.

A third experiment was carried out to measure the aromatase activity in the small ovarian follicles and residual ovary, using the assay described by Armstrong (1984; 1985). The assay was based on the knowledge that conversion of testosterone to oestradiol by aromatase results in the release of water. If 1β,2β-^{3}H testosterone is provided in the incubation medium then tritiated water is produced in proportion to the amount of testosterone converted to oestradiol. The production of oestrogens could therefore be compared between the traditional-line and the male line using this assay. If the male-line has a greater aromatase activity than the traditional-line this would support the hypothesis that the male-line turkeys were metabolising oestradiol into another active oestrogen that was not measured by the oestradiol radioimmunoassay.

METHODS

The Big 6 male-line and the traditional Nebraska spot strains were used in all three experiments. They were housed and reared as described in 2.1.

Experiment 1

Female turkeys from the two strains were arranged in twelve identical pens, in a randomized design of six blocks, with each pen containing 4-6 turkeys. For the first experiment two turkeys were randomly selected from each pen, six weeks after photostimulation. Each turkey was killed between 9 am and 12 noon, as described in 2.2. The total body weight was recorded and the ovaries were removed and washed in 0.01M phosphate buffered saline (Sigma Chemical Company, Poole, UK).

All ovarian follicles greater than 0.5g were classed as hierarchical follicles (2.4) and their individual weights were recorded. Any grossly atretic follicles were discarded. Each follicle from the hierarchy was placed in an individual polystyrene pot with incubation medium. The amount of medium used for each follicle was determined by the follicle weight as shown on Table 6.1 after a small trial to ensure that the volume of medium and size of container would ensure the follicle was free to move and completely covered by the medium.

The small follicles less than 0.5g were classified into 3 types, based on the classification used for domestic hens by Lee and Bahr (1994) but altered to account for the slightly larger size of turkey ovarian follicles. The classifications used were small white follicles, 1-3mm in diameter, large white, 3-5mm in diameter and small yellow, 5-8mm in diameter. Four follicles from each class were selected from each ovary and placed in a 5ml plasma tube containing 4ml incubation medium. The incubation medium used for all the follicles was Medium 199 (pH 7.2) containing 10mM HEPES and 0.1% bovine serum albumin (all purchased from Sigma Chemical Company, Poole, UK)

TABLE 6.1 Volume of incubation medium used for different weights of hierarchical follicles (>0.5g)

Follicle weight (g)	Volume of medium (ml)
0.5- 5.0	5
5.1-10.0	10
10.1-15.0	15
15.1-20.0	20
20.1-25.0	25
25.1-30.0	30

Each follicle was incubated for 3 hours at 39°C in a shaking water bath. At the end of the incubation period the pots were immediately placed on ice and the medium was removed and stored at –20°C. The oestradiol and progesterone concentration of the incubation medium was subsequently measured by radioimmunoassay as described in 2.6.1 and 2.6.2 respectively. The oestradiol extraction procedure was not necessary, as there were no lipids present in the incubation medium.

In a separate experiment the density of ovarian follicles was determined by measuring the weight and volume of individual follicles. Six male-line turkeys were killed five weeks after photostimulation and 12 follicles of various sizes were collected from each turkey. Each follicle was weighed and its volume was measured by placing the follicle in a measuring cylinder containing a known quantity of water and recording the volume of water displaced by the follicle. Ovarian follicle density was then calculated from the follicle weight and volume. The mean value for follicle density was used to calculate the volume and subsequently the surface area from the follicle weight of the individual follicles incubated, using the following equations;

$$Volume = mass/density$$

$$Radius^3 = 3 \times volume/4\pi$$

$$Surface\ area = 4\pi \times radius^2$$

Analysis of variance was used to test for effects of block, strain and follicle size on the steroid hormone output and to test the differences in total hormone output

between the two strains. The hormone output values were converted to natural logarithms prior to analysis to ensure normal distribution of the residuals.

Experiment 2

The second experiment was carried out seven weeks after photostimulation. Twelve male-line and 24 traditional-line turkeys were selected from the same twelve pens as used in the previous experiment. The turkeys were killed between 10am and 12 noon as described in 2.2. Each turkey was weighted, then the ovary was immediately removed and the number of hierarchical follicles (<0.5g) was recorded. The post ovulatory follicles were removed. The residual ovary was weighed and rinsed in 0.01M phosphate buffered saline. The small white follicles (1-3mm) were selected for this experiment as they are the most abundant follicle classification. Twenty small white follicles were harvested from each male-line turkey, while two traditional turkeys from the same block were paired together and 10 follicles collected from each bird were pooled.

Four follicles from each male-line turkey and pair of traditional-line turkeys were placed into 5ml plasma tubes with 4mls incubation medium containing 0, 1, 10, 100 or 1000 ng/ml ovine luteinizing hormone (Sigma Chemical Company, Poole, UK). Ovine luteinizing hormone is commonly used with success in avian experiments as neither turkey or chicken luteinizing hormone preparations are available. As before, the incubation medium used was Medium 199 (pH 7.2) with 10mM HEPES and 0.1% bovine serum albumin (Sigma Chemical Company, Poole, UK). The plasma tubes were then incubated in a water bath at 39∘C for 2 hours. At the end of the incubation period the tubes were places on ice and samples of the incubation medium were removed and stored at -20∘C. The oestradiol concentration of each sample of incubation medium was measured as described in 2.6.1 without prior sepharose extraction.

Regression analysis was used to test for strain and dose differences in oestradiol output from the small follicles.

Experiment 3

In the third experiment female turkeys from each strain were housed in 12 pens, with four turkeys in each pen, and reared as described in 2.1. Nine weeks after photostimulation two turkey from each strain were randomly selected from each pen. Each turkey was blood sampled and killed between 10am and 12 noon as described in 2.2. The body weight was recorded and the ovary was removed and rinsed in 0.01M phosphate buffered saline. The hierarchical follicles (>0.5g) were removed and counted. All the small ovarian follicles greater than 2mm were removed and classified into three groups, small white (2-3mm), large white (3-5mm) and small yellow (5-8mm). The number of follicles in each classification was recorded and 4 follicles from each group were selected for incubation. Each follicle was incubated individually in plasma tubes containing 4mls incubation medium.

The remainder of the ovary was divided into anterior and posterior sections at the site of the attachment of the ovarian stalk (Waddington and Walker 1988) and the two sections were weighed separately. All post ovulatory follicles were removed from both parts of the ovary and counted. Duplicate samples of anterior and posterior ovarian stroma (200-250mg) were removed and their weights were recorded. Each sample was placed in 4mls medium (250mmol/l sucrose, 1mmmol/l EDTA and 20% w/v glycerol) and homogenised for three passes of 10 seconds each. Samples were then centrifuged for 10 minutes at 600g and duplicate samples 100µl of supernatant were removed and placed in 4mls incubation medium.

All incubations from the third experiment were carried out in Medium 199 (pH7.2) containing 10mM HEPES and 0.1% BSA. Tritiated testosterone (1β,2β-^{3}H(N) testosterone, NEN Life Science Products, Inc. Boston, MA, USA) and testosterone (Sigma Chemical Company, Poole, UK) were added to the medium to achieve a final concentration of testosterone of 0.5µmol/l and a specific activity of 18.5 MBq/l. The incubations were carried out in a shaking water bath at 39°C for 2 hours.

At the end of the incubation period the samples were immediately placed on ice. Duplicate 500µl samples of medium were transferred to 1.5ml eppendorf tubes and 200µl of charcoal/water solution (5% charcoal w/v, Sigma Chemical Company, Poole, UK) was added. The tubes were placed on ice for 1 hour. Each tube was centrifuged for 15 minutes at 1200g and 300µl supernatant was transferred to a scintillation vial containing 3mls scintillation cocktail (Optiphase Hisafe 3, Fisher Scientific UK, Loughborough, UK). The amount of radioactivity present was counted using a liquid scintillation counter (Wallace 1410 Liquid Scintillation Counter, EG&G Wallac, Milton Keynes, UK). Control incubations, without any follicles or tissue, were also processed to calculate the amount of radioactivity carried over throughout the assay procedure and this was subtracted from the results. The total amount of radioactivity available was determined by measuring the amount of activity in 300µl of incubation medium containing tritiated testosterone.

The amount of oestradiol produced during the 2 hour incubation period was calculated by the following equation:

$$Oestradiol=((counts\ in\ sample - background\ counts) \times dilution\ factor / counts\ per\ nmol\ testosterone) \times formula\ weight\ of\ oestradiol$$

The blood samples were treated as described in 2.2. At a later date plasma oestradiol concentration was measured by radioimmunoassay after antibody-sepharose extraction, as described in 2.6.1.

Analysis of variance was used to test the effects of block, strain and follicle or ovarian tissue type on the aromatase activity, and to test the difference in total ovarian aromatase activity and activity/gram body weight between the two strains.

RESULTS

Experiment 1

The male-line turkeys had a greater body weight, a heavier residual ovary and a greater number of hierarchical follicles (>0.5g) than the traditional-line (Table 6.2). This is consistent with the results of previous experiments (Chapters 4 and 5).

TABLE 6.2 Mean body weight, residual ovary weight and number of hierarchical follicles (>0.5g) in traditional- and male-line turkeys

	Traditional-line	Male-line	SED	Significance
Body weight (g)	5.6	19.5	0.36	***
Residual ovary weight (g)	7.0	27.3	1.73	***
Follicle number	7.3	17.2	0.74	***

The mean follicle density, calculated from follicle weight and volume was 1.026±0.007mg/mm^3 (n=72). There was no significant difference in follicle density between the different turkeys (n=6). Regression analysis showed that follicle weight had no significant effect on follicle density. Yu *et al.* (1992b) used an egg yolk density of 0.781mg/mm^3 to calculate follicle surface area from follicle weight. A one-sample t-test showed that the measured density of 1.026mg/mm^3 for turkey follicles was significantly different (P<0.001) from the value used by Yu and co-workers, and the former was used to calculate follicular surface area in this experiment.

The results of the first experiment are presented as the total hormone output over the three hour incubation period.

The output of oestradiol from each individual follicle in the hierarchy (<0.5g) for the two strains is shown in Figure 6.1. with the follicles numbered in order by weight starting at number 1 for the heaviest follicle. Figure 6.2 shows the progesterone output from the follicular hierarchy presented in the same arrangement. It should be noted that as the number of follicles in the hierarchy varied for each individual turkey the number of samples contributing to each data point declines after follicle

numbers 4 and 8 respectively for the traditional- and the male-line. Due to the different numbers of mature yellow follicles from each bird it was not possible to test the significance of the results using analysis of variance.

The results presented on Figure 6.1 showed that the oestradiol output initially increased then decreased as the follicle number increased. The oestradiol output was greatest from the fourth largest follicle of the traditional-line and the sixth largest follicle of the male-line. The progesterone output from each follicle decreased as the follicle number increases (Figure 6.2) in both the traditional-line and the male-line. In the traditional line most of the progesterone was produced by the first and second largest follicles, while in the male-line the progesterone output was high from the four largest follicles.

As the theca and granulosa cells are located at the periphery of the follicles (Etches 1996d) the surface area of the follicles reflects the number of steroidogenic cells present. The surface area for each follicle was calculated from the follicle weight, using the measured value of 1.026 mg/mm^3 for follicle density. The output of progesterone and oestradiol from each follicle was divided by the surface area of the follicle to represent the cellular hormone output. Figures 6.3 and 6.4 respectively show the oestradiol and progesterone output per mm^2 follicle surface area from the individual follicles that make up the follicular hierarchy in the traditional- and male-line turkeys. As for Figures 6.1 and 6.2 the amount of data represented by each column decreased as the follicle number increased.

When the oestradiol output from the follicles was related to the number of steroidogenic cells present it was clear that the oestradiol output increased with follicle number, therefore the oestradiol production by the theca cells decreased as the follicles matured. When expressed in proportion to follicle surface area, the oestradiol output appeared to be greater in the traditional-line compared to the male-line.

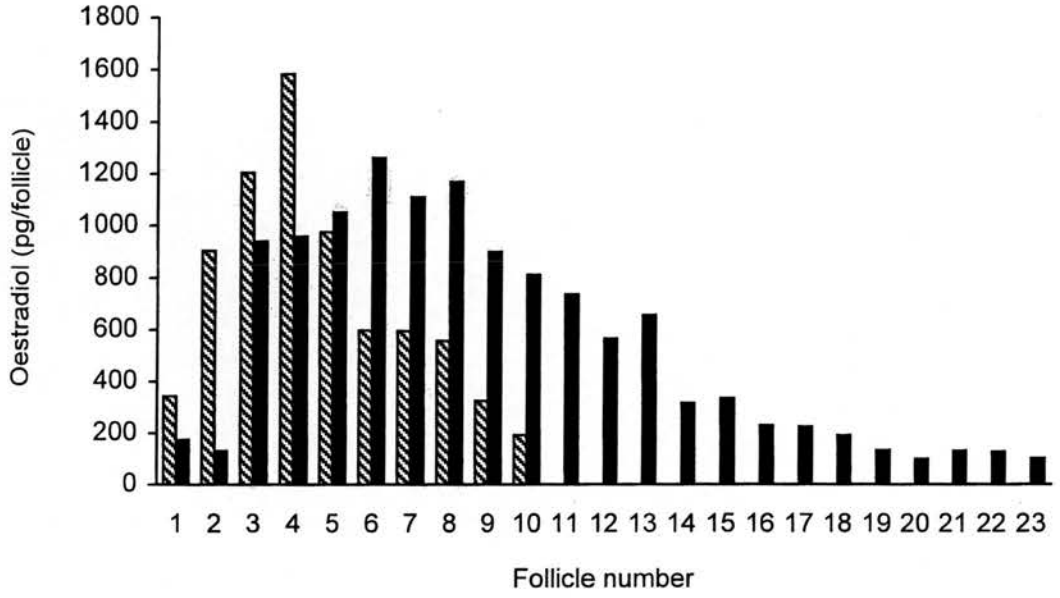


FIGURE 6.1 Oestradiol output per follicle from the follicular hierarchy arranged according to follicle weight in traditional- and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

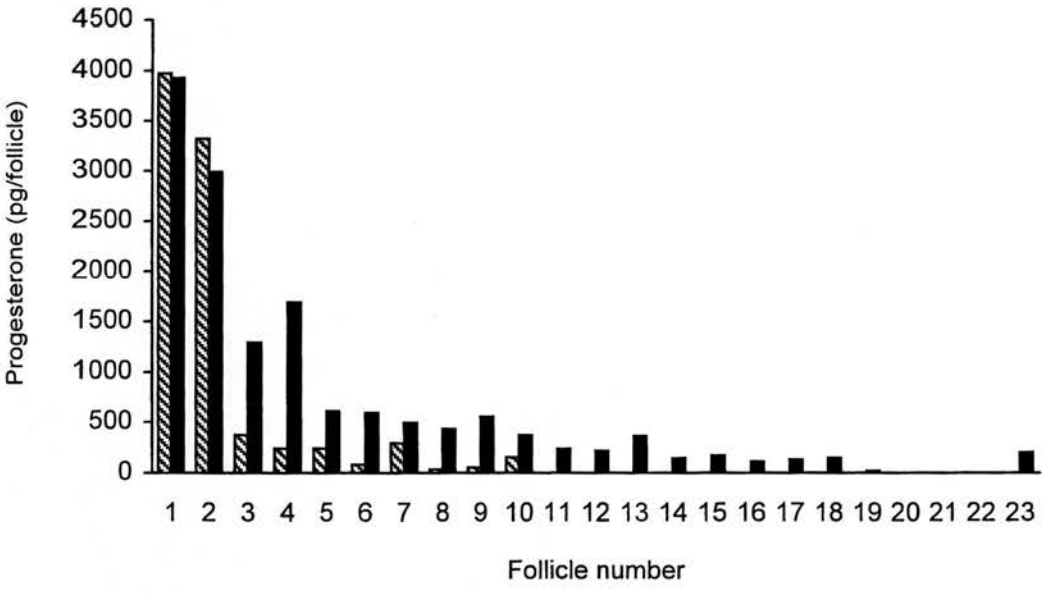


FIGURE 6.2 Progesterone output per follicle from the follicular hierarchy arranged according to follicle weight in traditional- and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

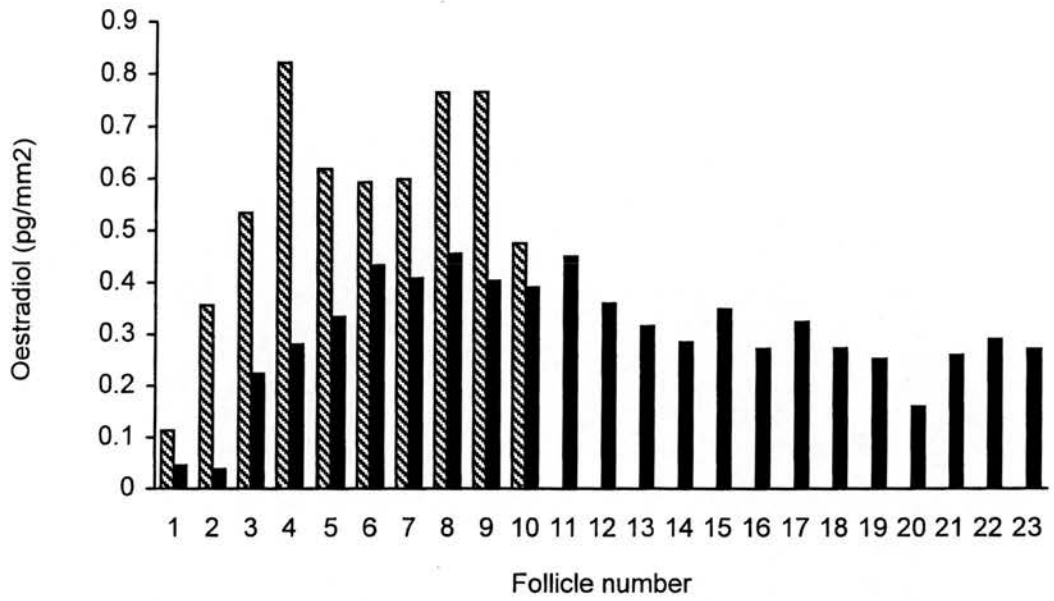


FIGURE 6.3 Oestradiol output per mm^2 follicle surface area from the follicular hierarchy arranged according to follicle weight in traditional- and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

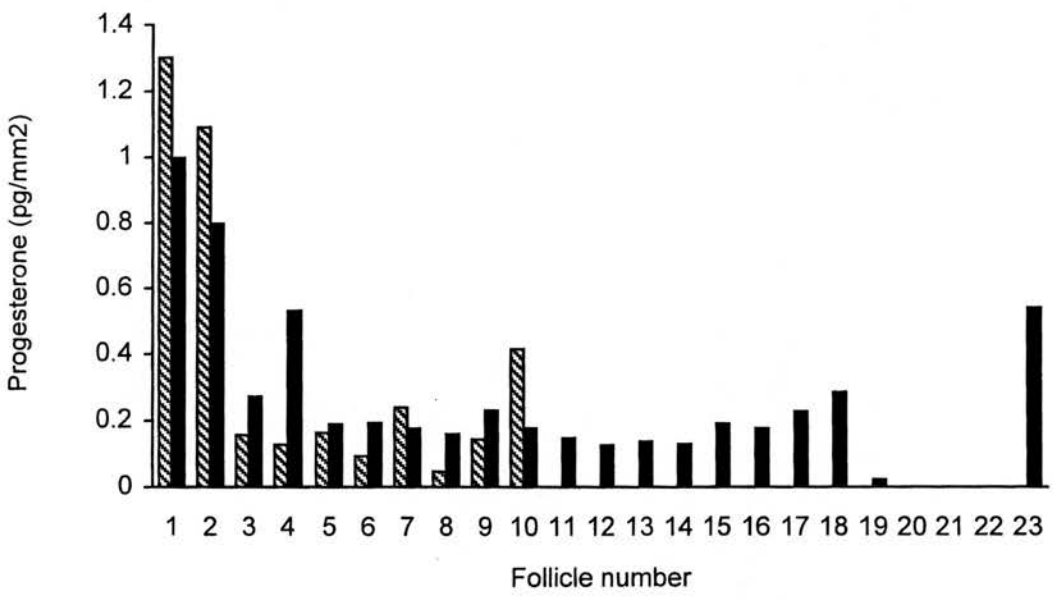


FIGURE 6.4 Progesterone output per mm^2 follicle surface area from the follicular hierarchy arranged according to follicle weight in traditional- and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

The progesterone output per mm^2 follicle surface area decreased with increasing follicle number in both strains (Figure 6.4), which showed that progesterone output increased as the follicles matured. The progesterone output from the first two follicles relative to surface area was greater in the traditional-line compared to the male-line.

The hierarchical follicles of the male-line are often arranged in groups of two or more follicles of a similar size, which are therefore thought to be at a similar stage of maturity. Based on fat soluble dye feeding experiments Hocking (1987) proposed that it takes 9-10 days for follicles to develop from recruitment into the hierarchy to ovulation. Therefore there should be no more than 10 follicles greater than 8mm (about 0.5g) in the hierarchy of turkeys laying an egg a day. To allow comparison of follicles at the same stage of maturation between the traditional- and male-lines the follicles for each individual turkey were arranged into 10 positions in the follicular hierarchy. The arrangement of the follicles into 10 positions was achieved by grouping together follicles of a similar size and leaving gaps for missing follicles where there was a substantial difference in consecutive follicle weights.

Arranging the follicles into a hierarchy of 10 positions resulted in 0.74±0.048 and 1.70±0.050 follicles at each position for the traditional- and male-line respectively, ($P<0.001$). The position in the hierarchy had no significant effect on the number of follicles at that position in either strain.

The mean follicle weight at each position in the hierarchy for the two strains is shown in Figure 6.5. There was a significant interaction between position in the hierarchy and strain on follicle weight ($P<0.001$). The follicles were heavier in the male-line compared to the traditional-line at every position in the hierarchy. The weight of the follicles decreased with increasing position in the hierarchy, which was expected as the hierarchy was arranged by follicle weight and the difference in weight between the two strains decreased with the decreasing follicle weight.

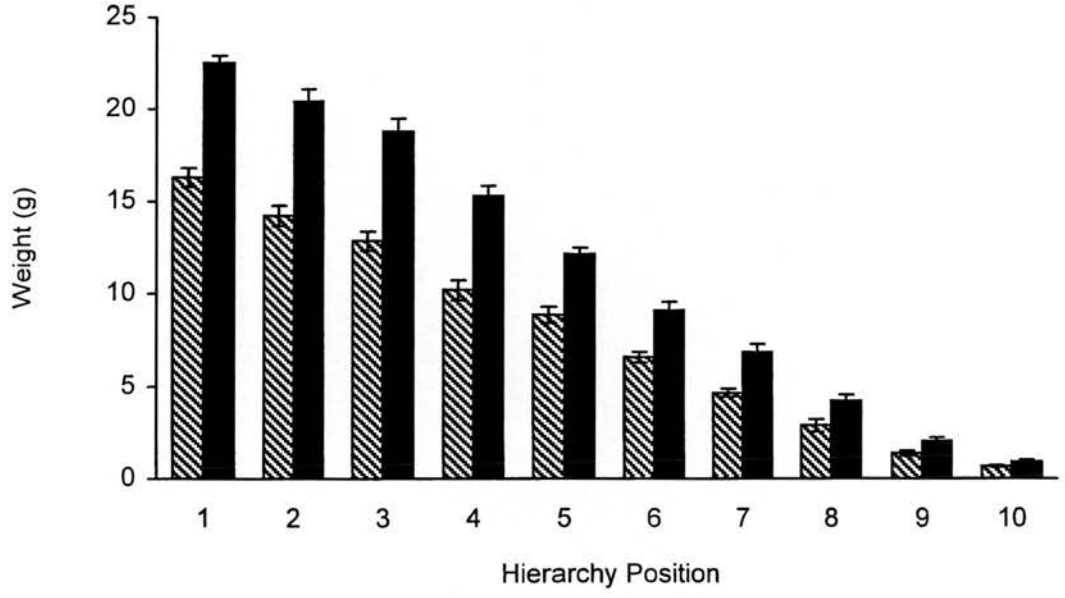


FIGURE 6.5 Mean (±SEM) follicle weight at each position in the follicle hierarchy in traditional- and male-line turkeys
Key: ▨ Traditional-line; ■ Male-line

Figure 6.6 shows the mean output of oestradiol per follicle at each of the 10 positions in the follicular hierarchy in the traditional- and male-line turkeys. There was no significant interaction between strain and hierarchy position for $\log_e$ oestradiol output. The position in the follicular hierarchy had a significant effect on the $\log_e$ oestradiol output from the follicle ($P<0.001$) and the $\log_e$ oestradiol output per follicle was significantly greater in the traditional-line compared to the male-line ($P<0.001$). The results show that the oestradiol output per follicle is greatest at positions 3-5 in the hierarchy or 3-5 days prior ovulation.

Figure 6.7 shows the progesterone output per follicle for each of the 10 positions in the follicular hierarchy. The interaction between the position in the hierarchy and strain was not significant, but hierarchy position had a significant effect on $\log_e$ progesterone output per follicle ($P<0.001$). There was also a significant effect of strain on $\log_e$ progesterone output ($P<0.001$) as the mean $\log_e$ output from the smaller follicles was greater in the male-line. In both the traditional- and male-lines the majority of the progesterone was produced by the follicles at positions 1 and 2 of the hierarchy. This shows that the main source of progesterone was the follicles that would have ovulated in the next 1 or 2 days.

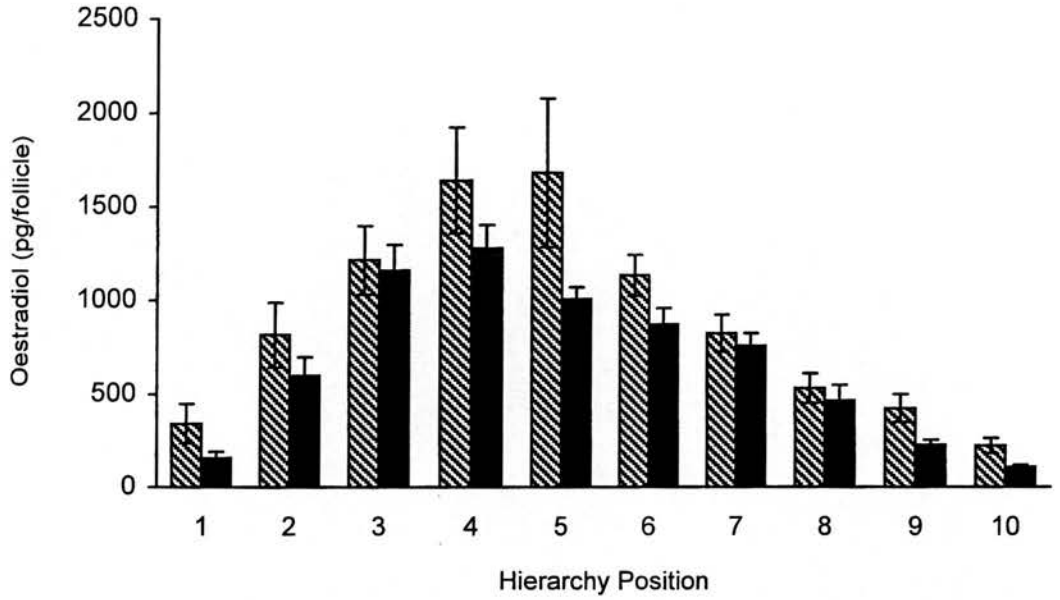


FIGURE 6.6 Mean (±SEM) oestradiol output per follicle at each position in the follicular hierarchy in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

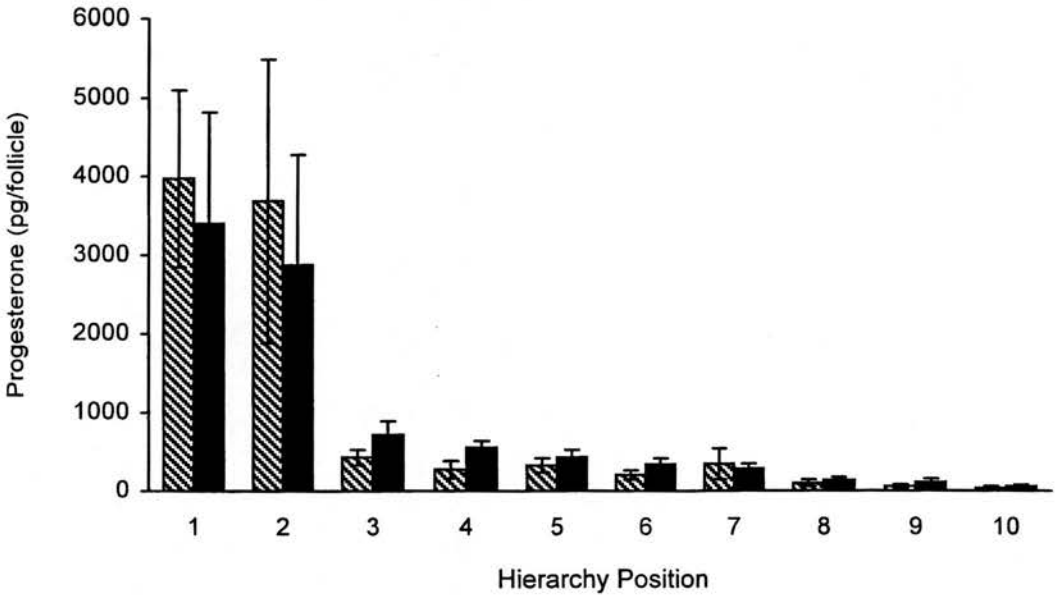


FIGURE 6.7 Mean (±SEM) progesterone output per follicle at each position in the follicular hierarchy in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

The oestradiol output per mm^2 follicle surface area for the 10 positions in the follicular hierarchy is shown on Figure 6.8. There was a significant interaction between strain and hierarchy position ($P<0.001$) as the traditional-line produced more oestradiol than the male-line at each position but the difference decreased as the follicles approached ovulation. The results show that oestradiol output/mm^2 decreased in the last 3-4 days of follicle maturation in both the traditional-line and the male-line.

Figure 6.9 shows the progesterone output per mm^2 follicle surface area for follicles at the 10 hierarchy positions in the traditional- and male-lines. The analysis of variance showed no significant interaction between strain and hierarchy position on $\log_e$ progesterone output/mm^2. There was a significant effect of position in the hierarchy on the $\log_e$ progesterone output/mm^2 ($P<0.001$), but there was no significant difference between the two strains. The progesterone output/mm^2 was greatest in follicles at the first and second positions in the hierarchy and was very low in the follicles at the other positions. The progesterone output from the follicles at positions 1 and 2 was greater in the traditional-line compared to the male-line although this was not statistically significant.

The total output of oestradiol from the follicular hierarchy of traditional- and male-line turkeys is shown on Figure 6.10. The total output was significantly greater ($P<0.001$) in the male-line compared to the traditional-line. Figure 6.11 shows the total progesterone output from the follicular hierarchy of the traditional- and male-line turkeys. There was no significant difference in total progesterone output from the hierarchy between the two strains. The male-line turkeys have larger plasma volume than the traditional-line. To account for the differences in plasma volume, the total output from the follicular hierarchy was divided by body weight. Figure 6.12 and 6.13 respectively show oestradiol and progesterone output per kg body weight. When considered relative to body weight the oestradiol output was significantly greater in the traditional-line than the male-line while there was no significant difference in progesterone output between the two strains.

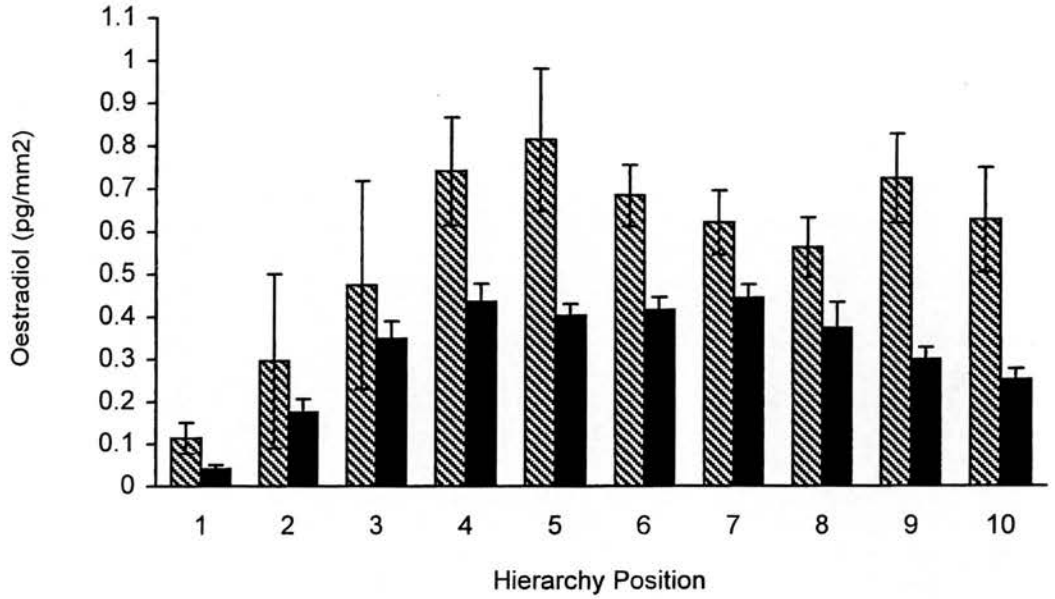


FIGURE 6.8 Mean (±SEM) oestradiol output per mm^2 follicle surface area at each position in the follicular hierarchy in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

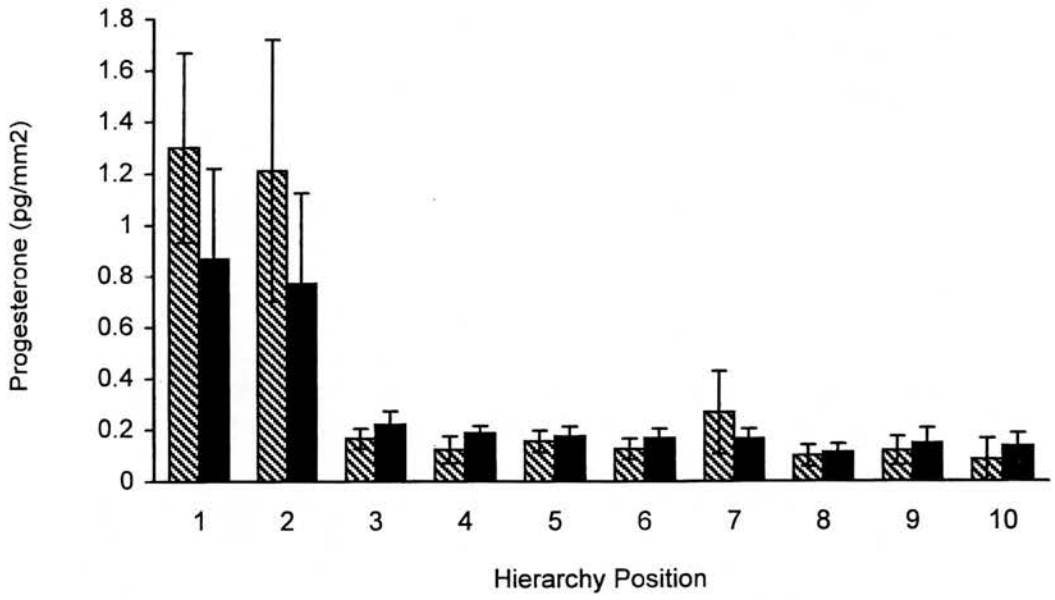


FIGURE 6.9 Mean (±SEM) progesterone output per mm^2 follicle surface area at each position in the follicular hierarchy in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

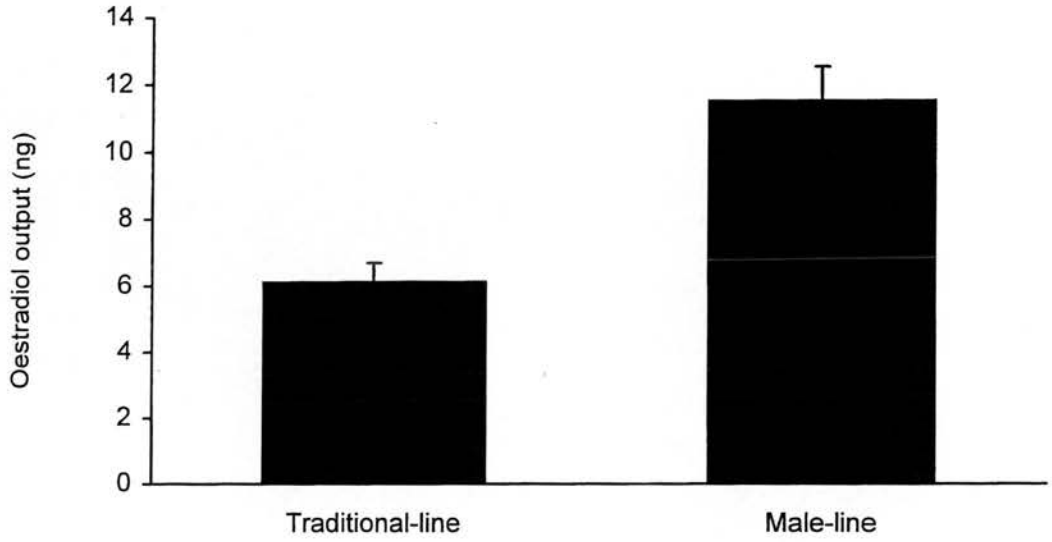


FIGURE 6.10 Mean (±SEM) total oestradiol output from the follicular hierarchy (>0.5g) in traditional-line and male-line turkeys (3 hour incubation)

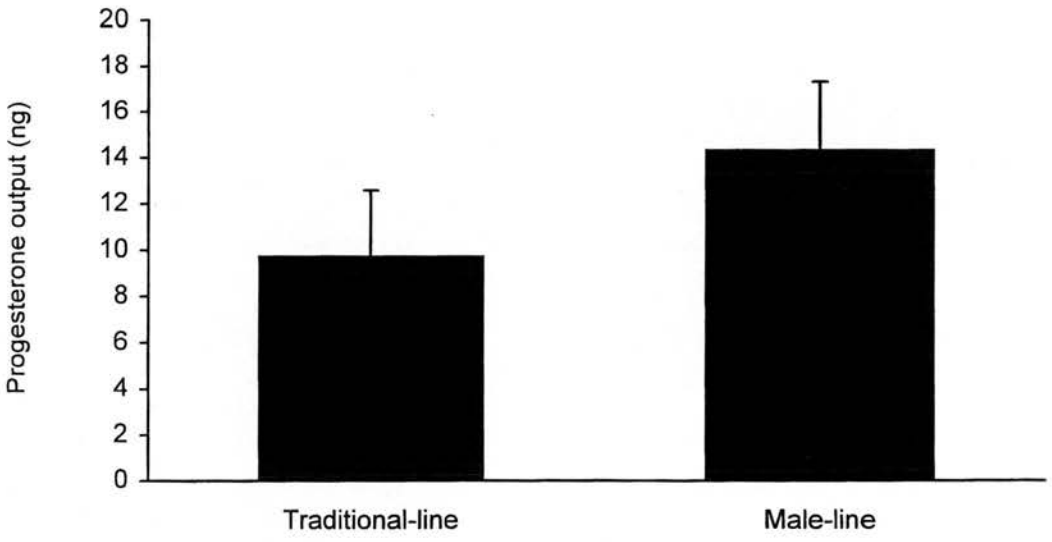


FIGURE 6.11 Mean (±SEM) total progesterone output from the follicular hierarchy (>0.5g) in traditional-line and male-line turkeys (3 hour incubation)

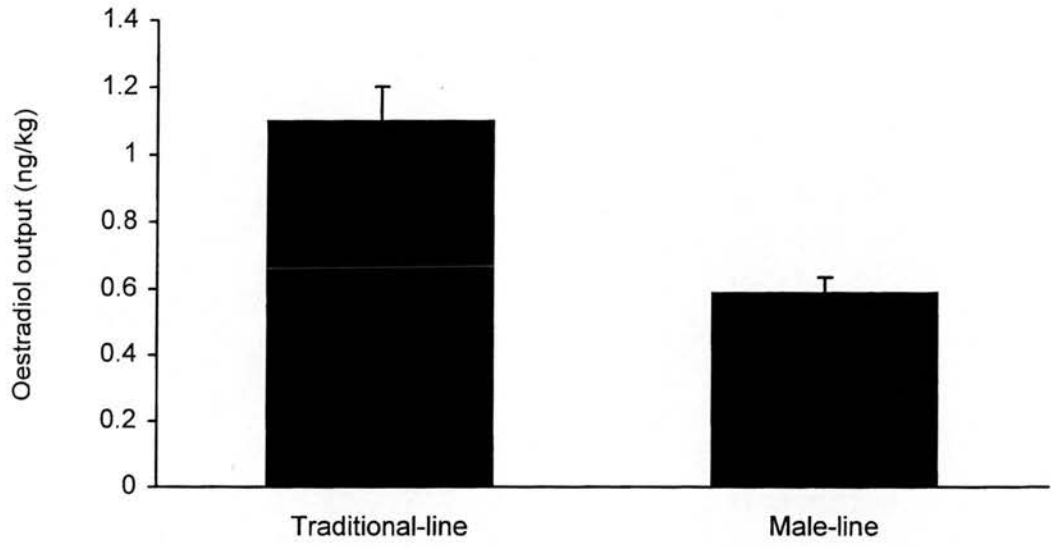


FIGURE 6.12 Mean (±SEM) total oestradiol output from the follicular hierarchy (>0.5g) relative to body weight in traditional-line and male-line turkeys (3 hour incubation)

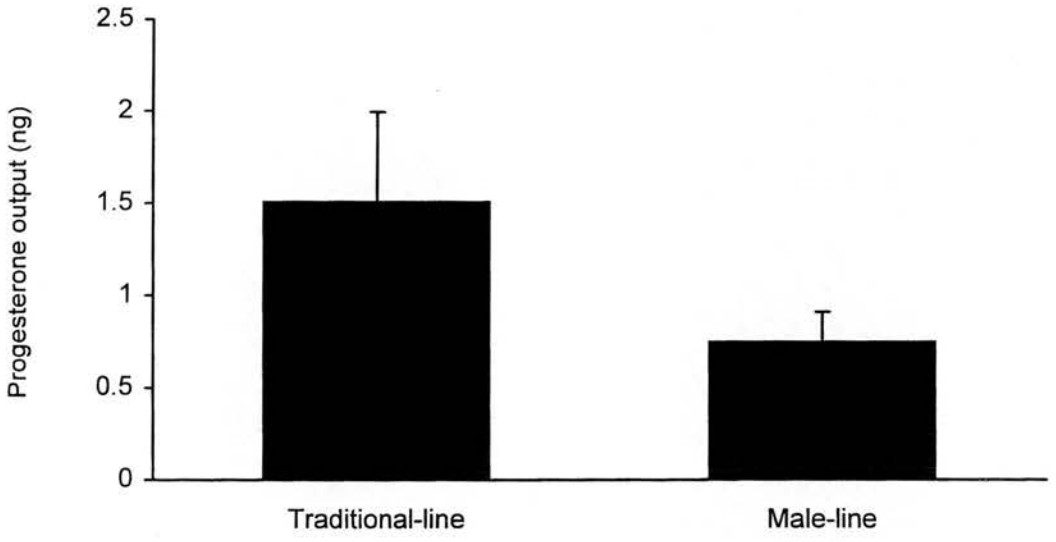


FIGURE 6.13 Mean (±SEM) progesterone output from the follicular hierarchy (>0.5g) relative to body weight in traditional-line and male-line turkeys (3 hour incubation)

The output of oestradiol from the three classes of small ovarian follicles in the two strains is shown in Figure 6.14. There was no significant interaction between strain and follicle class on $\log_e$ oestradiol output. There was a significant effect ($P<0.001$) of follicle classification on $\log_e$ oestradiol output as oestradiol output increased as follicle diameter increased. The output of oestradiol was significantly greater ($P<0.001$) from the small follicles of the traditional-line compared to the male line.

Figure 6.15 shows the progesterone output from the same three groups of small ovarian follicles for the two strains. There was no significant difference in $\log_e$ progesterone output between the different sizes of small follicle or between the traditional-line and the male-line. The progesterone output from the small ovarian follicles was very low in both strains in comparison to the progesterone output from the hierarchical follicles or relative to plasma progesterone concentrations.

Experiment 2

The mean values for total body weight, hierarchical follicle number and residual ovary weight of the traditional- and male-line turkeys used in this experiment are given on Table 6.3. Body weight, residual ovary weight and mature follicle number were all significantly greater in the male-line, as observed in previous experiments.

TABLE 6.3 Mean body weight, residual ovary weight and number of hierarchical follicles (>0.5g) in traditional- and male-line turkeys

	Traditional-line	Male-line	SED	Significance
Body weight (g)	5.3	19.0	0.28	***
Residual ovary weight (g)	6.5	24.9	1.09	***
Follicle number	7.5	18.7	0.63	***

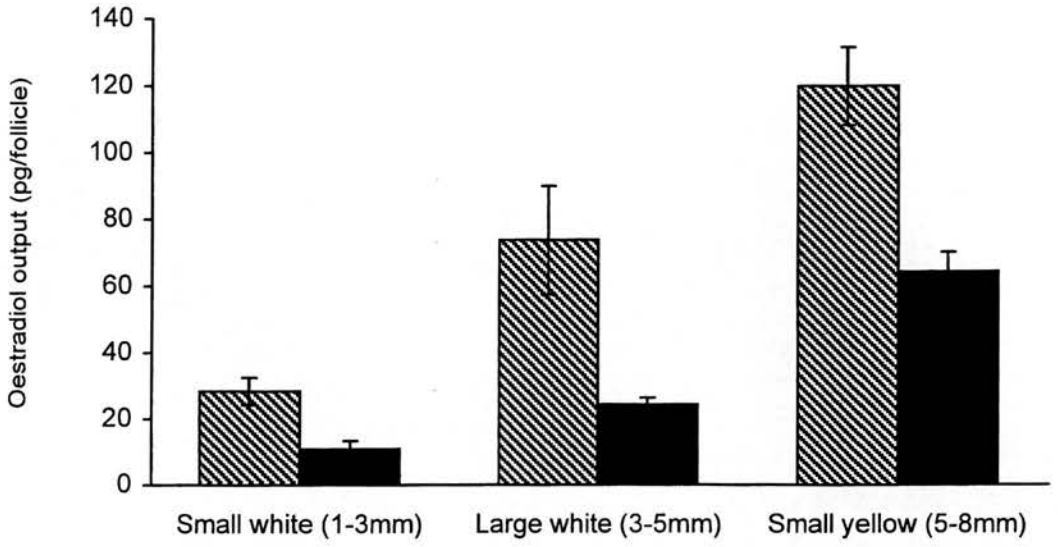


FIGURE 6.14 Mean (±SEM) oestradiol output per follicle from three classes of small ovarian follicles in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

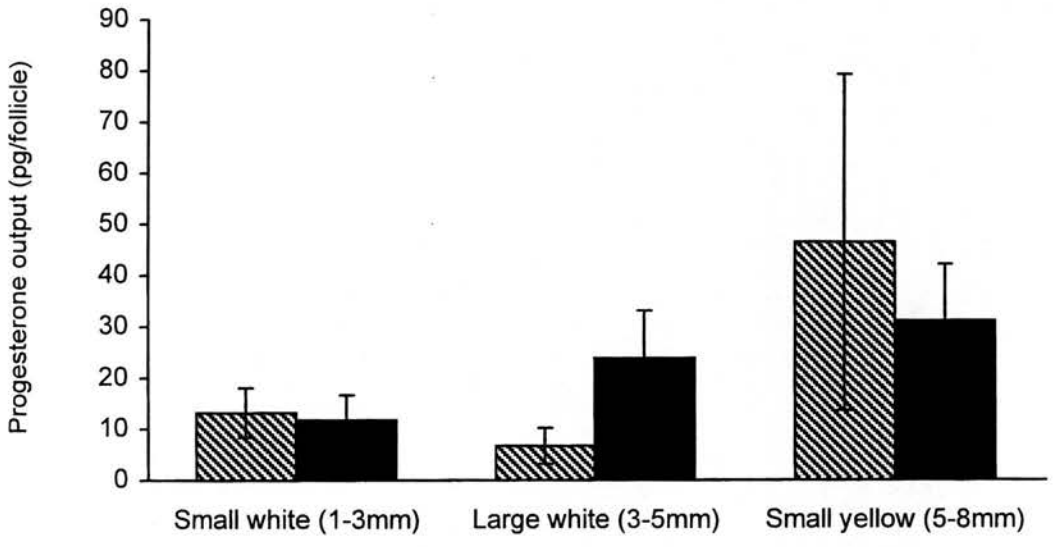


FIGURE 6.15. Mean (±SEM) progesterone output per follicle from three classes of small ovarian follicles in traditional-line and male-line turkeys (3 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

Figure 6.16 shows the oestradiol output from the small white follicles in response to stimulation by ovine luteinizing hormone, over the two hour incubation period, with the concentration of luteinizing hormone expressed on a logarithmic scale. There was a significant effect of both dose ($P<0.01$) and strain ($P<0.05$) on oestradiol output in response to luteinizing hormone, with output increasing in response to increasing concentrations of luteinizing hormone, and the traditional-line showing a greater response than the male-line. There was no significant interaction between dose and strain.

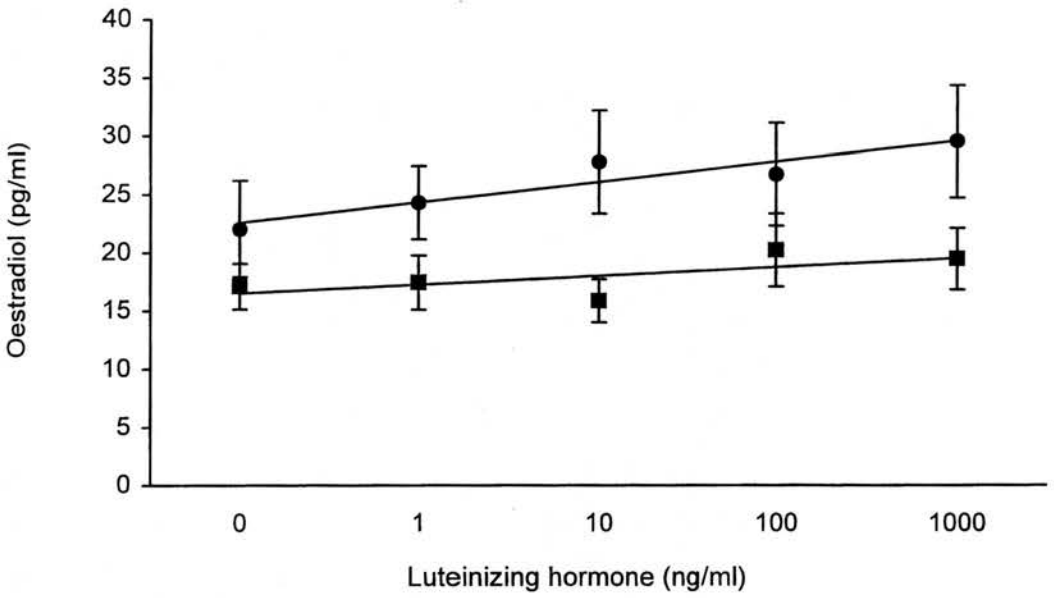


FIGURE 6.16 Oestradiol output from small white follicles in response to increasing concentrations of ovine luteinizing hormone in traditional- and male-line turkeys (2 hour incubation)
Key: ● Traditional-line; ■ Male-line

Mean body weight of the turkey used in this experiment were 5.4±0.09kg and 18.4±0.43kg respectively for the traditional- and male-lines.

The aromatase activity in the three different classifications of small ovarian follicles investigated is shown on Figure 6.17. The small follicles from the traditional-line had significantly greater aromatase activity than those from the male-line (TL = 43.9 pmol/follicle, ML = 35.8 pmol/follicle, SED = 3.92; $P<0.001$). There was no significant difference in the aromatase activity from the different classifications of small ovarian follicles or interaction between strain and follicle classification.

The aromatase activities from the anterior and posterior sections of the ovarian stroma are shown on Figure 6.18. There was no significant difference in aromatase activity between the anterior and posterior sections of ovarian stroma. There was also no significant difference in the activity of the stroma between the traditional- and male-line.

The total ovarian aromatase activity from the residual ovary (small follicles <0.5g and ovarian stroma) is presented on Figure 6.19. There was significantly more aromatase activity in the male-line compared to the traditional-line ($P<0.001$). The total ovarian aromatase activity per kg body weight is shown in Figure 6.20. When aromatase activity was considered relative to body weight there was no significant difference between the traditional- and male-line turkeys.

Plasma oestradiol concentration was significantly lower in the male-line (179.2 pg/ml) compared to the traditional-line (206.3pg/ml); SED=9.82, $P<0.05$.

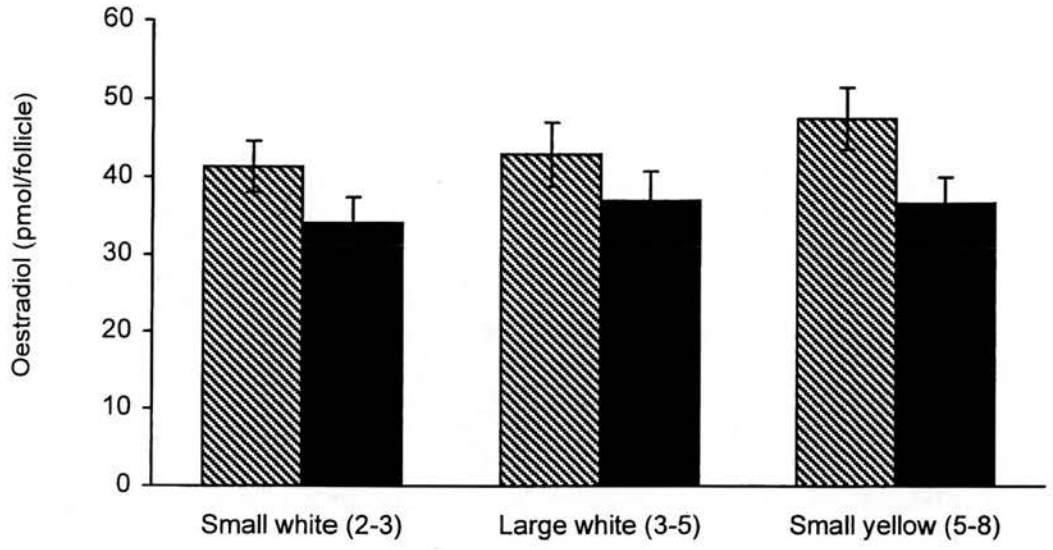


FIGURE 6.17 Mean (±SEM) aromatase activity per follicle from three classes of small ovarian follicles in traditional-line and male-line turkeys (2 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

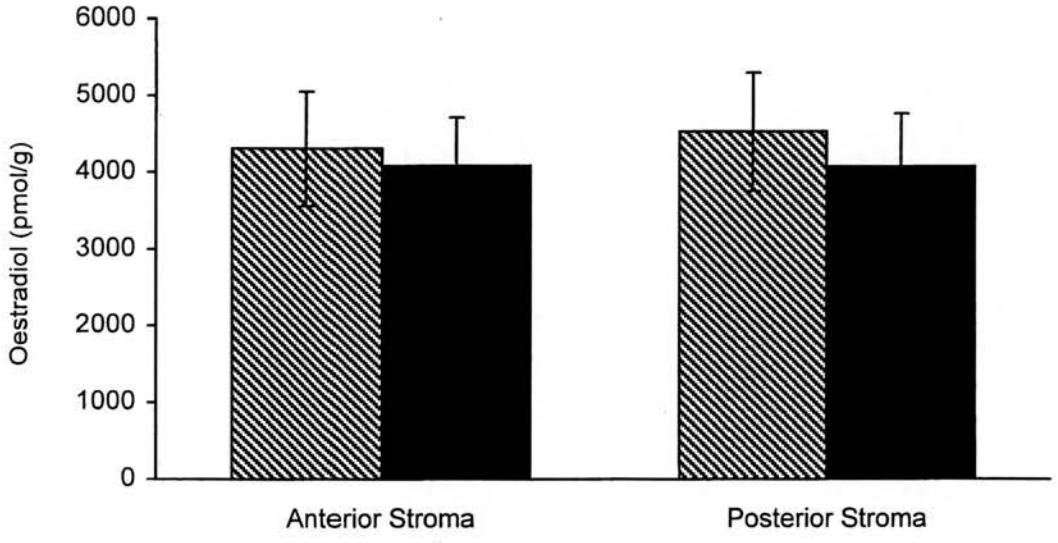


FIGURE 6.18 Mean (±SEM) aromatase activity from the anterior and posterior ovarian stroma in traditional-line and male-line turkeys (2 hour incubation)
Key: ▨ Traditional-line; ■ Male-line

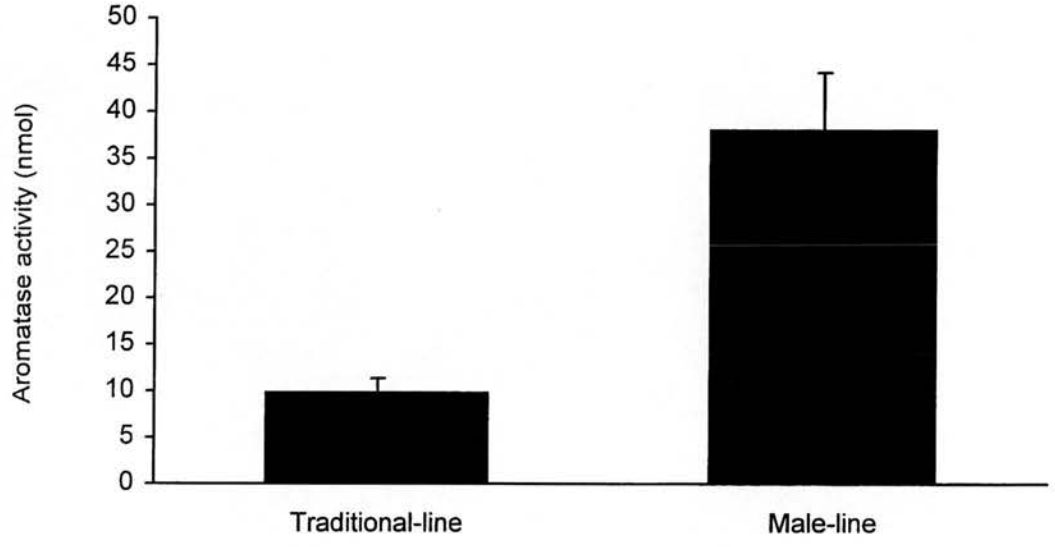


FIGURE 6.19 Mean (±SEM) aromatase activity of the residual ovary in traditional-line and male-line turkeys (2 hour incubation)

FIGURE 6.20 Mean (±SEM) aromatase activity of the residual ovary per kg body weight in traditional-line and male-line turkeys (2 hour incubation)

DISCUSSION

The results of Experiment 1 showed that follicular oestradiol output decreases as the follicles mature while the majority of progesterone is produced by the two largest follicles in the hierarchy. These results are similar to previous observations in turkeys (Porter *et al.* 1991a) and domestic hens (Marrone and Hertelendy 1985; Robinson and Etches 1986).

Yu *et al.* (1992b) incubated whole intact hierarchical follicles from domestic hens and compared the hormone outputs with those previously measured from cellular preparations. They concluded that whole large follicle incubations were a reliable method of investigating follicular steroid production without the complexity of separating the different cellular components and with the advantage that normal cellular interactions could continue.

The output from the hierarchical follicles measured in Experiment 1 were comparable to those measured by Yu *et al.* (1992b) with the exception of the progesterone output from the largest preovulatory follicle, which was much greater in the domestic hen. The progesterone output from all the follicles was very variable, as reflected by the large standard errors of the means. Plasma progesterone peaked at almost 3 times its baseline concentration prior to ovulation (Chapter 5). It is likely that this is due to a pre-ovulatory increase in follicular progesterone output.

Johnson *et al.* (1987) incubated the granulosa and theca layers together in the presence of luteinizing hormone. They found greater progesterone production from F1 follicles removed from domestic hens 8 hours prior to predicted ovulation compared to 32 hours prior to predicted ovulations. The high variability of progesterone is therefore probably due to the individual turkeys being at different stages in their ovulatory cycle. It was not possible to take this into account in the experimental protocol due to the low and variable egg production of the turkeys.

In Experiment 1 the second largest follicles also produced a considerable amount of progesterone, whereas Yu *et al.* (1992b) found the second largest follicle of domestic hens produced very little progesterone. These results suggest that, in turkeys, the follicles predominately produce progesterone for the final two of days of their maturation.

When the output of oestradiol and progesterone were compared between the traditional- and male-line, it was apparent that the multiple follicular hierarchy of the male-line was associated with a change in the pattern of steroidogenesis in the follicular hierarchy. In the traditional-line oestradiol output starts to decrease in the fourth largest follicle, while in the male-line it decreases from the sixth to eighth largest follicles. The majority of the progesterone production in the traditional-line was from the two largest follicles while in the male-line the four largest follicles all produced considerable amounts of progesterone. These results suggest that the multiple follicular hierarchy of the male-line is physiological as well as anatomical, with follicles of a similar weight being at a similar stage of maturation as shown by their similar steroidogenic properties.

In a review of the ovulatory cycle of the hen Etches (1990) defined the second largest follicle in the ovarian hierarchy as being immature and unable to be ovulated when the largest follicle is ovulated. He stated that the final stage of maturation of the second largest follicle, which makes it capable of ovulation, only starts after ovulation of the largest follicle. Clearly to apply this definition to the multiple hierarchy of follicles that exists in turkeys it is necessary first to arrange the follicles into a limited number of hierarchy positions.

The suggestion that the multiple hierarchy of the male-line has a physiological basis was supported by the results observed when the follicles of both strains were arranged into a fixed hierarchy of 10 positions. The pattern of changes in oestradiol and progesterone output were similar across the hierarchy for the two strains, with oestradiol production decreasing five days before ovulation and progesterone production increasing for two days before ovulation. From these results it was

concluded that the follicles of male-line turkeys develop similarly to the traditional-line turkeys and domestic hens. The follicles predominately produce oestradiol until about five days prior to ovulation, with the main production of progesterone occurring in the final two days before ovulation. However in the male-line the follicles are usually arranged in pairs or triples, which have similar weights and steroidogenic outputs.

The follicles that make up the hierarchy are often labelled F1, F2, F3 etc, according to their size with the F1 follicle being the largest pre-ovulatory follicle (Senior and Furr 1975; Huang and Nalbandov 1979; Bahr *et al.* 1983; Etches *et al.* 1983; Robinson and Etches 1986; Johnson *et al.* 1987; Porter *et al.* 1991a; Yu *et al.* 1992b; Rodriguez-Maldonado *et al.* 1996). However if the follicles of traditional- and male-line turkeys were labelled in this way then a direct comparison of F5 follicles, for example, between the two strains would be inaccurate as the follicles would be at very different maturation states.

Clearly the F1, F2, F3... nomenclature is unsuitable for labelling the multiple hierarchy of follicles that exist in turkeys and *ad libitum* fed broiler breeders. The follicles of birds with multiple follicular hierarchies should be arranged into physiological groups according to follicles of similar weight and labelled F1.1, F1.2, F1.3, F2.1 etc, starting with the heaviest follicle.

If comparisons are to be made between follicles of single and multiple follicular strains it is necessary to set the number of positions in the hierarchy and arrange the follicles of both strains into these set positions, such as was done in the first experiment. This was not done in the work of Yu *et al.* (1992a) investigating steroidogenesis of *ad libitum* and food restricted broiler breeder females. They showed that in food restricted birds only the F1 follicle produced progesterone while significant amounts of progesterone was produced by both the F1 and F2 follicles from *ad libitum* fed birds. They suggested that this showed that maturation of steroidogenesis was abnormal in *ad libitum* fed broilers. However it is not abnormal

development of steroidogenesis that is responsible for this effect, but simply the presence of multiple follicles at the various stages of steroidogenic development.

The significant effect of strain on $\log_e$ progesterone output reflects the greater output of progesterone from the male-line follicles at positions 3-6 in the hierarchy. However the progesterone output is very low from these follicles in both strains and the difference between the strains is a consequence only of the size of the follicles. When the $\log_e$ progesterone per mm^2 follicle surface area was compared there were no significant differences between the strains.

The mean oestradiol output per mm^2 at each of the 10 positions in the hierarchy was lower in the male-line compared to the traditional-line. This suggests that steriodogenesis in the follicular hierarchy of the male-line is impaired.

Oestradiol output from the small ovarian follicles, expressed on a per follicle basis, was highest from the small yellow follicles and lowest from the small white follicles in both the traditional- and male-line turkeys, which was in agreement with findings of Lee and Bahr (1994). However the small yellow follicles are the largest of the small ovarian follicles, and it is likely that if the oestradiol output had been expressed on a cellular basis, output would have been greatest from the small white follicle. This was demonstrated by Lee and Bahr (1994) who expressed the output of oestradiol per μgram protein. The oestradiol output per follicle from the small ovarian follicles of the traditional-line turkeys was similar to the amount of oestradiol measured by Lee and Bahr in domestic hens. The male-line turkeys had a lower output of oestradiol from the small ovarian follicles, which may be due to an impairment of ovarian steroidogenesis in this strain.

Porter *et al.* (1991b) measured oestradiol output in 2-5 mm diameter small ovarian follicles from large white turkeys. The basal oestradiol output determined by them was slightly greater than that measured in Experiment 1, however they had a longer incubation period and they also cut the follicles in half prior to incubation which

could increase the accessibility of the follicular luteinizing hormone receptors and therefore increase the response to luteinizing hormone stimulation.

The low oestradiol output from both the follicular hierarchy and the small ovarian follicles of the male-line explains the low plasma oestradiol concentration found in Chapters 4 and 5. Although the male-line has a larger hierarchy of follicles and a heavier residual ovary the reduced oestradiol output from these components results in the total oestradiol output/kg body weight being significantly lower in the male line compared to the traditional-line. These results are therefore consistent with the lower plasma concentration of oestradiol observed in the male-line (Chapter 5).

The first experiment measured the follicular steroid hormone production in the absence of any additional luteinizing hormone stimulation. *In vivo* there would always be some luteinizing hormone in the plasma and the luteinizing hormone concentration is elevated for several hours prior to ovulation (Chapter 5). Plasma luteinizing hormone stimulates production of oestradiol and progesterone from the ovarian follicles (Huang *et al.* 1979; Hammond *et al.* 1981a; Asem *et al.* 1983; Calvo and Bahr 1983). The responsiveness of the follicles to luteinizing hormone would therefore influence their steroid production, and it is possible that the sensitivity of the ovarian follicles to luteinizing hormone varies between the two strains.

The second experiment investigated the possibility that the traditional- and male-lines differ in their sensitivity to luteinizing hormone. The response of the small white follicles to stimulation by luteinizing hormone was chosen as these follicles are the most abundant and therefore follicles from each bird could be incubated at various concentrations of luteinizing hormone.

The results of the second experiment showed that ovine luteinizing hormone stimulates production of oestradiol from the small white follicles in both the traditional-line and the male-line. The magnitude of the response to luteinizing hormone was smaller than that observed in domestic hens by Robinson and Etches (1986). These authors used bovine luteinizing hormone, and it is possible that turkey

ovarian follicles are less sensitive to ovine luteinizing hormone than bovine luteinizing hormone. The effect of ovine luteinizing hormone on small follicles from domestic hens was measured by Wells *et al.* (1985) and they observed a much greater response but at higher concentrations of luteinizing hormone than was used in this experiment and after a longer incubation period. Ovine luteinizing hormone has been used to stimulate oestradiol production in cellular preparations of turkey small white follicles (Porter *et al.* 1989b; Porter *et al.* 1991c) at the same concentrations as in this experiment. However it is not possible to compare the magnitude of their response to that of this experiment due to the different preparations used.

The significant effect of luteinizing hormone on oestradiol production by the small white follicles demonstrates the viability of the incubated follicles. The cells were clearly still able to response to stimulation by luteinizing hormone. The measurement of different amounts of oestradiol at the various concentrations of luteinizing hormone shows that the oestradiol measured was not simply escaping from the follicular tissue. This indicates that the controlled production and release of oestradiol by the follicular cells was still occurring during the incubation, after the follicles were removed from the turkeys.

The small white follicles of the male-line turkeys produced less oestradiol at every concentration of luteinizing hormone used. The fitted line produced by the regression analysis was steeper in the traditional-line which could suggest that the male-line turkeys are less responsive to luteinizing hormone stimulation than the traditional-line, although this observation is not supported by any significant interaction effect between strain and dose of luteinizing hormone.

It would have been interesting to investigate the response of other ovarian follicles to luteinizing hormone stimulation, in terms of both oestradiol and progesterone production. However due to the much smaller numbers of the other follicles it would require many turkeys to obtain enough follicles. The other possibility would have been to prepare cellular suspensions from the theca and granulosa cells but this would have altered the interactions between the three cell layers. If such an

experiment was to be carried out it would be a good opportunity to investigate the responsiveness of the follicular cells to other stimuli, such as the effect of progesterone or testosterone on oestradiol production.

It was shown in Chapter 5 that the mean luteinizing hormone concentration over 36 hours was significantly lower in the male-line compared to the traditional-line. The basal output of oestradiol from the ovarian follicles in the absence of luteinizing hormone stimulation was lower in the male-line; and the response of small white follicles from this strain to luteinizing hormone was also lower. The combination of the lower plasma luteinizing hormone concentration and the lower oestradiol output in the presence of luteinizing hormone will result in much lower ovarian oestradiol production in the male-line compared to the traditional-line.

The first experiment did not address the possibility that the male-line turkeys metabolise the majority of their oestradiol to another active oestrogen that is not measured by the oestradiol radioimmunoassay. The third experiment measured the aromatase activity of the residual ovary in the traditional- and male-line turkeys to investigate this possibility. Aromatase is the enzyme responsible for the conversion of testosterone to oestradiol. If oestrogen production in male-line turkeys occurs to the same degree as in the traditional-line then the male-line turkeys would produce oestradiol relative to the size of their ovarian tissue and then metabolise most of the oestradiol to another active oestrogen. This would be reflected as a greater aromatase activity in the male-line.

The third experiment showed that the aromatase activity of the small ovarian follicles was lower in the male-line compared to the traditional-line. The aromatase activity was of a similar magnitude to that measured by Armstrong (1985) in small ovarian follicles of domestic hens. In domestic hens aromatase activity was found to be proportional to follicle diameter. In these turkeys there were no significant difference in aromatase activity between the small follicles classified by their diameter. However there was a non significant increase in the aromatase activity of follicles from the traditional-line as the diameter of the follicles increased. Had follicle

diameter been recorded for each individual follicle instead of simply used to classify each follicle it is possible that a significant relationship between aromatase activity and follicle size would have been observed.

It has been shown that the development of the ovarian follicles differs between the anterior and posterior sections of the ovary (Waddington and Walker 1988; Waddington and Hocking 1993) and ovarian enzyme activity can vary in relation to the area of the ovary (Armstrong 1987). However in the third experiment there was no difference in the aromatase activity of the anterior and posterior ovarian stromal tissue.

The greater total aromatase activity of the male-line residual ovary suggests that there would be more oestrogen produced by the residual ovary of the male-line. However the volume of plasma that this oestrogen is released into would also be greater in the male-line as the body weight of this strain is much greater. When the aromatase activity of the residual ovary was compared per kg body weight there was no difference between the two strains. The suggests that there would be no significant difference in the concentration of oestrogens in the plasma between the two strains.

Plasma oestradiol concentration was lower in the male-line turkeys, which suggests that the male-line turkeys may be metabolising a small amount of oestradiol to another active oestrogen. However, in this experiment only the aromatase activity of the residual ovary was measured. In domestic hens about 50% of the total ovarian aromatase activity comes from the hierarchical follicles.

The aromatase activity of the follicular hierarchy was not measured in this experiment as it would have involved large quantities of radioactivity. It is possible that the aromatase activity of these follicles would have been lower in the male-line compared to the traditional-line as was observed in the small ovarian follicles. This could result in the male-line strain having a lower aromatase activity/kg body weight overall and therefore account for the lower plasma oestradiol concentration of the

strain. Clearly the results of this experiment do not provide any evidence for the male-line strain having higher active oestrogen levels than the traditional-line that could be involved in predisposing the male-line to prolapse of the oviduct.

These three experiments have demonstrated that the low plasma oestradiol concentration of the male-line compared to the traditional-line is due to lower steroidogenesis in the ovarian tissue of the male-line, even in the presence of additional luteinizing hormone stimulation. It is clear that the relative amount of steroidogenesis occurring in the male-line is not proportional to the amount of ovarian tissue present. There is no evidence to suggest that the male-line has a higher total plasma oestrogen concentration than the traditional-line. The multiple follicular hierarchy of the male-line has been shown to be physiological as well as anatomical. This should be considered when comparing properties of follicles in birds with multiple hierarchies such as commercial breeding turkeys and *ad libitum* fed broiler breeders.

7. Effects of food restriction or delayed photostimulation on the follicle hierarchy, plasma oestradiol concentration and vaginal collagen content in male-line turkeys

INTRODUCTION

Male-line turkeys have more ovarian follicles in their follicular hierarchy compared to traditional- and female-line turkeys (Hocking 1992b; Hocking and Bernard 1998). These are arranged in a multiple hierarchy of follicles of similar weight and physiological maturity (Chapter 6). The multiple hierarchy results in the ovulation of several follicles each day that reduces egg production through the loss of follicles as internal ovulations into the body cavity and through the production of double-yolked, soft-shelled and mis-shapen eggs.

Food restriction has been used for many years to improve reproduction of broiler breeders. Hocking *et al.* (1987) showed that the *ad libitum* fed broiler breeder was characterised by the over production of ovarian follicles resulting in a multiple hierarchy that was reduced by food restriction. It was later suggested that food restriction should be continued to photostimulation (Hocking *et al.* 1989). Recently it was demonstrated that *ad libitum* feeding of previously restricted broilers from photostimulation increased follicle numbers compared to broiler breeders restricted to the onset of lay (Hocking 1996) which suggested that food restriction should be continued during lay to control the over development of ovarian follicles.

Food restriction has been investigated as a tool for controlling the number of developing ovarian follicles in turkeys. Nestor *et al.* (1981) found no effect of mild food restriction on hierarchical follicle number of turkeys selected for increased meat yield. Hocking (1992b) showed that food restriction decreased follicle number in some strains of turkey but this effect was not significant in the male-line strain. It is possible that the degree of restriction (body weight 70% of *ad libitum*) of the male-line was not sufficient to control the overproduction of the ovarian follicles.

More severe food restriction of male-line turkeys resulted in a small decrease in the number of hierarchical follicles (Hocking and Bernard 1998). The food intake of these turkeys was high around the onset of lay compared to *ad libitum* fed controls and the authors postulated that greater control of the food intake around sexual maturity would have resulted in greater control of ovarian follicle production.

The age at photostimulation also affects the number of follicles in the hierarchy in turkeys. Turkeys photostimulation at 18 or 24 weeks have significantly greater numbers of hierarchical follicles than those photostimulated at 30 weeks (Hocking *et al.* 1988; Hocking *et al.* 1992). If early photostimulation results in increased numbers of hierarchical follicles it is possible that delaying the onset of photostimulation would decrease the number of follicles in the ovarian hierarchy of male-line females.

Prolapse of the oviduct is more common in male-line turkeys than traditional- or female-line turkeys. It was originally thought that there might be a connection between the multiple follicular hierarchy of the male-line and the high incidence of prolapse in this line. Prolapse has been shown to be associated with a decrease in the collagen content of the vagina (Chapter 4). If some management factor could be identified that altered the collagen content of the vagina, it may be possible to increase the vaginal collagen content and reduce the incidence of prolapse in the male-line strain.

The aim of this experiment was to test the hypothesis that manipulation of the multiple hierarchy could alter the vaginal collagen content in the male-line. The effects of restricted feeding throughout sexual maturity or delayed photostimulation on the over production of ovarian follicles in the male-line was tested and the vaginal collagen contents and plasma oestradiol concentrations were measured.

METHODS

Sixty Big 6 male-line turkeys were housed and reared as described in 2.1. At 18 weeks of age they were arranged in 12 pens with 5 turkeys in each pen. Each pen was assigned to one of three treatments in a randomised block design. The treatments were as follows.

1. Control. Turkeys were fed *ad libitum* throughout and were photostimulated at 29 weeks and 4 days.
2. Restricted. Turkeys were food restricted from 18 weeks and were photostimulated at 29 weeks and 4 days. The aim of the food restriction was to reduce body weight to 0.6 of the *ad libitum* fed turkeys at photostimulation and to maintain that body weight until termination of the experiment.
3. Delayed photostimulation. Turkeys were fed *ad libitum* throughout and were photostimulated at 34 weeks and 4 days.

Each turkey was weighed at the start of the experiment (18 weeks), at the start of photostimulation and 5 weeks after photostimulation. The restricted turkeys were weighed weekly, as necessary, to monitor their body weight and food allowance. The restricted turkeys were fed a set daily food allowance that was placed in the food hoppers at 08.00.

At 29 weeks and 4 days of age the four pens of turkeys that were on the delayed photostimulation treatment were moved to identical pens within the same hut where the lighting schedule could be controlled separately from the other pens. They were maintained on 7 hours light for an extra five weeks before photostimulation at 34 weeks and 4 days of age.

Five weeks after photostimulation the turkeys were blood sampled and killed as described by 2.2. The oviduct was removed and dissected (2.4) and the weights of the uterus, vagina and total oviduct were recorded. The vaginas were stored in individual plastic bags at -20°C for future collagen analysis (2.5). The ovary was

removed and the follicles were classed as described in 2.4. The post ovulatory follicles were removed and counted, and the residual ovary was weighed.

The blood samples were treated as described in 2.2 and plasma oestradiol concentration was measured in a single radioimmunoassay after antibody-sepharose extraction (2.6.1).

RESULTS

Table 7.1 shows the body weights of the control, restricted and delayed photostimulation turkeys at the start of the dark period, at photostimulation and 5 weeks after photostimulation.

TABLE 7.1 Mean total body weight (kg) of control, restricted and delayed photostimulation turkeys at the start of the dark period, photostimulation and 5 weeks after photostimulation

	Control	Restrict fed	Delayed Photostimulation	SED	Sig.
Dark period	15.3	15.0	14.9	0.31	NS
Photostimulation	21.4	13.4	20.8	0.36	***
Five weeks post photostimulation	20.8	13.2	20.0	0.39	***

There were no significant differences in body weight of the different treatment groups at the start of the dark period when the restricted feeding began. There were significant differences in body weight at the start of photostimulation and at 5 weeks after photostimulation due to the food restriction. The 5 week delay in photostimulation had no significant effect on body weight at photostimulation. By five weeks after photostimulation body weight was significantly lower in both the restricted ($P<0.001$) and the delayed photostimulation ($P<0.05$) groups compared to the control group.

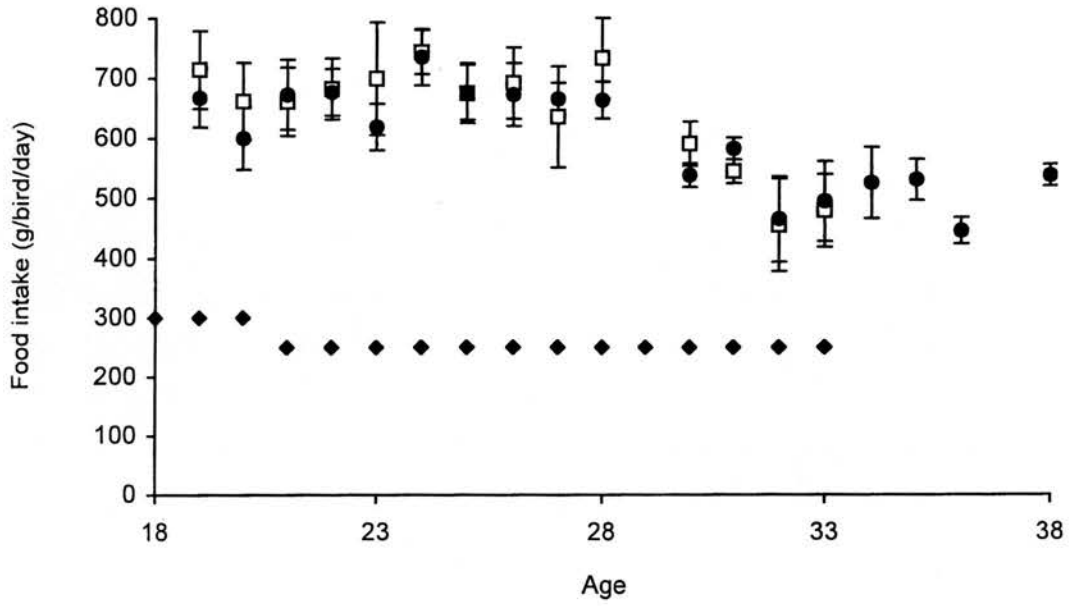


FIGURE 7.1 Mean (±SEM) daily food intake per bird for control, food restricted and delayed photostimulation turkeys from the start of the experiment at 18 weeks of age until 5 weeks after the onset of photostimulation
Key: □ Control; ◆ Restricted; ● Delayed photostimulation

Figure 7.1 shows the food intake of the three treatment groups. The food intake of the restricted turkeys was significantly lower than the control or delayed photostimulation groups ($P<0.001$). There was no significant difference in food intake between the delayed photostimulation and control groups during the first 16 weeks of the experiment before the control group were killed.

In both the control group and the delayed photostimulation group there was a significant effect of week on food intake ($P<0.001$) as food intake decreased in both groups after 29 weeks of age. This decrease in food intake coincided with the increase in photoperiod for the control group but also occurred in the delayed photostimulation group, which did not receive the increased photoperiod until about 34 weeks.

After 5 weeks of photostimulation the number of turkeys that had ovulated, determined by the presence of post ovulatory follicles on the ovary, was recorded

and the proportion in each treatment is shown on Table 7.2. Food restriction significantly reduced the number of birds that had ovulated after 5 weeks of photostimulation. Within the restricted group, the turkeys that had ovulated were found to have a significantly heavier body weight (14.0kg) than those that had not ovulated (12.4kg, $P<0.05$, SED=0.65). All turkeys that had not ovulated were excluded from the analysis.

TABLE 7.2 Mean percentage of birds that had ovulated and mean number of eggs per bird in lay after 5 weeks of photostimulation for male-line turkeys subjected to either control, restricted feeding or delayed photostimulation treatments

	Control	Restricted	Delayed photostimulation	SED	Sig.
Ovulated (%)	87.4	47.4	83.3	13.24	**
Number eggs/bird in lay	3.3	2.6	5.9	0.962	*

Egg production was recorded for each pen and divided by the number of birds in the pen that had ovulated by 5 weeks after photostimulation. The total number of eggs per bird is given on Table 7.2. The significant effect of treatment on the number of eggs per bird was due to the difference between the restricted and delayed photostimulation groups. Neither the restricted nor the delayed photostimulation groups were significantly different from the control group.

The effects of the restricted feeding and delayed photostimulation on the weights of the oviduct, uterus, vagina and residual ovary and the number of mature, atretic and post ovulatory follicles are shown on Table 7.3.

TABLE 7.3 Mean oviduct, uterus, vagina, and residual ovary weight and number of hierarchical, atretic and post ovulatory follicles in male-line turkeys subjected to either control, restricted feeding or delayed photostimulation treatments, that had ovulated at least once

	Control	Restricted	Delayed photostimulation	SED	Sig.
Oviduct (g)	143.6	116.5	145.2	7.29	***
Uterus (g)	37.9	34.0	37.7	2.23	NS
Vagina (g)	12.6	12.0	12.7	1.14	NS
Residual ovary (g)	22.9	17.4	27.7	2.64	***
Hierarchical follicles (>0.5g)	18.8	16.4	16.8	1.45	NS
Atretic follicles	0.86	0.08	0.22	0.441	NS
POFs	10.0	6.7	11.7	1.80	*

The significant effect of treatment on oviduct weight was due to the lighter oviduct of the restricted group compared to the control group. There was no difference in oviduct weight between the control group and the delayed photostimulation group. The weight of the uterus and vagina were unaffected by either treatment. The residual ovary of the restricted group was significantly lighter ($P<0.05$) than that of the control group, while delayed photostimulation resulted in a significantly heavier ($P<0.05$) residual ovary than the control.

There were no significant effects of either restricted feeding or delayed photostimulation on the number of maturing yellow follicles or on the number of atretic follicles.

Although treatment had a significant effect on the number of post ovulatory follicles present, this was due to the difference between the restricted and delayed photostimulation groups. Comparison of either the restricted feeding or the delayed photostimulation group with the controls did not result in any significant differences in the number of post ovulatory follicles.

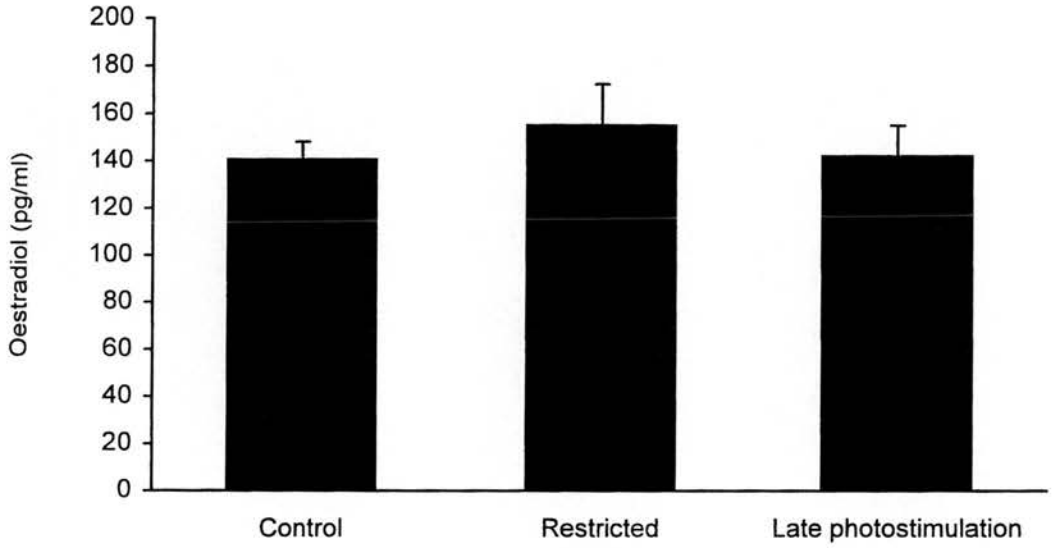


FIGURE 7.2 Mean (±SEM) plasma oestradiol concentration 5 weeks after photostimulation in control, food restricted and delayed photostimulation male-line turkeys

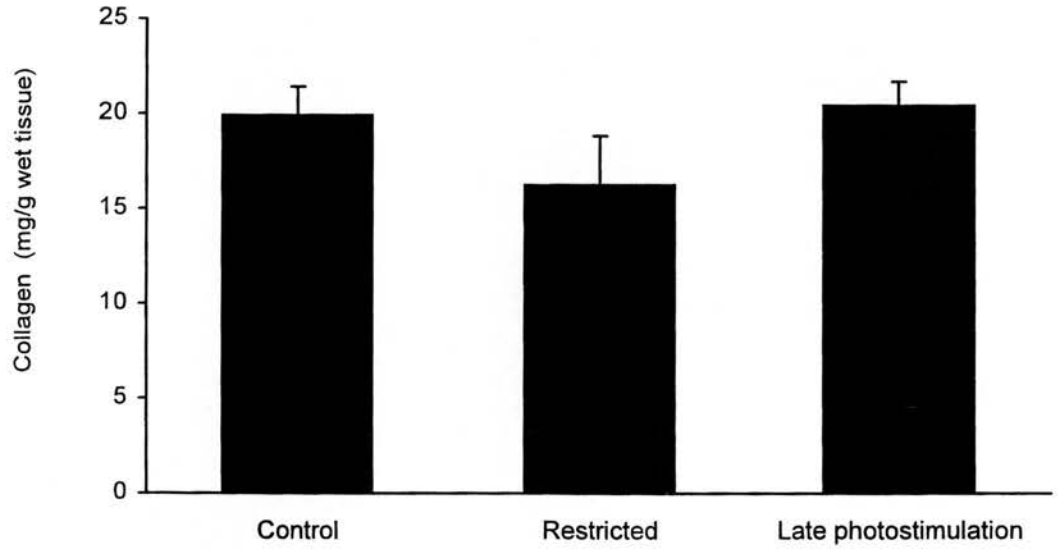


FIGURE 7.3 Mean (±SEM) vaginal collagen content 5 weeks after photostimulation in control, food restricted and delayed photostimulation male-line turkeys

Figure 7.2 shows the mean plasma oestradiol concentration of the control, restricted and delayed photostimulation groups. There were no significant differences in plasma oestradiol concentration between the three groups. The mean vaginal collagen content per gram of wet tissue for the three treatment groups is presented on Figure 7.3. The vaginal collagen content showed no significant differences between the control, restricted and delayed photostimulation groups.

DISCUSSION

The results show that neither restricted feeding nor delayed photostimulation had any effect on mature follicle number, plasma oestradiol or vaginal collagen in male-line turkeys.

The food restriction in this experiment was severe, resulting in a body weight of 63% of the controls. This restriction was continued for 5 weeks after photostimulation and while the *ad libitum* food intake did decrease after photostimulation it was still much greater than the food allowance of the restricted turkeys. Food restriction to this extent prevented half of the restricted turkeys from reaching sexual maturity by five weeks after photostimulation. The restricted turkeys that did start egg production were the heavier individuals within the group. It seems likely that increasing the degree of food restriction beyond the extent used in this experiment would prevent the turkeys from reaching sexual maturity. From the results of this experiment and previous work investigating the effects of food restriction on turkeys (Hocking 1992b; Hocking *et al.* 1992; Hocking and Bernard 1998) it seems likely that food restriction does not control the over production of yellow follicles in male-line turkeys.

Hocking (1992b) demonstrated a linear relationship between body weight and yellow follicle number in turkeys, and suggested that a hierarchy of 9 follicles was associated with a body weight of 9.6kg. Clearly male-line turkeys restricted to 9.6kg

would not come into lay therefore these results confirm the suggestion that restricted feeding is not suitable for controlling the multiple follicular hierarchy in turkeys. The lack of effect of food restriction on the number of yellow follicles explains why food restriction does not improve egg production (Hocking 1992a).

Food restriction is effective in controlling the over production of ovarian follicles in broiler breeders (Hocking *et al.* 1987; Hocking *et al.* 1989; Hocking 1993; Hocking 1996). It is possible that different mechanisms are involved in controlling the development of the ovarian hierarchy in turkeys and broiler breeders. Food restriction has been common practice in breeding companies for many years to improve egg production and fertility in broiler breeders. Restricted feeding is not generally used in the turkey industry. It is possible that the use of food restriction in the broiler breeder industry over many years has resulted in the selection of birds that do control the production of ovarian follicles in response to food restriction. This could explain the different response of turkeys and broiler breeders to food restriction.

The five week delay in the onset of photostimulation did not reduce the number of hierarchical follicles in the experiment. This suggests that the lack of control of the follicular development present in turkeys photostimulated at 29 weeks and 4 days is not due to any immaturity of the turkeys. However it has been shown that photostimulation of turkeys 6 or 12 weeks earlier than usual increases the number of hierarchical follicles (Hocking *et al.* 1988; Hocking *et al.* 1992). This suggests that there may be an immaturity of the mechanisms controlling follicular development at 18 or 24 weeks of age.

The current knowledge of the effects of altering the onset of photostimulation suggests that around 30 weeks is the most suitable age for photostimulation in turkeys. Early photostimulation decreases initial egg size and the early rate of egg production although the laying period is extended and a greater total number of eggs can be produced. However early photostimulation can result in early termination of

lay or intermittent pauses in egg production (Hocking 1992a) while there are no obvious benefits in delaying the onset of photostimulation.

Restricted feeding resulted in a lighter oviduct and residual ovary at 5 weeks after photostimulation. This result was based on turkeys that had ovulated and were considered to be laying but did not account for how long they had been in lay. It is probable that the restricted turkeys had not been in lay for as long as the controls and therefore it is likely that the oviduct and ovary were not fully developed at this point. The residual ovary was heavier in the delayed photostimulation group compared to the controls. This is possibly due to the greater age of these turkeys. Alternatively they may have come into lay slightly earlier than the control group.

The lack of effect of either restricted feeding or delaying the onset of photostimulation on the vaginal collagen content suggests that altering either of these management factors would not reduce the incidence of prolapse in male-line turkeys.

8. General Discussion

Comparison of male-line turkeys with a high incidence of prolapse and traditional-line turkeys with a low incidence of prolapse was carried out to highlight differences between the two lines that could be involved in predisposing the male-line to prolapse of the oviduct.

The development of the reproductive system at first egg and for seven weeks following the onset of photostimulation was investigated. No differences in maturity of the ovary, oviduct or its supporting structures were found between the traditional- and male-lines. It was concluded that there was no evidence to support previous suggestions (Lilburn and Nestor 1993; Melnychuk *et al.* 1997) that oviducts of male-line females are immature at the onset of lay.

The male-line ovaries were characterised by a multiple hierarchy of developing follicles as has been described previously (Hocking 1992b; Melnychuk *et al.* 1997; Hocking and Bernard 1998). There was no difference in the number of hierarchical follicles between the prolapsed and non-prolapsed male-line turkeys and therefore no evidence of an association between the size of the multiple hierarchy and the propensity to prolapse.

Histological comparison of the oviduct and its supporting structures between traditional-, male-line and prolapsed male-line turkeys did not reveal any abnormalities associated with prolapse. Furthermore, over development of the ventral ligament that could result in increased torsion in the oviduct was not associated with prolapse in turkeys, as has been suggested in domestic hens (Rao *et al.* 1985).

It was concluded that the experiments described in Chapter 3 gave no evidence of any anatomical factors that could be involved in the high incidence of prolapse in male-line turkeys, and supported the suggestion of Hocking (1993b) that there is a physiological basis to the high incidence of prolapse in male-line turkeys.

It was originally hypothesised that male-line turkeys might have high plasma oestradiol due to oestrogen production by their large ovaries, and that the high oestradiol concentration would stimulate collagen degradation in the oviduct and predispose the male-line oviduct to prolapse. It was shown, in Chapter 4, that low vaginal collagen was associated with prolapse in turkeys. The prolapsed male-line turkeys had a lower vaginal collagen content than their non-prolapse contemporaries and the vaginal collagen content of the male-line was lower than the traditional-line. It was suggested that the low vaginal collagen content of the male-line turkeys reduced the structural integrity of the vagina and predisposed the strain to prolapse.

The vaginal collagen of the prolapsed turkeys had fewer mature cross-links compared to non-prolapsed turkeys, which would have further reduced the structural integrity of the vagina in these individuals. The results suggest that total collagen turnover may be increased in the prolapsed turkeys, as the vaginal collagen was less mature, with a slightly increased proportion of immature cross-links. It is possible that collagen synthesis could increase following prolapse in an attempt to improve the structural strength of the vagina, although clearly degradation must also be increased as the total collagen content was reduced.

It would be desirable to measure the collagen content and proportion of cross-links in individual turkeys before and after prolapse but such an experiment is not possible due to the invasive nature of the sampling for vaginal collagen measurement. If an association between prolapse and increased plasma concentrations of matrix metalloproteases such as collagenase could be established then it may be possible to demonstrate whether increased collagen turnover occurs as a result of prolapse or prior to it.

The collagen contents of the gut and uterus were similar in traditional-, male- and prolapsed male-line turkeys. The reduction in collagen associated with prolapse was limited to the vaginal section of the oviduct, and supported the observations on prolapsed turkeys, that prolapse occurred as a result of a failure of the vaginal structure.

The hypothesis that low vaginal collagen is involved in prolapse could explain the increase in the incidence of prolapse within flocks associated with stresses such as turkey rhinotracheitis and hot weather. These stresses increase the intra-abdominal pressure of the turkeys through increased respiration and coughing. The raised intra-abdominal pressure would strain the structural capabilities of the vagina and result in prolapse of turkeys with lower vaginal collagen contents. It is conceivable that there is a proportional relationship between the intra-abdominal pressure and the amount of vaginal collagen required to be present to prevent prolapse of the oviduct.

The variable nature of the vaginal collagen content of turkeys suggests that there may be scope for selection of turkeys with high vaginal collagen that would have increased resistance to prolapse. If it was established that the vaginal collagen content was a heritable trait then it may be possible to develop some type of marker for high vaginal collagen that could be measured non-invasively. This would allow selection for high vaginal collagen to be applied to the male-line breeding flocks with the expectation that the incidence of prolapse in male-line flocks would decline. Techniques for indirect selection based on DNA markers or measurement of a collagen metabolite in plasma or excreta could be utilised for this purpose.

It was concluded that low vaginal collagen is associated with prolapse in turkeys, as has previously been demonstrated in humans (Jackson *et al.* 1996). The differences in vaginal collagen content were not visible from histological investigation using Van Gieson's stain for muscle and collagen or through immunohistological staining with collagen type-specific antibodies (Chapter 3). Measurement of the total collagen content using chemical procedures was necessary to establish differences associated with prolapse. Ayem and Noakes (1998) attempted to investigate the relationship between vaginal collagen and prolapse in sheep using histological staining techniques and found no significant difference in collagen content associated with prolapse. If the total collagen content of the tissue had been measured it is possible that they would have found a significant reduction in vaginal collagen associated with prolapse in ewes, as in turkeys and humans.

Experiments described in Chapters 3, 4 and 5 have shown that male-line turkeys do not have high plasma oestradiol concentrations. In fact the male-line had significantly lower plasma oestradiol concentrations than the traditional-line after 5 weeks of photostimulation and there was no evidence to suggest that male-line plasma oestradiol concentration was higher prior to the onset of lay or at any point during the ovulatory cycle. The results did not support the original hypothesis that high plasma oestradiol concentrations in the male-line would be associated with prolapse but does support the suggestion of Hocking (1993b), based on research in domestic hens, that male-line turkeys may have low plasma oestrogen levels.

There was no difference in plasma oestradiol or progesterone concentration between the prolapsed and non-prolapsed male-line turkeys. Clearly prolapse in male-line turkeys is not associated with low oestradiol concentrations, as has been suggested for hens (Shemesh *et al.* 1982). The lack of difference in plasma concentrations of oestradiol and progesterone between prolapsed and non-prolapsed turkeys does not suggest an involvement of either progesterone or oestradiol with prolapse in male-line turkeys.

Elevating plasma oestradiol concentration through administration of oestradiol injections did not result in a decrease in vaginal collagen content, suggesting that high plasma oestradiol does not stimulate vaginal collagen degradation in turkeys. Raising plasma progesterone through progesterone administration also had no effect on vaginal collagen in turkeys. Raised plasma progesterone and oestradiol concentrations do not appear to be involved in controlling collagen metabolism in the vagina of the turkey, unlike the cervix of rats and guinea-pigs where oestradiol stimulates and progesterone inhibits collagen degradation (Pastore *et al.* 1989; Rajabi *et al.* 1991a; Rajabi *et al.* 1991b; Bienkiewicz *et al.* 1996).

It seems unlikely that plasma concentrations of either oestradiol or progesterone are involved in prolapse in turkeys, as prolapse was associated with low vaginal collagen

and increased plasma concentrations of progesterone or oestradiol did not appear to affect vaginal collagen metabolism in turkeys.

It is possible that a synergistic action of oestradiol and progesterone acts on vaginal collagen in the male-line and both are required to alter the vaginal collagen content. However in the hormone administration experiments the endogenous hormones would be present in addition to the exogenous administered hormone and any synergistic effect on vaginal collagen should still have been visible. The results did not suggest that any such dual action of progesterone and oestradiol on vaginal collagen was likely and, as the administration of progesterone resulted in the death of several turkeys, it was decided that there was no evidence to warrant conducting an experiment co-administrating oestradiol and progesterone.

The cause of the low vaginal collagen in the male-line has not been defined. Clearly it was not caused by high plasma oestradiol stimulating collagen degradation as was originally hypothesised. It is possible that other factors could be stimulating collagen degradation or inhibiting collagen synthesis, such as growth factors like growth hormone or insulin-like growth factor 1. Hocking *et al.* (1994) showed that growth hormone and insulin-like growth factor 1 were low in association with rapid growth of broiler breeders. It may be that they are also low in male-line turkeys and do not stimulate sufficient collagen production during the development of the oviduct. Picaper *et al.* (1986) found that plasma growth hormone was significantly higher in fat-line broilers compared to lean-line broilers at 5 weeks of age, whereas Williams *et al.* (1986) found no significant difference in plasma growth hormone concentration between fat- and lean-line broilers, although plasma growth hormone concentration increased in both lines in association with the onset of egg production.

It is possible that collagen metabolism was not being altered by some controlling factor in the male-line and that vaginal collagen is simply inherently lower in the male-line compared to the traditional-line. Although no differences were seen in uterine collagen content, the collagen content of uterine tissue was lower than vaginal tissue, and it is possible that there was insufficient collagen present for a

significant difference between the two lines to be seen. The collagen content of the gut was compared between the two strains, as gut tissue has a similar structure to vaginal tissue, and has a much higher collagen content than the uterus. There was no difference in the gut collagen content between the traditional- and male-line turkeys which showed that low collagen content was not a characteristic of all endothelial tissues in the male-line. Selection for meat yield could indirectly select for certain characteristics in the gut as it is involved in the efficiency of digestion. The collagen of the gut may therefore have been actively maintained by selection while vaginal collagen was not. It would be interesting to measure the vaginal collagen content during development of the reproductive system following photostimulation, to determine if the vaginal collagen is always lower in the male-line or whether it falls around the onset of lay. If the latter was the case it would indicate that decreased collagen is likely to be a result of some factor affecting collagen degradation.

The relationship between collagen content and the stability of the vaginal tissue was not determined. However it is difficult to assess what forces would be involved in prolapse and therefore whether to test for tissue shear strength or elasticity in response to an applied load. If a positive relationship between tissue strength and collagen content could be established for turkey vaginal tissue it would substantiate the conclusion that low vaginal collagen impairs the structural integrity of the vagina and predisposes the male-line to prolapse.

Measurement of the ratio of type I to type III collagen in association with prolapse would be interesting, as the different collagen types lend different structural properties to the tissue. Norton *et al* (1992) reported a decrease in type I:type III ratio associated with prolapse in women, although no difference in this ratio was found by Jackson *et al* (1996). Measurement of the vaginal tissue activity of matrix metalloproteases, such as collagenases, would also be valuable, as it is likely that the activity of the collagen degrading enzymes would be increased in association with prolapse. Electron microscopy could also be used to investigate the structural properties of the collagen in the vagina as there may be a breakdown in the organisation of the collagen associated with prolapse. Investigations like these would

increase understanding of the possible defects in collagen structure that could combine to reduce the stability of vaginal tissue in the male-line. However they would be unlikely to reveal any underlying cause of these changes nor suggest any mechanism for improving the vaginal stability of the male-line.

It was surprising that the male-line turkeys did not have higher plasma concentrations of either progesterone or oestradiol due to steroidogenesis by their larger ovaries in comparison to the traditional-line. The oestradiol output from the male-line ovarian follicles was lower than from follicles of the traditional-line, even in the presence of luteinizing hormone stimulation. The results suggested that steroidogenesis was reduced in the male-line ovary and explained why the male-line turkeys had lower plasma oestradiol concentrations in spite of their larger ovaries. The progesterone output from the follicles was too variable, due to the variation of progesterone output during the ovulatory cycle, for any significant differences between the traditional- and male-line ovaries to be detected.

The presence of luteinizing hormone in the incubation medium increased the oestradiol output from small ovarian follicles, with a greater response in the small follicles from the traditional-line. The plasma luteinizing hormone concentration was lower in male-line turkeys compared to the traditional-line. The combination of low plasma luteinizing hormone and a low responsiveness of the ovarian follicles to luteinizing hormone would further accentuate the low oestradiol output from the male-line ovaries *in vivo*.

It has previously been demonstrated that turkey plasma contains several different oestrogen metabolites (Brown *et al.* 1979; Bacon *et al.* 1980; Brown 1982) that may not be detected by the oestradiol radioimmunoassay. The aromatase activity of the residual ovary (ovarian stroma and small follicles) was measured to assess whether there was another active oestrogen present in large quantities in the male-line that could account for the low plasma oestradiol concentration and low follicular oestradiol output of the male-line (Chapter 6). The results provided no evidence to suggest that there was a higher concentration of total oestrogen in the plasma of the

male-line. The aromatase activity of the hierarchical follicles was not measured and, in domestic hens, accounts for about 50% of the ovarian aromatase activity (Armstrong 1984). However from the results it was hypothesised that the hierarchical follicles of the male-line would have decreased aromatase activity compared to the traditional-line, as was demonstrated for the small ovarian follicles. If the aromatase activity of the hierarchical follicles was also reduced then the total ovarian aromatase activity of the male-line would be lower, relative to body weight, compared to the traditional-line, which would account for the lower plasma oestradiol concentration of the strain.

Administration of oestradiol in quantities proportional to body weight caused a greater increase in plasma oestradiol in the male-line compared to the traditional-line. If the male-line turkeys metabolised large quantities of oestradiol to some other active oestradiol metabolite not detected by the oestradiol radioimmunoassay, the administration of oestradiol would not have raised plasma oestradiol in the male-line to the same extent as the traditional-line. The greater increase in the plasma oestradiol concentration of the male-line following oestradiol administration supported the conclusion that the male-line turkeys were not metabolising oestradiol to another active oestrogen.

In these experiments body weight was used to correct for the large differences in blood volume between the traditional- and male-line turkeys. Blood volume is proportional to body weight, but it is not known whether the large muscle mass of the male-line results in an altered relationship between body weight and blood volume. Therefore the use of body weight to correct for differences in blood volume may be inaccurate. Measurement of blood volume could be carried out through indicator dilution experiments, however the accessible compartment for oestradiol also includes body fat and therefore measurement of blood volume would still not provide an accurate measure of the dilution pool size. There was no alternative measurement that would have been any more reliable than body weight. It was concluded that using body weight to correct for strain differences in plasma volume

was sufficiently accurate for the purpose of comparing the results of these experiments.

The pattern of oestradiol and progesterone output from the individual follicles within the hierarchy of the two strains (Chapter 6) clearly demonstrated that the multiple hierarchy of the male-line has a physiological basis, with multiple follicles at each stage of maturation. It was expected that the multiple follicle groups would be at similar maturation stages as several follicles have to be at a mature stage at the same time for multiple ovulations to occur. The results showed that once a follicle has been recruited into the multiple hierarchy of developing follicles maturation appears to progress as normal resulting in ovulation 9-10 days later. The results suggest that the excessive number of hierarchical follicles in the male-line arises due to recruitment of too many follicles into the hierarchy and not due to any increase in the time required for follicular maturation. However, at present, it is not understood what factors control the recruitment of follicles into the hierarchy and it is not possible to speculate what mechanisms may be defective in the male-line to cause the multiple hierarchy.

The preovulatory peaks of progesterone and luteinizing hormone that are thought to control ovulation were no more frequent in association with multiple ovulation (Chapter 5). This suggested that multiple ovulations occur around the same time in the male-line, rather than at various times throughout the ovulatory cycle. Ovulation of more than one follicle at a time is not conducive to the production of properly formed hard shelled eggs suitable for incubation. Double yolked eggs do occur in turkeys but they are not a predominant feature of the male-lines and it seems likely that a large proportion of male-line follicles are lost as internal ovulations. The observation that many of the male-line turkeys had recently ovulated follicles within the body cavity, including one turkey that had 4 intact ova in the body cavity when killed, supported this suggestion.

The lack of a consistent pattern of oestradiol changes during the ovulatory cycle of the turkey agreed with previous finding (Opel and Arcos 1978) and supports the

suggestion that changes in plasma oestradiol concentrations are not directly involved in the control of ovulation. Elevation of plasma oestradiol beyond physiological levels by oestradiol administration had no significant effect on egg production or the follicular hierarchy. Administration of progesterone rapidly prevented ovulation and oviposition from occurring and resulted in atresia of the follicular hierarchy. It is likely that the blocking of ovulation is due to negative feedback of progesterone preventing increased luteinizing hormone release and increased progesterone production by the mature follicle to trigger ovulation. The results support the hypothesis that it is progesterone and not oestradiol that is directly involved in controlling ovulation (Sharp 1983).

It is not known why selection for increased meat yield should result in a multiple hierarchy and low ovarian steroidogenesis in the male-line although it is possible to hypothesise a link between the two characteristics. If a surge in progesterone production from the mature follicle is required to trigger ovulation, and steroidogenesis is reduced in the male-line then it is possible that several mature follicles are required to provide sufficient progesterone to trigger ovulation. This would result in selection for turkeys with a propensity for multiple ovulations. However it is unclear at present whether progesterone production is lower in the male-line or whether only oestradiol production is reduced. Oestradiol and progesterone are both required to prime the negative feedback responses involved in ovulation. The multiple hierarchy could therefore have developed because turkeys with increased numbers of small ovarian follicles produced sufficient amounts of oestradiol to prime the positive and negative feedback systems required to trigger ovulation.

Prolactin, the anterior pituitary hormone thought to be involved in stimulating broodiness in turkeys, has been found to reduce steroidogenesis (Lien *et al.* 1989). It is possible that the low ovarian oestradiol production in the male-line could be due to increased secretion of prolactin inhibiting steroidogenesis, as broodiness is a common problem with male-line turkeys in comparison to female-lines. However prolactin also stimulates ovarian regression (Etches 1996c). Ovarian regression is not

a characteristic of male-line turkeys, compared to the traditional-line, and plasma prolactin concentrations are unlikely to be higher in the male-line than the traditional-line.

The finding of reduced ovarian steroidogenesis in the male-line may have implications on the reproductive performance of both turkeys and broiler breeders. Further investigation into the functioning of the male-line ovary would reveal whether the reduced oestradiol production is due to reduced availability of substrates or enzymes involved in steroid metabolism. The results of the experiment carried out to measure the aromatase activity of the residual ovary suggested that ovarian aromatase activity was lower in the male-line. However it is possible that the low oestradiol output is caused by a combination of lower enzyme activity, responsiveness to luteinizing hormone stimulation and substrate availability.

The lack of effect of either severe food restriction or delayed photostimulation on hierarchical follicle number (Chapter 7) did not support the hypothesis that food restriction or age at onset of photostimulation could be used to control the overproduction of hierarchical follicles in turkeys. It seems unlikely that there is any management factor that could be altered to control ovarian growth in the male-line. However one possible factor that could be considered would be gradual lighting patterns, such as the step-down, step-up lighting regime used by Melnychuk *et al* (1996). A small decrease in the number of hierarchical follicles was associated with the alternative lighting regime although the significance of this difference was not reported.

The lower collagen content of the male-line vagina was not due to an immaturity of the turkeys at photostimulation, as delaying photostimulation by 5 weeks had no effect on the total collagen content of the vagina. However although the turkeys were 5 weeks older, the vaginal tissue was of a similar age in the two groups as the majority of the oviduct is developed in the first 3-4 weeks of photostimulation. It is possible that the rapid growth of the oviduct does not allow sufficient time for the vaginal collagen to develop. A step-down, step-up lighting regime could cause the

oviduct to develop over a longer time period and may allow greater stability of the vaginal collagen to develop. However the oviduct of the traditional-line turkeys undergoes the same rapid growth as the male-line and is not characterised by low collagen or a predisposition to prolapse.

The investigations carried out on prolapsed male-line turkeys have provided circumstantial evidence that suggest it is unlikely that prostaglandins and arginine vasotocin are involved in prolapse. Prolapse in turkeys develops gradually over several days, which would suggest involvement of long term factors. Plasma concentrations of prostaglandins and arginine vasotocin are only elevated for short periods of time, in association with oviposition, and therefore are unlikely to be involved in the development of prolapse. In addition several of the prolapsed turkeys investigated in Chapter 4 had no post ovulatory follicles and had therefore never laid an egg. This shows that oviposition is unlikely to be directly involved in prolapse, and hyper-stimulation of the oviduct by prostaglandins and arginine vasotocin is not likely to be the cause of prolapse.

In conclusion, the high incidence of prolapse in male-line turkeys was associated with a low vaginal collagen content that probably impaired the structural integrity of the vagina. The cause of the low vaginal collagen in the male-line has not been identified but it was not caused by either high plasma oestradiol or low plasma progesterone stimulating collagen degradation. Prolapsed male-line turkeys also had fewer mature cross-links in the vaginal collagen, which would further impair the structural integrity of the vagina and may indicate increased turnover of collagen in these individuals.

The multiple follicular hierarchy of the male-line consisted of several follicles at each hierarchical position that had similar hormone outputs. However, the large ovary of the male-line did not result in high concentrations of steroid hormones in the plasma as ovarian steroidogenesis was lower in the male-line compared to the traditional-line.

Neither food restriction nor delayed photostimulation had any effect on either the multiple follicular hierarchy or the low vaginal collagen content of the male-line turkeys. Development of a system of selection for turkeys with a higher vaginal collagen content may decrease the incidence of prolapse in male-line turkeys.

Further research into prolapse in male-line turkeys should aim to establish a direct relationship between collagen content and strength of vaginal tissue in order to confirm that low vaginal collagen is involved in the predisposition to prolapse. Determination of whether vaginal collagen is lower in the male-line throughout the development of the oviduct or whether it decreases around the onset of lay is necessary to establish whether some factors stimulate increased collagen degradation or whether vaginal collagen is intrinsically low in the male-line. Investigation into the role of growth factors in oviduct growth and collagen turnover in traditional- and male-line turkeys may also reveal differences that could be involved in the low vaginal collagen content and predisposition to prolapse that characterises male-line turkeys.

Appendix 1. Immunohistological identification of oestrogen receptors

INTRODUCTION

Comparison of plasma oestradiol concentration between the two strains only supplies part of the information involved in the effect of oestradiol on the reproductive tract. The number, sensitivity and location of oestrogen receptors in the strain would also alter the response of the tissues to oestradiol stimulation. Oestrogens stimulate development of the oviduct (Etches 1996a), and the oviduct is therefore likely to contain oestrogen receptors.

At present two types of oestrogen receptor have been identified in mammals and they are known as ER-α and ER-β (Rosenfeld *et al.* 1999). It is thought that both receptors are present in certain tissues but are expressed in different proportions, for example, ERα may be expressed more than ERβ in the mammalian uterus (Rosenfeld *et al.* 1999). Any functional significance of the different types of oestrogen receptors has not yet been discovered. The binding domain of the oestrogen receptor has been shown to be 100% homologous in its DNA sequence between cows, rats, mice and humans (Rosenfeld *et al.* 1999), but comparison of the avian oestrogen receptor to that of mammals has not been reported.

There are various methods for investigating the presence of hormone receptors. Measurement of the production of receptor mRNA is commonly used as a measure of the quantity of receptors present. However this method does not take into account post transcriptional modifications which are often required to form functional receptors. Binding of radiolabelled oestradiol in the presence and absence of an excess of unlabelled oestradiol can be used to measure the maximum specific binding of oestradiol to tissue. This exchange procedure has been used to demonstrate oestrogen binding sites on hen ovarian follicles (Kamiyoshi *et al.* 1986), hen hypothalamus and pituitary (Kawashima *et al.* 1993) and quail oviduct (Turner *et al.* 1993).

Another possibility is immunohistological investigation using specific antibodies raised against the receptor. This allows the location of the receptors within the tissue to be observed and the number of receptors present could be quantified using image analysis.

There is no antibody available that has been raised against chicken or turkey oestradiol receptors. The antibodies that are commercially available have been raised against human oestradiol receptors and may not bind to turkey or chicken oestrogen receptors.

A commercially available kit has been used on uterine tissue from quail and hens (Ohashi *et al.* 1991; Yoshimura *et al.* 1995) and was reported to successfully highlight oestrogen receptors. Baris *et al.* (1998) investigated the effectiveness of three different antibodies raised against human oestrogen receptors for use on hen oviduct and bone. They tested receptor positive human breast tissue and hen oviduct tissue as controls to develop the staining method. They concluded that the best identification of oestrogen receptors in hen oviduct was achieved using antibody 1D5 at a dilution of 1/20, with an incubation time of 4-24 hours and using a Cy3 fluorescence visualisation system. Other immunohistological investigations of oestradiol receptors have successfully used peroxidase or FITC fluorescence visualisation systems (Braidman *et al.* 1995; Zhao *et al.* 1999).

The aim of this experiment was to compare the location and quantity of oestradiol receptors in uterine and vaginal tissue from traditional- and male-line turkeys. The 1D5 anti-human oestrogen receptor antibody was used in this trial as it had reportedly been used with success in hen oviduct.

METHODS

Traditional- and male-line (Big 6) turkeys were reared as described in 2.1. After five weeks of photostimulation 6 turkeys from each strain were killed with an overdose of sodium pentobarbitone (2.2). Immediately after death the uterus and vagina were

dissected from each bird as described in 2.4. Duplicate samples roughly 1cm^2 in size were taken from each tissue and treated as described in 2.7.

The initial staining procedure used was based on the procedure used by Baris *et al.* (1998). The phosphate buffered saline (PBS, Sigma Chemical Company, Poole, UK) used contained 1% bovine serum albumin (Sigma Chemical Company, Poole, UK). Sections were fixed in buffered neutral formalin for 12 minutes followed by 5 minute incubations in methanol and subsequently acetone (both at -20°C). After washing sections with 3 cycles of 5 minute incubations in PBS, sections were incubated in normal goat serum (SAPU) diluted 1:40 in PBS. Sections were washed with 3 × 5 minutes PBS and incubated for 2 hours in 1D5 (Biomen, Berkshire, UK) at a 1:20 dilution in PBS. After washing with 3 × 5 minutes in PBS, sections were incubated in FITC conjugated goat anti mouse IgG (Sigma Chemical Company, Poole, UK) diluted 1:80 for 45 minutes. Sections were washed again with 3 × 5 minutes of PBS and mounted under glycerol (Merck Ltd, Leicestershire, UK).

Several variations on the basic procedure were carried out in an attempt to obtain positive staining. The variations included altering incubation time in primary antibody (2, 4, 12 and 24 hours) altering incubation time in secondary antibody (45, 90 and 120 minutes), fixing slides overnight in acetone at -60°C instead of the BNF, methanol and acetone stages and using a third antibody amplification system. Visualisation of the staining with Cy3 fluorescence (sheep anti rabbit, 1:1000 and 1:500 in PBS, Sigma Chemical Company, Poole, UK) and peroxidase (rabbit anti mouse, 1:500 for 2 hours, Sigma Chemical Company, Poole, UK, with a DAB substrate kit for peroxidase, Vector Laboratories, Peterborough, UK) visualisation systems were also tested.

Sections of hen oviduct were stained as a positive control, as the 1D5 antibody had been reported to work in hens (Baris *et al.* 1998). Negative controls, replacing the primary antibody incubation for incubation with the relevant secondary antibody were included in each trial.

RESULTS

Identification of positive staining via the fluorescence visualisation systems was hampered by the presence of auto-fluorescence in the tissue. An example of this auto-fluorescence is shown on Figure A1.1. This fluorescence was visible in sections that had no treatment and were simply fixed in BNF and mounted in glycerol.

No positive staining was visible in uterine or vaginal sections from either the traditional-line or the male-line stained by any of the various combinations of incubation times, fixing procedures and visualisation systems used. The staining procedures were also tested on uterine and vaginal sections prepared from a laying hen without any positive results.

FIGURE A1.1 Traditional-line turkey vaginal cross-section that has not been stained, showing autofluorscent staining. $_{\times}$330 magnification.

DISCUSSION

The 1D5 antibody did not reveal any positive staining in the uterus or vagina of traditional- and male-line turkey or from a laying hen. Baris *et al.* (1998) reported positive staining of oestradiol receptors in tissue sections from the magnum of laying hens. However they do not report whether they tested for the presence of auto-fluorescence in the tissue. Auto-fluorescence was observed in the uterine and vaginal tissue of turkeys and also in the laying hen that was tested. The magnum tissue of the turkeys or the laying hen was not tested for either positive staining or auto-fluorescence.

The 1D5 antibody has also been investigated for its affinity to the chicken oestrogen receptor without any success (Prof. P J Sharp, Roslin Institute, personal communication).

From the results of this experiment it seems likely that the 1D5 anti-human oestrogen receptor antibody does not bind to turkey oestrogen receptors.

Appendix 2. Solutions used in hydroxyproline assay

Stock Buffer:

0.24 M citric acid[δ]

0.88 M sodium acetate trihydrate[σ]

0.88 M anhydrous sodium acetate[δ]

0.21 M acetic acid[δ]

0.85 M sodium hydroxide[δ]

pH 6.1 (with hydrochloric acid[ϕ])

Assay Buffer:

3 mls *n*- propanol[ω]

2 mls distilled water

10 mls stock buffer

Chloramine-T Reagent:

0.282g chloramine-T[δ]

1ml *n*-propanol[ω]

1ml distilled water

8mls stock buffer

DMBA reagent:

2g dimethylaminobenzaldehyde[δ]

1.25 ml *n*-propanol[ω]

2.75 ml perchloric acid[δ]

[δ] Merck Ltd, Leicestershire, UK; [σ] Sigma Chemical Company, Poole, UK;

[ϕ] Fisons Scientific Equipment, UK; [ω] May & Baker Ltd, Dagenham, UK

Appendix 3. Solutions used in radioimmunoassays

Coupling buffer:

0.1M sodium carbonate[δ] (pH 8.3)

0.1M sodium hydrogen carbonate[δ]

0.5M sodium chloride[δ]

Acetate buffer:

0.1M sodium acetate[δ]

pH 4.0 with glacial acetic acid[δ]

Phos-gel assay buffer

18g sodium chloride[σ]

2g swine skin gelatin[σ]

100mg thimerosal[σ]

200ml 0.5M phosphate[σ] (pH 7.5)

Made up to 2l with distilled water

Assay buffer (Luteinizing hormone assay)

40 mM sodium phosphate[σ] (pH7.0)

0.9% sodium chloride[σ]

0.1% sodium azide[σ]

2.5% horse serum[ρ]

[δ] Merck Ltd, Leicestershire, UK; [σ] Sigma Chemical Company, Poole, UK;

[ρ] Scottish Antibody Production Unit, Law Hospital, Lankarkshire, UK

Appendix 4. Immunostaining for Collagen Type I and III

Wash sections in 0.01 M PBS (phosphate buffered saline tablets$^{\sigma}$) for at least 5 minutes

Cover sections with solution of normal goat serum$^{\rho}$ diluted 1 in 5 with PBS for 30 minutes

Remove the normal goat serum and cover sections with primary antibody solution

Collagen I diluted 1 in 160

Collagen III diluted 1 in 40

Negative control sections were incubated with Goat anti rabbit FITC$^{\sigma}$ conjugate diluted 1 in 200

All diluted in 0.01M PBS containing 0.1% bovine serum albumin$^{\sigma}$

Leave to incubate overnight at 4◦C

Wash by rinsing in 0.01M PBS for 5 minutes three times

Cover sections in goat anti rabbit FITC$^{\sigma}$ diluted 1 in 200 in 0.01M PBS containing 0.1% BSA

Leave to incubate for one hour at room temperature

Wash by rinsing in 0.01M PBS for 5 minutes three times

Mount with Fluoromount Mountant$^{\delta}$

$^{\delta}$ Merck Ltd, Leicestershire, UK; $^{\sigma}$ Sigma Chemical Company, Poole, UK;

$^{\rho}$ Scottish Antibody Production Unit, Law Hospital, Lanarkshire, UK

Appendix 5. Histological staining procedures

2.1 Haematoxylin and eosin stain

Place slides in xylene[δ] for 5 minutes

Hydrate with 97% alcohol followed by 94% alcohol (industrial methalyated spirits[τ]) followed by washing in water

Place in haematoxylin (Mayer's Haematoxylin (Stevens and Wilson 1996)) for 5 minutes

Wash slides in water for 5 minutes and counterstain in 2.5% eosin[δ] for 2 minutes

Wash with 97% alcohol and return to xylene[δ]

Mount slides using DPX Mountant for Microscopy[δ]

2.2 Van Gieson's stain

Place slides in xylene[δ] for 5 minutes

Hydrate with 97% alcohol followed by 94% alcohol (industrial methalyated spirits[τ]) followed by washing in water

Stain for 10 minutes with Weigert's Haematoxylin (equal mix of solutions A and B; solution A: 1g haematoxylin[ε] in 100ml absolute alcohol[τ]; solution B: 4ml 30% ferric chloride[δ], 1ml hydrochloric acid[ϕ] and 95 ml water)

Wash slides in water and differentiate with 3 dips in acid alcohol (1% hydrochloric acid[ϕ] in absolute alcohol[τ]), followed by water; then 3 dips in a saturated solution of lithium carbonate[δ] in water, followed by water

Counterstain for 3 minutes with Van Gieson's solution (10 ml 1% fuchsin acid[δ] and 100 ml saturated picric acid[ε]

Wash with 97% alcohol and return to xylene[δ]

Mount slides using DPX mounting medium[δ]

[δ] Merck Ltd, Leicestershire, UK; [τ] C. Tennant & Co. Blantyre, UK;

[ε] Hopkin & Williams, Essex, UK; [ϕ] Fisons Scientific Equipment, UK

REFERENCES

Andrews, L. D., & Morrow, H. D. (1978). Restricted feeding regimes and subsequent reproductive performance in cage and floor housed turkey hens. *Poultry Science*, **57**, 17-21

Armstrong, D. G. (1984). Ovarian aromatase activity in the domestic fowl (*Gallus domesticus*). *Journal of Endocrinology*, **100**, 81-86

Armstrong, D. G. (1985). Changes in aromatase activity in small ovarian follicles of the domestic fowl (*Gallus domesticus*) during growth and atresia. *Journal of Endocrinology*, **105**, 297-301

Armstrong, D. G. (1987). Ornithine decarboxylase activity in small ovarian follicles from the laying hen (*Gallus domesticus*): a comparison of follicles from several regions of the ovary. *Journal of Endocrinology*, **112**, 183-187

Asem, E. K., Lintner, F., Biellier, H. V., Burke, W. H., & Hertelendy, F. (1983). Comparison of turkey luteinizing hormone (LH)- and ovine LH-induced progesterone production in granulosa cells of the turkey (*Meleagris gallopavo*) and of the domestic fowl (*Gallus domesticus*). *General and Comparative Endocrinology*, **52**, 445-451

Ayen, E., & Noakes, D. E. (1998). Distribution of collagen in the vaginal wall of ewes. *The Veterinary Journal*, **155**, 213-215

Bacon, W. L., Brown, K. I., & Musser, M. A. (1980). Changes in plasma calcium, phosphorus, lipids, and estrogens in turkey hens with reproductive state. *Poultry Science*, **59**, 444-452

Bahr, J. M., & Johnson, A. L. (1984). Regulation of the follicular hierarchy. *The Journal of Experimental Zoology*, **232**, 495-500

Bahr, J. M., Wang, S. C., Huang, M. Y., & Calvo, F. O. (1983). Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. *Biology of Reproduction*, **29**, 326-334

Baris, C., Carter, D. H., Freemont, A. J., Thorp, B. H., & Braidman, I. P. (1998). A method for immunofluorescent localization of oestrogen receptors in bone sections from an egg-laying poultry strain. *Avian Pathology*, **27**, 121-128

Bienkiewicz, A., Welfel, J., & Kus, E. (1996). The influence of estrogens, progesterone and their antagonists on the collagen content in the pregnant rat uterus. *Hormone and Metabolic Research*, **28**, 592-594

Borron, D. C., McCartney, M. G., & Fuller, H. L. (1974). The effects of restricted energy feeding during the growing period on the reproductive performance of turkey breeder females. *Poultry Science*, **53**, 1485-1493

Braidman, I. P., Davenport, L. K., Carter, D. H., Selby, P. L., Mawer, E. B., & Freemont, A. J. (1995). Preliminary in situ identification of estrogen target cells in bone. *Journal of Bone and Mineral Research*, **10**, 74-80

Brown, K. I. (1982). Four turkey plasma and ovarian peaks detected with anti-17β-estradiol -6BSA serum. *Poultry Science*, **61**, 1427

Brown, K. I., Long, D. W., Bacon, W. L., & Braselton, W. E. (1979). Evidence for the presence of 15-hydroxylated estrogens in the peripheral plasma of the laying turkey. *General and Comparative Endocrinology*, **39**, 552-560

Burmester, B. M. (1948). The influence of sex hormones upon the occurrence of prolapse in chickens. *Poultry Science*, **27**, 745-750

Calvo, F. O., & Bahr, J. M. (1983). Adenylyl cyclase system of the small preovulatory follicles of the domestic hen: Responsiveness to follicle-stimulating hormone and luteinizing hormone. *Biology of Reproduction*, **29**, 542-547

Carey, M. P., & Slack, M. C. (1994). Vaginal vault prolapse. *British Journal of Hospital Medicine*, **51**, 417-420

Cobb, R. (1986) The influence of management and husbandry systems on the incidence and severity of disease:- Layer birds. In: Proceedings no. 92. *Poultry Health - Australian Veterinary Poultry Association*, 333

Creemers, L. B., Jansen, D. C., Veen-Reurings, A. v., Bos, T. v. d., & Everts, V. (1997). Microassay for the assessment of low levels of hydroxyproline. *BioTechniques*, **22**, 656-658

Day, S. L., & Nalbandov, A. V. (1977). Presence of prostaglandin F (PGF) in hen follicles and its physiological role in ovulation and oviposition. *Biology of Reproduction*, **16**, 486-494

DeLancey, J. O. L. (1993). Anatomy and biomechanics of genital prolapse. *Clinical Obstetrics and Gynecology*, **36**, 897-909

De Maaar, T. W. (1996). Uterine prolapse in a camel. *Journal of Camel Practice and Research*, **3**, 147-149

Etches, R. J. (1990). The ovulatory cycle of the hen. *Critical Reviews in Poultry Biology*, **2**, 293-318

Etches, R. J. (1996a). "Growth and Sexual Maturation." In: Reproduction in poultry, CAB International, Oxon, 74-105

Etches, R. J. (1996b). "Management of reproduction using photoschedules." In: Reproduction in poultry, CAB International, Oxon, 263-278

Etches, R. J. (1996c). "Nutrition, food consumption and reproduction." In: Reproduction in poultry, CAB International, Oxon, 279-297

Etches, R. J. (1996d). "The Ovary." In: Reproduction in Poultry, CAB International, Oxon, 125-165

Etches, R. J., & Cheng, K. W. (1981). Changes in the plasma concentrations of luteinizing hormone, progesterone, oestradiol and testosterone and in the binding of follicle-stimulating hormone to the theca of follicles during the ovulation cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology*, **91**, 11-22

Etches, R. J., & Cunningham, F. J. (1976). The interrelationship between progesterone and luteinizing hormone during the ovulatory cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology*, **71**, 51-58

Etches, R. J., MacGregor, H. E., Morris, T. F., & Williams, J. B. (1983). Follicular growth and maturation in the domestic hen (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **67**, 351-358

Etches, R. J., Petitte, J. N., & Anderson-Langmuir, C. E. (1984). Interrelationships between the hypothalamus, pituitary gland, ovary, adrenal gland, and the open period for LH release in the hen (*Gallus domesticus*). *The Journal of Experimental Zoology*, **232**, 501-511

Etches, R. J., & Schoch, J. P. (1984). A mathematical representation of the ovulatory cycle of the domestic hen. *British Poultry Science*, **25**, 65-76

Follett, B. K., Scanes, C. G., & Cunningham, F. J. (1972). A radioimmunoassay for avian luteinizing hormone. *Journal of Endocrinology*, **52**, 359-378

Furr, B. J. A., Bonney, R. C., England, R. J., & Cunningham, F. J. (1973). Luteinizing hormone and progesterone in peripheral blood during the ovulatory cycle of the hen *Gallus domesticus*. *Journal of Endocrinology*, **57**, 159-169

Gilbert, A. B., Evans, A. J., Perry, M. M., & Davidson, M. H. (1977). A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **50**, 179-181

Gilbert, A. B., Perry, M. M., Waddington, D., & Hardie, M. A. (1983). Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **69**, 221-227

Giplin, S. A., Gosling, J. A., Smith, A. R. B., & Warrell, D. W. (1989). The pathogenesis of genitourinary prolapse and stress incontinence of urine. A histological and histochemical study. *British Journal of Obstetrics and Gynaecology*, **96**, 15-23

Goto, K., Nakanishi, Y., & Ogawa, K. (1985). The effect of arginine vasotocin and prostaglandins on oviposition in the domestic fowl. *Japanese Poultry Science*, **22**, 55-62

Graber, J. W., & Nalbandov, A. V. (1976). Peripheral estrogen levels during the laying cycle of the hen (Gallus domesticus). *Biology of Reproduction*, **14**, 109-114

Hammond, R. W., Burke, W. H., & Hertelendy, F. (1981a). Influence of follicular maturation on progesterone release in chicken cells in response to turkey and ovine gonadotrophins. *Biology of Reproduction*, **24**, 1048-1055

Hammond, R. W., Koelkebeck, K. W., Scanes, C. G., Biellier, H. V., & Hertelendy, F. (1981b). Plasma prostaglandin, LH, and progesterone levels during the ovulation cycle of the turkey (*Meleagris gallopavo*). *General and Comparative Endocrinology*, **44**, 400-403

Hammond, R. W., Olson, D. M., Frenkel, R. B., Biellier, H. V., & Hertelendy, F. (1980). Prostaglandins and steroid hormones in plasma and ovarian follicles during the ovulation cycle of the domestic hen. *General and Comparative Endocrinology*, **42**, 195-202

Harvey, E. B., Kaiser, H. E., & Rosenberg, L. E. (1968). "Trunk and torso." In: An atlas of the domestic turkey (*meleagris gallopavo*) myology and osteology, U. S. Government Printing Office, 56

Hocking, P. M. (1987). Observations on the size of the follicular hierarchy in the turkey hen and a case of arrested follicular growth. *British Poultry Science*, **28**, 755-757

Hocking, P. M. (1992a). Effects of photostimulation at 18, 24 and 30 weeks of age on the productivity of female turkeys fed *ad libitum* or restricted until point of lay. *British Poultry Science*, **33**, 253-269

Hocking, P. M. (1992b). Genetic and environmental control of ovarian function in turkeys at sexual maturity. *British Poultry Science*, **33**, 437-448

Hocking, P. M. (1993a). Effects of body weight at sexual maturity and the degree and age of restriction during rearing on the ovarian follicular hierarchy of broiler breeder females. *British Poultry Science*, **34**, 793-801

Hocking, P. M. (1993b). Relationships between egg size, weight and pelvic dimensions in turkeys. *Animal Production*, **56**, 145-150

Hocking, P. M. (1996). Role of body weight and food intake after photostimulation on ovarian function at first egg in broiler breeder females. *British Poultry Science*, **37**, 841-851

Hocking, P. M., & Bernard, R. (1998). Comparative development of the ovary and production, fertility and hatchability of eggs from traditional turkeys and a contemporary male-line fed *ad libitum* or restricted. *British Poultry Science*, **39**, 291-297

Hocking, P. M., Bernard, R., Wilkie, R. S., & Goddard, C. (1994). Plasma growth hormone and insulin-like growth factor-I (IGF-I) concentrations at the onset of lay in *ad libitum* and restricted broiler breeder fowl. *British Poultry Science*, **35**, 299-308

Hocking, P. M., Gilbert, A. B., Walker, M., & Waddington, D. (1987). Ovarian follicular structure of white leghorns fed *ad libitum* and dwarf and normal broiler breeders fed *ad libitum* or restricted until point of lay. *British Poultry Science*, **28**, 493-506

Hocking, P. M., Gilbert, A. B., Whitehead, C. C., & Walker, M. A. (1988). Effects of age and of early lighting on ovarian function in breeding turkeys. *British Poultry Science*, **29**, 223-229

Hocking, P. M., Waddington, D., & Walker, M. A. (1992). Changes in ovarian function of female turkeys photostimulated at 18, 24 or 30 weeks of age and fed *ad libitum* or restricted until point of lay. *British Poultry Science*, **33**, 639-647

Hocking, P. M., Waddington, D., Walker, M. A., & Gilbert, A. B. (1989). Control of the development of the ovarian follicular hierarchy in broiler breeder pullets by food restriction during rearing. *British Poultry Science*, **30**, 161-174

Hosie, B. D., Low, J. C., Bradley, H. K., & Robb, J. (1991). Nutritional factors associated with vaginal prolapse in ewes. *The Veterinary Record*, **128**, 204-208

Huang, E. S., Kao, K. J., & Nalbandov, A. V. (1979). Synthesis of sex steroids by cellular components of chicken follicles. *Biology of Reproduction*, **20**, 454-461

Huang, E. S., & Nalbandov, A. V. (1979). Steroidogenesis of chicken granulosa and theca cells: in vitro incubation system. *Biology of Reproduction*, **20**, 442-453

Itoh, H., Kono, T., Kuwayama, T., & Ichinoe, K. (1988). The relationship between plasma concentrations of progesterone, oestradiol and testosterone and the ages of first egg laying in maturing pullets. *Japanese Poultry Science*, **25**, 86-92

Jackson, S. R., Avery, N. C., Tarlton, J. F., Eckford, S. D., Abrams, P., & Bailey, A. J. (1996). Changes in metabolism of collagen in genitourinary prolapse. *The Lancet*, **347**, 1658-61

Johnson, A. L. (1990). Steroidogenesis and actions of steroids in the hen ovary. *Critical Reviews in Poultry Biology*, **2**, 319-346

Johnson, A. L., & Tienhoven, A. v. (1980). Hypothalamo-hypophyseal sensitivity to hormones in the hen. 1. Plasma concentrations of LH, progesterone, and testosterone in response to central injections of progesterone and R5020. *Biology of Reproduction*, **23**, 910-917

Johnson, P. A., Stoklosowa, S., & Bahr, J. M. (1987). Interaction of granulosa and theca layers in the control of progesterone secretion in the domestic hen. *Biology of Reproduction*, **37**, 1149-1155

Kamiyoshi, M., Niwa, T., & Tanaka, K. (1986). Nuclear estrogen receptor binding in granulosa cells and estradiol-17β contents in follicular membranes of the ovary

of the hen during the ovulatory cycle. *General and comparative endocrinology*, **61**, 428-435

Kawashima, M., Kamiyoshi, M., & Tanaka, K. (1993). Estrogen receptor binding in the hen hypothalamus and pituitary during the ovulatory cycle. *Poultry Science*, **72**, 839-847

Kleissl, H. P., Rest, M. v. d., Naftolin, F., Glorieux, F. H., & Leon, A. d. (1978). Collagen changes in the human uterine cervix at parturition. *American Journal of Obstetrics and Gynecology*, **130**, 748-753

Krueger, K. K., Owen, J. A., Krueger, C. E., & Ferguson, T. M. (1978). Effect of feed and light regimes during the growing period on subsequent reproductive performance of broad breasted white turkeys fed two protein levels. *Poultry Science*, **57**, 27-37

Kuhn, K., & Glanville, R. W. (1980). "Molecular structure and higher organization of different collagen types." In: Biology of Collagen, A. Viidik and J. Vuust, eds., Academic Press, London, 1-14

Law, A. S., Baxter, G., Logue, D. N., O'Shea, T., & Webb, R. (1992). Evidence for the action of bovine follicular fluid factor(s) other than inhibin in suppressing follicular development and delaying oestrus in heifers. *Journal of Reproduction and Fertility*, **96**, 603-616

Lee, K. A., & Bahr, J. M. (1994). Utilization of substrates for testosterone and estradiol-17β production by small follicles of the chicken ovary. *Domestic Animal Endocrinology*, **11**, 307-314

Lien, R. J., Pusztai, A., & El Halawani, M. E. (1989). Influence of the administration of ovine prolactin (oPrl) to laying turkeys on subsequent ovarian steroidogenesis in vitro. *Poultry Science*, **68**, 86

Lilburn, M. S., & Nestor, K. E. (1993). The relationship between various indices of carcass growth and development and reproduction in turkey hens. *Poultry Science*, **72**, 2030-2037

Makinen, J., Kahari, V., Soderstrom, K., & Vuorio, E. (1987). Collagen synthesis in the vaginal connective tissue of patients with and without uterine prolapse. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, **24**, 319-325

Makinen, J., Soderstrom, K., Kiilholma, P., & Hirvonen, T. (1986). Histological changes in the vaginal connective tissue of patients with and without uterine prolapse. *Archives of Gynecology*, **239**, 17-20

Marrone, B. A., & Hertelendy, F. (1983a). Steroid metabolism by avian ovarian cells during follicular maturation. *Biology of Reproduction*, **29**, 953-962

Marrone, B. L., & Hertelendy, F. (1983b). Steroidogenesis by avian ovarian cells: effects of luteinizing hormone and substrate availability. *American Journal of Physiology*, **244**, E487-E493

Marrone, B. L., & Hertelendy, F. (1985). Decreased androstenedione production with increased follicular maturation in theca cells from the domestic hen (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **74**, 543-550

Martindale. (1989). "Progesterone." In: The Extra Pharmacopoeia, J. E. F. Reynolds, ed., The Pharmaceutical Press, London, 1411

Mashaly, M. M., Birrenkott, G. P., El-Begearmi, M. M., & Wentworth, B. C. (1976). Plasma LH and progesterone concentrations in the turkey hen during the ovulatory cycle. *Poultry Science*, **55**, 1226-1234

McCartney, M. G., Borron, D. C., & Brown, H. B. (1977). Reproductive performance of turkey females as affected by grower and pre-breeder diets. *Poultry Science*, **56**, 985-991

McIntush, E. W., & Smith, M. F. (1998). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function. *Reviews of Reproduction*, **3**, 23-30

Melnychuk, V. L., Renema, R. A., Robinson, F. E., & Emmerson, D. A. (1994) Reproductive development in the turkey hen: comparison between male and female lines. *Proceedings of the 9th European Poultry Conference*, Glasgow, UK, 318-319

Melnychuk, V. L., Robinson, F. E., Robinson, N. A., & Hardin, J. R. T. (1996) Sexual maturation of turkey females: The effects of strain and photostimulation program. *Proceedings of the 19th Technical Turkey Conference*, 1-6

Melnychuk, V. L., Robinson, F. E., Renema, R. A., Hardin, R. T., Emmerson, D. A., & Bagley, L. G. (1997). Carcass traits and reproductive development at the onset of lay in two lines of female turkeys. *Poultry Science*, **76**, 1197-1204

Menge, H., Frobish, L. T., Weinland, B. T., & Geis, E. G. (1979). Effect of dietary protein and energy on reproductive performance of turkey hens. *Poultry Science*, **58**, 419-426

Meyer, G. B., Props, C. F., Leighton, A. T., Krey, H. P. V., & Potter, L. M. (1980). Influence of dietary protein during the pre-breeder period on subsequent reproductive performance of large white turkeys. 1. Growth, feed consumption, and female sex-limited reproductive traits. *Poultry Science*, **59**, 352-357

Nestor, K. E., Cantor, A. H., Bacon, W. L., & Brown, K. I. (1981). The influence of body weight restriction during the growing and holding periods on reproduction

of turkey females from strains differing in body weight. *Poultry Science*, **60**, 1458-1467

Norton, P., Baker, J., Sharp, H., & Warenski, J. (1990). Genitourinary prolapse: Relationship with joint mobility. *Neurourology and Urodynamics*, **9**, 321-322

Norton, P., Boyd, C., & Deak, S. (1992). Collagen synthesis in women with genital prolapse or stress urinary incontinance. *Neurourology and Urodynamics*, **11**, 300-301

Ohashi, T., Kusuhara, S., & Ishida, K. (1991). Immunoelectron microscopic demonstration of estrogen receptors in osteogenic cells of Japanese quail. *Histology*, **96**, 41-44

Opel, H., & Arcos, M. (1978). Plasma concentration of progesterone and estradiol during the ovulatory cycle of the turkey. *Poultry Science*, **57**, 251-260

Owings, W. J., & Sell, J. L. (1980). Effect of restricted feeding from 6 to 20 weeks of age on reproductive performance of turkeys. *Poultry Science*, **59**, 77-81

Pastore, G. N., DiCola, L. P., Dollahon, N. R., & Gardiner, R. M. (1989). The effect of estradiol on collagen structure and organization in the immature rat uterus. *Proceedings of the Society for Experimental Biology and Medicine.*, **191**, 69-77

Picaper, G., Leclercq, B., Saadoun, A., & Mongin, P. (1986). A radioimmunoassay of chicken growth hormone using growth hormone produced by recombinant DNA technology: validation and observations of plasma hormone variations in genetically fat and lean chickens. *Reproduction Nutrition Development*, **26**, 1105-1114

Porter, T. E., Hargis, B. M., Silsby, J. L., & El Halawani, M. E. (1989a). Differential steroid production between theca interna and theca externa cells: A three-cell

model for follicular steroidogenesis in avian species. *Endocrinology*, **125**, 109-116

Porter, T. E., Hargis, B. M., Silsby, J. L., & El Halawani, M. E. (1989b). Enhanced progesterone and testosterone secretion and depressed estradiol secretion *in vitro* from small white follicle cells of incubating turkey hens. *General and Comparative Endocrinology*, **74**, 400-405

Porter, T. E., Hargis, B. M., Silsby, J. L., & El Halawani, M. E. (1991a). Characterization of dissimilar steroid productions by granulosa, theca interna and theca externa cells during follicular maturation in the turkey (*Meleagris gallopavo*). *General and Comparative Endocrinology*, **84**, 1-8

Porter, T. E., Silsby, J. L., Behnke, E. J., Knapp, T. R., & El Halawani, M. E. (1991b). Ovarian steroid production in vitro during gonadal regression in the turkey. I. Changes associated with incubation behaviour. *Biology of Reproduction*, **45**, 581-586

Porter, T. E., Silsby, J. L., Behnke, E. J., Knapp, T. R., & El Halawani, M. E. (1991c). Ovarian steroid production in vitro during gonadal regression in the turkey. II. Changes induced by forced molting. *Biology of Reproduction*, **45**, 587-591

Potter, L. M., Shelton, J. R., & Meyer, G. B. (1977). Effects of controlled feeding of turkeys during the pre-breeder period on subsequent performance. *Poultry Science*, **57**, 478-484

Pratt, D. A., Daniloff, Y., Duncan, A., & Robins, S. P. (1992). Automated analysis of the pyridinium crosslinks of collagen in tissue and urine using solid-phase extraction and reversed-phase high performance liquid chromatography. *Analytical Biochemistry*, **207**, 168-175

Rajabi, M., Solomon, S., & Poole, A. R. (1991a). Hormonal regulation of interstitial collagenase in the uterine cervix of the pregnant guinea pig. *Endocrinology*, **128**, 863-871

Rajabi, M. R., Dodge, G. R., Solomon, S., & Robin, A. (1991b). Immunochemical and immunohistochemical evidence of estrogen-mediated collagenolysis as a mechanism of cervical dilation in the guinea pig at parturition. *Endocrinology*, **128**, 371-378

Rajabi, M. R., Solomon, S., & Poole, A. R. (1991c). Biochemical evidence of collagenase-mediated collagenolysis as a mechanism of cervical dilation at parturition in the guinea pig. *Biology of Reproduction*, **45**, 764-772

Rajabi, M. R., Solomon, S., & Poole, A. R. (1992). Activation of protein kinasae C stimulates collagenase production by cultured cells of the cervix of the pregnant guinea pig. *American Journal of Obstetrics and Gynaecology*, **167**, 194-200

Ramadan, R. O., & Hafez, A. M. (1993). Uterine prolapse in a cat. *The Veterinary Record*, **132**, 568

Rao, A. G., Das, L. N., Chanda, S. K., Bose, V. S. C., & Mishra, S. C. (1985). Prolapse of the oviduct in a newhampshire strain. *Indian Journal of Poultry Science*, **20**, 303-306

Richardson, G. F., Klemmer, A. D., & Knudsen, D. B. (1981). Observation on uterine prolapse in beef cattle. *Canadian Veterinary Journal*, **22**, 189-191

Risco, C. A., Reynolds, J. P., & Hird, D. (1984). Uterine prolapse and hypocalcemia in dairy cows. *Journal of the American Veterinary Medical Association*, **185**, 1517-1519

Robins, S. P. (1976). "The separation of cross-linking components from collagen." In: The Methodology of Connective Tissue Research, D. A. Hall, ed., Joynson-Bruvvers Ltd, Oxford, 37-52

Robins, S. P. (1988). Functional properties of collagen and elastin. *Bailliere's Clinical Rheumatology*, **2**, 1-36

Robins, S. P. (1999). "Fibrillogenesis and maturation of collagens." In: Dynamics of Bone and Cartilage Metabolism, M. J. Seibel, S. P. Robins, and J. P. Bilezikian, eds., Academic Press, 31-42

Robinson, F. E., & Etches, R. J. (1986). Ovarian steriodogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). *Biology of Reproduction*, **35**, 1096-1105

Rodriguez-Maldonado, E., Velazquez, P. N., Juarez-Oropeza, M. A., & Pedernera, E. (1996). Steroid metabolism in theca externa cells from preovulatory follicles of domestic hen. *General and Comparative Endocrinology*, **101**, 173-179

Rosenfeld, C. S., Yuan, X., Manikkam, M., Calder, M. D., Garverick, H. A., & Lubahn, D. B. (1999). Cloning, sequencing and localization of bovine estrogen receptor-β within the ovarian follicle. *Biology of Reproduction*, **60**, 691-697

Saito, N., & Koike, T. I. (1992). Alterations in uterine contractility during the oviposition cycle in domestic hens. *British Poultry Science*, **33**, 671-676

Savory, C. J. (1995). Feather peaking and cannibalism. *World's Poultry Science Journal*, **51**, 215-219

Senior, B. E. (1974). Oestradiol concentration in the peripheral plasma of the domestic hen from 7 weeks of age until the time of sexual maturity. *Journal of Reproduction and Fertility*, **41**, 107-112

Senior, B. E., & Cunningham, F. J. (1974). Oestradiol and luteinizing hormone during the ovulatory cycle of the hen. *Journal of Endocrinology*, **60**, 201-202

Senior, B. E., & Furr, B. J. A. (1975). A preliminary assessment of the source of oestrogen within the ovary of the domestic fowl, *Gallus domesticus*. *Journal of Reproduction and Fertility*, **43**, 241-247

Shahabi, N. A., Bahr, J. M., & Nalbandov, A. V. (1975). Effect of LH injection on plasma and follicular steroids in the chicken. *Endocrinology*, **96**, 969-972

Sharp, P. J. (1983). "Hypothalamic control of gonadotrophin secretion in birds." In: Progress in nonmammalian brain research, G. Nistico and C. Bolis, eds., CRC Press Inc., Boca Raton, 123-176

Sharp, P. J., Beuving, G., & Middlekoop, J. H. (1975). Plasma luteinizing hormone and ovarian structure in multiple ovulating hens. *Proceedings of the 5th European Poultry Conference*, Malta, 1259-1267

Sharp, P. J., Dunn, I. C., & Talbot, R. T. (1987). Sex differences in the LH responses to chicken LHRH-I and II in the domestic fowl. *Journal of Endocrinology*, **115**, 323-331

Sharp, P. J., Lea, R. W., Chadwick, A., & Lake, P. E. (1981). Concentrations of plasma luteinising hormone, prolactin, progesterone and androgens during the ovulatory cycle of the turkey. *British Poultry Science*, **22**, 375-383

Shemesh, M., Shore, L., Bendheim, U., Lavi, S., & Weisman, Y. (1982). Correlation between serum estrogen concentrations, light and prolapsed uterus in laying hens. *Advances in Pathology*, **1**, 273-276

Shemesh, M., Shore, L., Lavi, S., Ailenberg, M., Bendheim, U., Totach, A., & Weisman, Y. (1984). The role of 17β-estradiol in the recovery from oviductal prolapse in layers. *Poultry Science*, **63**, 1638-1643

Shimada, K., & Asai, I. (1978). Uterine contraction during the ovulatory cycle of the hen. *Biology of Reproduction*, **19**, 1057-1062

Shimada, K., & Asai, I. (1979). Effects of prostaglandin $F_{2\alpha}$ and indomethacin on uterine contraction in hens. *Biology of Reproduction*, **21**, 523-527

Shimada, K., Neldon, H. L., & Koike, T. I. (1986). Arginine vasotocin (AVT) release in relation to uterine contractility in the hen. *General and Comparative Endocrinology*, **64**, 362-367

Shimada, K., Olson, D. M., & Etches, R. J. (1984). Follicular and uterine prostaglandin levels in relation to uterine contraction and the first ovulation of a sequence. *Biology of Reproduction*, **31**, 76-82

Shimada, K., Saito, N., Itogawa, K., & Koike, T. I. (1987). Changes in plasma concentrations of arginine vasotocin after intrauterine injections of prostaglandin F-2α and acetylcholine at various times during the oviposition cycle of the domestic hen (Gallus domesticus). *Journal of Reproduction and Fertility*, **80**, 143-150

Shore, L. S., Shemesh, M., Bendheim, U., & Totah, A. (1984). The effect of pharmacological doses of oestrogen on oviductal prolapse in the hen. *Refuah Veterinarith*, **41**, 34-37

Stevens, A., & Wilson, I. (1996). "The haematoxylins and eosin." In: Theory and practice of histological techniques, J. D. Bancroft and A. Stevens, eds., Churchill Livingstone, Hong Kong, 99-112

Stryer, L. (1988). "Connective-tissue proteins: collage, elastin, and proteoglycans." In: Biochemistry, W. H. Freeman and Company, New York, 185-204

Turner, R. T., Bell, N. H., & Gay, C. V. (1993). Evidence that estrogen binding sites are present in bone cells and mediate medullary bone formation in Japanese quail. *Poultry Science*, **72**, 728-740

Verma, O. P., & Cherms, F. L. (1964). Observations on the oviduct of turkeys. *Avian Diseases*, **8**, 19-26

Waddington, D., & Hocking, P. M. (1993). Modification of intra-ovarian follicular distributions in broiler breeder hens by *ad libitum* or restricted feeding. *British Poultry Science*, **34**, 777-784

Waddington, D., & Walker, M. A. (1988). Distribution of follicular growth, atresia and ovulation in the ovary of the domestic hen (*Gallus domesticus*) at different ages. *Journal of Reproduction and Fertility*, **84**, 223-230

Webb, R., Baxter, G., McBride, D., Nordblom, G. D., & Shaw, M. P. K. (1985). The measurement of testosterone and oestradiol-17β using iodinated tracers and incorporating an affinity chromatography extraction procedure. *Journal of Steroid Biochemistry*, **23**, 1043-1051

Wechsung, E., & Houvenaghel, A. (1978). Effect of prostaglandins on oviduct tone in the domestic hen in vivo. *Prostaglandins*, **15**, 491-505

Weiss, J. B., Sedowofia, K., & Jones, C. (1980). "Collagen degradation : a defended multi-enzyme system." In: Biology of Collagen, A. Viidik and J. Vuust, eds., Academic Press, London, 113-133

Wells, J. W., Culbert, J., Gilbert, A. B., Walker, M. A., & Davidson, M. F. (1985). LH stimulation of oestradiol production *in vitro* by small ovarian follicles in the hen (*Gallus domesticus*). *IRCS Medical Science*, **13**, 1091

Wheeler, R. S., & Hoffman, E. (1948). Prolapse and "pickout" in diethylstilbestrol-treated cockerels. *The Cornell Veterinarian*, **38**, 89-92

Wilcox, B. D., Rydelek-Fitzgerald, L., & Jeffrey, J. J. (1992). Regulation of collagenase gene expression by serotonin and progesterone in rat uterine smooth muscle cells. *The Journal of Biological Chemistry*, **267**, 20752-20757

Williams, J., Harvey, S., & Leclercq, B. (1986). Plasma levels of luteinizing hormone, growth hormone, and estradiol from six weeks of age to sexual maturity in two lines of chickens selected for low or high abdominal fat content. *Poultry Science*, **65**, 1782-1786

Williams, J. B., & Sharp, P. J. (1977). A comparison of plasma progesterone and luteinizing hormone in growing hens from eight weeks of age to sexual maturity. *Journal of Endocrinology*, **75**, 447-448

Wilson, S. C., & Sharp, P. J. (1973). Variations in plasma LH levels during the ovulatory cycle of the hen, *Gallus domesticus*. *Journal of Reproduction and Fertility*, **35**, 561-564

Wilson, S. C., & Sharp, P. J. (1975). Changes in plasma concentrations of luteinizing hormone after injection of progesterone at various times during the ovulatory cycle of the domestic hen (*Gallus domesticus*). *Journal of Endocrinology*, **67**, 59-70

Wilson, S. C., & Sharp, P. J. (1976). Induction of luteinizing hormone release by gonadal steroids in the ovariectomized domestic hen. *Journal of Endocrinology*, **71**, 87-98

Woessner, J. F. (1989). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB Journal*, **5**, 2145-2154

Yang, J., Long, D. W., & Bacon, W. L. (1997). Changes in plasma concentrations of luteinizing hormone, progesterone, and testosterone in turkey hens during the ovulatory cycle. *General and Comparative Endocrinology*, **106**, 281-292

Yoshimura, Y., Okamoto, T., & Tamura, T. (1995). Changes in localization of ovarian immunoreactive estrogen receptor during follicular development in hens. *General and comparative endocrinology*, **100**, 368-374

Yu, M. W., Robinson, F. E., & Etches, R. J. (1992a). Effect of feed allowance during rearing and breeding on female broiler breeders. 3. Ovarian steroidogenesis. *Poultry Science*, **71**, 1762-1767

Yu, M. W., Robinson, F. E., & Etches, R. J. (1992b). Quantification of ovarian steriodogenesis in the domestic fowl by incubation of intact large follicles. *Poultry Science*, **71**, 346-351

Zhao, Y., Williams, L. M., Hannah, L. T., Ross, A. W., McKelvey, W. A. C., & Robinson, J. J. (1999). Oestrogen and progesterone receptor immunoreactivity and c-*fos* expression in the ovine cervix. *Journal of Reproduction and Fertility*, **115**, 287-292